Responsiveness of Isolated Human Airways

Modulation by Inflammatory Cells, Mediators, and Physical Stimuli

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Jongejan, Roberto Cornelis

Responsiveness of isolated human airways : modulation by inflammatory cells, mediators and physical stimuli / Roberto Cornelis Jongejan. - [S.l. : s.n.]. - Ill.
Thesis Rotterdam. - With ref. - With summary in Dutch.
ISBN 90-9003865-5
SISO 605.14 UDC 616.2(043.3)
Subject heading: airway hyperresponsiveness.

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Omslagontwerp: R.C. Jongejan en H. Derksen.

Omslagillustratie: Doorsneden door astmatische en niet astmatische bronchi met bijbehorende dosis-werkingscurven.

Druk: ICG Printing b.v., Dordrecht.

Responsiveness of Isolated Human Airways

Modulation by Inflammatory Cells, Mediators, and Physical Stimuli

Reactiviteit van Humane Luchtwegen in Vitro

Modulatie door Ontstekingscellen, Mediatoren, en Fysische Prikkels

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. C.J. RIJNVOS
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 20 FEBRUARI 1991 OM 13.45 UUR

DOOR

ROBERTO CORNELIS JONGEJAN

GEBOREN TE BUENOS AIRES

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The printing of this thesis and the studies described in it were financially supported by The Netherlands Asthma Foundation.

Additional financial support for the printing of this thesis was provided by Glaxo bv, Boehringer Ingelheim bv, and the Inpharzam Zambon Group.

Fisons Pharmaceuticals (Leusden, The Netherlands) did the excellent job of making this thesis broadly available to all who are interested.

Dessins de SEMPE extraits de l'album – Les Vacances du Petit Nicolas – Editions Denoel, Paris, avec l'autorisation de M. J.J. Sempé.

Dit boek is voor mijn vader, mijn moeder en voor Jet

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Preface

One of the exciting challenges in asthma research is to clarify the mechanisms that make the airways of atopic asthmatics so remarkably responsive to a wide variety of stimuli. Clarification of these mechanisms is important because hyperresponsiveness of the airways may well be the abnormality that underlies asthma. A thorough understanding of these mechanisms might therefore facilitate the development of strategies to improve the treatment of atopic asthma.

This thesis compiles the results of studies on the function of isolated human airways. The studies were aimed at elucidating the possible role of the airway muscle in bronchial hyperresponsiveness in asthma.

Chapters 1 to 7 provide background information which will facilitate the interpretation of the results. *Chapters* 1 and 2 review some clinical aspects of airway hyperresponsiveness together with the structural changes that are seen in the wall of hyperresponsive airways. Having read these chapters you will probably agree that airway hyperresponsiveness is due to a faulty regulation of airway caliber which is related to specific inflammatory changes in the airway wall. With the information of *Chapters* 1 and 2 at hand you may understand the schematic representation given in *Chapter* 3. This scheme summarizes the factors that determine the extent to which inhaled stimuli will contract airway muscle and the extent to which smooth muscle contraction will change airway caliber. These factors are reviewed in more detail in *Chapters* 4 to 7. You will see that inflammatory cells, the airways mucosa, the innervation of the airways, airway smooth muscle, and airway muscle mechanics, may all contribute to the faulty regulation of airway patency in airway hyperresponsiveness. *Chapter* 8 (and also *Chapter* 3) show that functional studies of isolated human airways provide a good model to evaluate the possible role of the airway muscle in the pathogenesis of airway hyperresponsiveness. Based on the putative importance of the airway muscle in the regulation of airway caliber the questions to be answered in this thesis are derived:

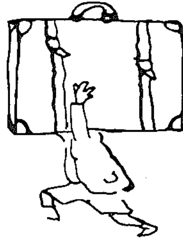
1. Can inflammatory changes as observed in the airway wall in asthma increase the cholinergic responsiveness of human airway muscle?
2. How do physical stimuli to which asthmatics are hyperresponsive such as cold air, hypotonic saline and hypertonic saline, interact with human airway muscle and its regulation?

The model used to answer these questions is described in *Chapter* 9. *Chapters* 10 to 13 contain the results of investigations into the effect of inflammatory changes on the airway muscle. *Chapters* 14 to 16 describe the studies on the possible role of airway muscle in airway hyperresponsiveness to physical stimuli.

In *Chapter* 17 you will find a summary of the results and the main conclusions. To give these conclusions a more general perspective, their contribution to the current awareness of the mechanisms of bronchial hyperresponsiveness in atopic asthma is discussed. The thesis ends with some suggestions for further research.

Part I

Introduction



Chapter 1

Clinical Aspects of Airway Hyperresponsiveness in Asthma and Chronic Obstructive Pulmonary Disease (COPD)*

1.1. Definitions of asthma and COPD

It has proven difficult to reach consensus on a working definition of the term asthma, although over the years several congresses have been organised to address this very issue[16]. In the most recent guidelines of the American Thoracic Society[1] asthma is defined as a clinical syndrome characterized by increased responsiveness of the tracheobronchial tree of which the primary manifestation is variable airways obstruction. The major clinical symptoms of asthma are paroxysms of dyspnea, wheezing and cough. These symptoms may vary from mild and almost undetectable to severe and unremitting (status asthmaticus). The increased responsiveness of the tracheobronchial tree mentioned in the above definition is usually referred to as airway or bronchial hyperresponsiveness, which is defined as an increased tendency of the airways to narrow to a variety of chemical, pharmacological or physical stimuli[22]. Some of these stimuli are nonspecific since they will affect any subject with asthma (e.g. methacholine), while others are specific (e.g. allergens), because they only affect a subgroup of asthmatics[41]. Atopy, which is an inherited tendency to produce IgE antibodies to common environmental antigens[74], occurs in some 80% of the children with childhood asthma[67]. There seems to be a crude dose-response relationship between the severity of atopy and that of asthma[107,108]. Also, in young children atopy is a very strong predictor of bronchial hyperresponsiveness, but the association fades with age[13,23]. So, in children with diagnosed asthma, bronchial hyperresponsiveness is almost always present and some 80% of these asthmatics seem to be atopic. The reverse, however, is not true: not all children with bronchial hyperresponsiveness and/or atopy have asthma.

In an adult population the situation is more complex because here the relation between asthma, atopy, and bronchial hyperresponsiveness may be influenced by smoking and other environmental factors. Smoking is a major risk factor in the development of chronic obstructive pulmonary disease (COPD)[75] and a substantial proportion of patients with COPD is hyperresponsive[106]. According to the definitions of the American Thoracic Society[1], COPD comprises three

* Adapted from: Kerrebijn KF, Howell JBL, Jongejan RC, Postma DS, Sears MR, Woolcock AJ. Clinical presentation. In: Holgate ST ed. The role of inflammatory processes in airway hyperresponsiveness. Oxford: Blackwell Scientific Publications, 1989; 1-35. Printed with permission of Blackwell Scientific Publications.

disorders: emphysema, peripheral airways disease, and chronic bronchitis. In any patient one or all of these may be present, as may bronchial hyperresponsiveness. Specific causes such as localised disease of the upper airways, bronchiectasis, and cystic fibrosis are excluded from the definition. Emphysema is defined as a condition of the lung characterized by abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls, and without obvious fibrosis. Peripheral airways disease refers to pathological changes in the distal airways of persons at risk to develop COPD (e.g. cigarette smokers) which appear to precede the development of emphysema. Chronic bronchitis refers to the condition of subjects with chronic or recurrent excess mucus secretion into the bronchial tree occurring on most days for at least three months of the year for at least two successive years.

Asthma and COPD are often regarded as two distinct entities with different aetiologies and pathogeneses[79]. Patients with COPD, however, may attain clinically important reversibility after treatment: those with asthma may develop COPD with little or no reversibility. The differentiation between these two groups is often arbitrary and difficult. Orie and co-workers have hypothesised that asthma and COPD are two aspects of the same basic process, and that patients share a common allergic constitution and bronchial hyperresponsiveness (the Dutch hypothesis)[72,103].

Given the broad range of patients which may be included in the above definitions and the overlap that may exist between asthma and COPD it has been recommended that, whenever studies are reported, a comprehensive description of the patients should be included[16]. Patients or patient groups should be defined by objective data such as age, gender, the type of symptoms and improvement after treatment, airflow limitation, degree of bronchodilatation after an inhaled β-agonist, allergy and bronchial hyperresponsiveness to various stimuli.

1.2. Bronchial hyperresponsiveness

Bronchial hyperresponsiveness can be quantified by measuring the effects of inhaled pharmacological stimuli such as methacholine (acetylcholine), histamine or propranolol[19,31]. Physical stimuli such as hyperventilation with cold air and inhalation of hyper- or hypotonic aerosols[87], chemical stimuli such as sulphur dioxide or physiological stimuli such as exercise can also be used to quantitate bronchial hyperresponsiveness. As shown in *Figure* 1.1 the dose response curves to inhaled methacholine or histamine in normals have a sigmoid shape. The maximal response is referred to as the plateau. The dose or concentration of the agonist causing a 20% reduction of the FEV_1 is called PD_{20} and PC_{20} respectively. In bronchial hyperresponsiveness in asthma and COPD the dose response curve may be shifted to the left (i.e. hypersensitivity: low PC_{20} or PD_{20}),

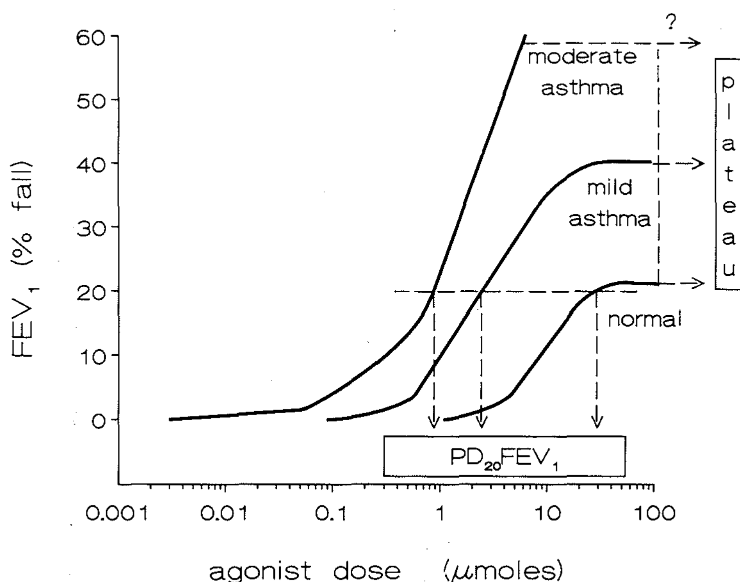


Figure 1.1. Dose response curves to inhaled agonists in normals and asthmatics. *Abbreviations*: FEV_1, forced expiratory volume in 1 second. $PD_{20}FEV_1$, dose of the agonist that causes a 20% reduction of the FEV_1. Modified from[105].

have a steeper slope and/or a higher plateau. This is also shown in *Figure* 1.1.

The presence of a plateau has been recognised as a characteristic feature of the dose response curve in non-asthmatics[105]. In moderate asthma a plateau can usually not be measured, because it lies beyond the maximal reduction in FEV_1 that can be provoked without endangering the patient. Generally, only the position (i.e. PC_{20} or PD_{20}) of the dose response curve is used as the indicator of bronchial hyperresponsiveness. However, the importance of the plateau as an indicator for the severity of asthma is becoming increasingly evident[7,8]. It seems plausible that the plateau more accurately reflects the patient's potential for developing (life-threatening) airflow limitation than the PC_{20} or PD_{20} do.

1.3. Characteristics of bronchial hyperresponsiveness in asthma and COPD

1.3.1. Position and shape of the dose response curve

As a group, asthmatics have a lower PD_{20} and a higher plateau than non-asthmatics, independent of baseline FEV_1. Although subjects with moderate to

severe COPD may have a PD_{20} to histamine within the range found in asthmatics, their responsiveness is often less pronounced[5,52,106]. In most patients with COPD the dose response curve to inhaled methacholine reaches a plateau at a relatively mild degree of airway narrowing; in asthmatics the plateau may be too high to measure without endangering the patient[64,93,105].

1.3.2. Responsiveness to different stimuli

The incidence of bronchial hyperresponsiveness to different stimuli is not the same in patients with asthma or COPD. Asthmatics have a higher incidence of bronchial hyperresponsiveness to methacholine, histamine, propranolol, sulphur dioxide, and isocapnic hyperventilation in cold air; the incidence of bronchial hyperresponsiveness to fog is higher in patients with COPD[25,26,78], although reports are conflictive[92]. There are no studies that directly compare the incidence of bronchial hyperresponsiveness to hypertonic saline in asthma and COPD, but the available data suggest that in asthmatics[92] the incidence is somewhat higher than in patients with COPD[4]. These differences suggest that different hyperresponsiveness profiles exist in these two groups of patients.

Patients with asthma and COPD also differ in their susceptibility to different stimuli. Asthmatics tend to be equally responsive to histamine and methacholine (acetylcholine)[26,49]; whereas patients with COPD are more responsive to histamine than to methacholine and acetylcholine[30]. The correlation of the methacholine responsiveness with the response to hyperventilation in cold air is very good in asthmatics but non-existent in patients with COPD[26,82,85].

1.3.3. Bronchial hyperresponsiveness and airway caliber

If the FEV_1 is reduced, airway responsiveness is almost invariably increased. This is a consequence of the way in which bronchial hyperresponsiveness is usually expressed, i.e. the percentage fall from baseline FEV_1 on provocation. If the initial size of the airways is reduced any given decrease in airway radius will cause a proportionally greater increase in measured resistance. In patients with asthma, Yan *et al.* found that the provocative concentration of histamine that causes a 20% reduction in FEV_1 (PC_{20} histamine) did not correlate with baseline FEV_1[106]. However, in patients with COPD with an FEV_1/FVC ratio below 70% the PC_{20} histamine correlated significantly with the FEV_1/FVC[106]. Furthermore, in these patients the fall in FEV_1 with a fixed dose of histamine (39 μmol) was related to the initial FEV_1 but, again, only if FEV_1 was below 70% of the predicted value. Ramsdale *et al.* found a correlation between PC_{20} methacholine and FEV_1 both in 'asthmatic' and 'bronchitic' subjects[83]. In keeping with the observations of Yan *et al.*[106], Ramsdale *et al.* also found that the airway obstruction in the patients with bronchitis explained about 75 % of the variation in the response to methacholine, but only 35 % in the asthmatics.

Patients with chronic bronchitis[1], may have bronchial hyperresponsiveness

even when no airway obstruction is present[30]. This may imply that the expression of bronchial hyperresponsiveness in patients with COPD does not depend solely on airway caliber before challenge. This is also supported by the observation that in some subjects with COPD with abnormal FEV_1/FVC ratios there is no fall in FEV_1 even with high doses of histamine. Yan *et al.* also showed that in 11 subjects with COPD and no increase in FEV_1 after the administration of fenoterol, a significant decrease in bronchial hyperresponsiveness occurred after fenoterol pretreatment[106]. Thus, initial airway caliber is important, but is not the only factor determining the degree of bronchial hyperresponsiveness in patients with COPD.

Differences in characteristics of bronchial hyperresponsiveness in asthma and COPD can thus be summarized as follows:

1. In most patients with COPD the dose response curve to inhaled methacholine reaches a plateau at a relatively mild degree of airway narrowing; in asthmatics a plateau can often not be reached[64,93,105].
2. In patients with COPD the response to methacholine is less than to equimolar doses of histamine[30], whereas the response in patients with asthma is similar.
3. In contrast to patients with asthma, those with COPD do not bronchoconstrict in response to hyperventilation with cold dry air[26,82,85].
4. In patients with COPD the severity of bronchial hyperresponsiveness is often related to the starting airway caliber; this is less pronounced in asthmatics[106].

The above differences suggest that the mechanisms of bronchial hyperresponsiveness in asthma and COPD may not be the same.

1.4. Modulation of bronchial hyperresponsiveness in asthma

Little is known about the exogenous factors that modulate the characteristics (shape of the dose response curve, responses to different provoking stimuli) of bronchial hyperresponsiveness. In most published studies, increased and decreased levels of bronchial hyperresponsiveness refer to changes in the position of the dose response curves to histamine or methacholine, after the airways have been exposed to the modulating factor. Of the many factors that may (transiently) increase the severity of bronchial hyperresponsiveness in asthmatic subjects the following are relevant for the studies described in this thesis.

1.4.1. Inflammation

Inhalation of aero-allergens by allergic asthmatic subjects often leads to an immediate fall in FEV_1 which resolves spontaneously in about 2 hours. In a proportion of subjects this early asthmatic reaction is followed by a late fall in FEV_1 after 6 to 8 hours the so-called late asthmatic reaction[11]. Inhalation of allergen also transiently increases bronchial hyperresponsiveness[20,70,84] and removal of patients sensitive to house dust mites from the allergen source has

been shown to lead to an improvement in bronchial hyperresponsiveness[76,77]. After a single early asthmatic reaction there is no increase in bronchial hyperresponsiveness, but after a dual response there is[18]. The late asthmatic reaction after exposure to allergens is characterized by an influx of inflammatory cells into the airway wall. Increases in numbers of eosinophils, neutrophils, pulmonary macrophages and lymphocytes have been described in bronchoalveolar lavage fluid obtained 6-48 hours after allergen challenge[24,27,33,63]. The magnitude of the late asthmatic reaction is related to the length and severity of the subsequent development of bronchial hyperresponsiveness, which may worsen for a period of several days to weeks[14]. In dual responders bronchial hyperresponsiveness has been reported to increase shortly or some hours after the resolution of the early reaction, but well before the clinical appearance of the late response. Reports, however, are conflicting[20,97,101]. This indicates that at this point there may already be changes in the lung which lead to increased bronchial hyperresponsiveness and precede the clinical appearance of the late response.

Studies into the cellular contents and the mediator levels of bronchoalveolar lavage fluids from symptom-free asthmatics with bronchial hyperresponsiveness have shown that the histamine level in bronchoalveolar lavage fluid is related to both the levels of prostaglandin (PG) $F_{2\alpha}$, PGD_2 and thromboxane (Tx) B_2 and to the number of mast cells in the same fluid specimen[38,104]. The number of mast cells and the histamine levels were both found to be strongly related to the PD_{20} to methacholine or histamine[38,51]. This suggests that in symptomatic asthmatics an ongoing production of inflammatory mediators by mast cells is related to the sensitivity of the airways to inhaled methacholine.

1.4.2. Fog/hypotonic saline

In asthmatics inhalation of ultrasonically nebulised water (fog) causes a small, transient decrease in PC_{20} which can be prevented with sodium cromoglycate[10,91].

1.4.3. Hypertonic saline

Inhalation of hypertonic saline does not increase methacholine responsiveness[91].

1.4.4. Exercise

The situation is obscure. During exercise the PD_{20} to methacholine seems to increase in asthmatics[45]. Most studies have found no change in bronchial hyperresponsiveness to histamine or methacholine after exercise but two studies have found some decrease in PD_{20}[57,94].

1.5. Effect of drugs on bronchial hyperresponsiveness in asthma

Analysis of the protective effects of drugs on bronchial hyperresponsiveness provides information about the mechanisms by which different stimuli cause airway narrowing in asthma.

1.5.1. Responsiveness to methacholine and histamine

In patients with asthma inhaled β_2-agonists in therapeutic doses protect, in a dose-dependent way, against airway narrowing caused by provoking stimuli[86]. Anticholinergics only protect against methacholine-induced bronchoconstriction[105]. The magnitude of the change in PD_{20} to inhaled methacholine or histamine seems to be related to the dose of the β_2-agonist[86]. The increase in FEV_1 and the reduction of bronchial hyperresponsiveness are therefore both dose-related. When the protective effects of sympathomimetics and anticholinergics are compared in the same asthmatic patient, the change in the position of the dose-response curve for a given degree of bronchodilatation seems to be greater after the sympathomimetic ($\pm$ 3 doubling doses) than after the anticholinergic drug ($\pm$ 1.5 doubling doses)[15,17,40]. This observation suggests that the effect of a β-agonist on the position of the dose-response curve is not due to bronchodilatation alone.

Although β_2-agonists reduce bronchial hyperresponsiveness as measured by the PC_{20} to histamine or methacholine they do not seem to reduce the maximal degree of airway narrowing to these agonists, suggesting that they only partially protect against excessive airway narrowing[8].

Inhaled corticosteroids can progressively decrease bronchial hyperresponsiveness as measured by PD_{20} or maximal airway narrowing in asthmatic patients[7,47,53,54,50], but do not normalize airway reactivity within 3 to 6 months. A recent study by van Essen-Zandvliet *et al.* suggests that the PD_{20} of moderately asthmatic children increases linearly towards normal during two years of treatment with inhaled steroids[102]. This occurs independently of changes in the FEV_1 during the course of treatment. The effects also appear to be dependent on the dose[54].

Sodium cromoglycate[56] and nedocromil sodium[29] have also been shown to reduce the severity of bronchial hyperresponsiveness or to inhibit the increase in bronchial responsiveness that may occur during the pollen season. Their effect on maximal airway narrowing is not known.

1.5.2. Responsiveness to allergen

The effect of several drugs on the responses to allergen and to the increase in bronchial hyperresponsiveness which follows allergen exposure are summarized in *Table* 1.1.

Table 1.1. Effect of drugs on the early and late asthmatic reaction after inhalation of allergen and on the allergen-induced increase in bronchial hyperresponsiveness

	EAR	LAR	BHR
β-agonists[21,42]	+++	++[a]	–
cromoglycate/nedocromil[21,55,56]	++	++	++
steroids[21,68,69]	+/–	++	++
histamine antagonists[44,81]	+	–	–
lipoxygenase inhibitors[9]	+	+/–	–
cyclooxygenase inhibitors/ TxA_2-receptor antagonists[6,51,59,90]	+/–	–	+

Abbreviations: EAR: early asthmatic reaction; LAR: late asthmatic reaction; BHR: prevention of allergen induced increase in bronchial hyperresponsiveness.
[a] Only the long acting β_2 agonists such as salmeterol[101].

1.5.3. Responsiveness to exercise, hyper- and hypotonic aerosol

The effects of different drugs on the responses to exercise and hyper- and hypotonic saline are summarized in *Table* 1.2. It can be seen that many different drugs have been tested for their effects against exercise induced asthma but that relatively few of these have been tested for their efficacy against bronchoconstriction after inhalation of hyper- or hypotonic saline. In general the drugs are more effective when given as aerosols[3].

The above data suggest that the airway muscle and inflammation of the airways are crucially important in bronchial hyperresponsiveness in asthma: almost any bronchoconstrictor stimulus can be antagonized with airway smooth muscle relaxants and anti-inflammatory drugs.

Table 1.2. Effect of drugs on exercise induced asthma and on hypo- and hypertonic aerosol-induced bronchoconstriction

	exercise	hypertonic aerosol	hypotonic aerosol
anticholinergics[12,32,37,88,95,98]	++	++	++
β-agonists[12,37,48,65,66]	++	++	++
cromoglycate/ nedocromil[2,12,37,49,95,98,99]	++	++	++
steroids[43]	+	ns	ns
histamine antagonists[58,34,35,71]	+	+	+
lipoxygenase inhibitors[46,60]	+	ns	ns
cyclooxygenase inhibitors[34,35,61,69]	–[a]	–	ns
calcium channel blockers[2,36,62,73,80]	+	–	–
α-agonists[28]	+	ns	ns
local anesthetics[39,89,98]	–	ns	–

Abbreviations: ns, not studied
[a] Inhibit refractoriness to second exercise challenge.

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Chapter 2

Structure of Human Airways: Anatomy, Histology and Ultrastructure

2.1. Introduction

In this chapter the anatomy, histology and ultrastructure of human airways is described going from the luminal side to the serosal side. The morphological changes of the airway wall in asthma and chronic obstructive pulmonary disease (COPD) are described.

2.2. Anatomy

The human bronchial tree can be modelled as a system of airways branching by relatively symmetric dichotomy[6,87,115]. Each division has a number designating the order of the generation (z) that branches from it[116]. Each generation will therefore contain 2^z airway segments. The wall of the conducting airways has three major components: (1) a mucosa which consists of epithelium and a lamina propria, (2) a smooth muscle sleeve and, (3) an enveloping layer of connective tissue partly provided with cartilage[117]. Taking the trachea as generation number 0 the conductive part of the bronchial tree has some 14 to 17 generations before reaching the respiratory bronchioles[87,116]. *Table* 2.1 describes the different types of bronchi, their diameters and the generation(s) in which they are found. The data are based on measurements on a plastic cast of the bronchial tree of an average adult[115].

2.3. Epithelium

The structure of the epithelium differs between the different types of bronchi. From the cartilaginous bronchi down to the bronchioles the epithelium is pseudostratified, and built up of high-columnar cells most of which are ciliated. Several epithelial cell types may be distinguished. The most common basally situated cell, the "basal cell", has been proposed as a progenitor cell of the epithelium in the central bronchi[1,98], although others suggest that the airway secretory cell is the primary progenitor cell[31,89,91] and that the basal cell acts as a bridge between columnar cells and the basal lamina[31]. In the bronchioles, where basal cells are absent, the secretory clara cell is the progenitor cell[2,30,91].

Table 2.1. Dimensions of human airways based on a model of an average adult human lung at 3/4 maximal inflation.

generation	number of airways in generation	diameter of airway (mm)		
0	1	18.0	trachea	
1	2	12.2	main br.	
2	4	8.3	lobar br.	
3	8	5.6	segmental br.	
4	16	4.5		cartilage
5	32	3.5	subsegmental br.	
6	64	2.8		
7	128	2.3		
8	256	1.86	terminal br.	
9	512	1.54		
10	1024	1.30		variable cartilage
11	2048	1.09		
12	4096	0.95	bronchioles	
13	8192	0.82		
14	16384	0.74		no cartilage
15	32768	0.66		
16	65536	0.60		

There are two types of secretory cells: goblet or mucous cells in the proximal airways[47] and clara cells in the peripheral airways[14,47,90].

In non-diseased human airways 0 to 2% of all cells in the epithelial layer are mast cells[64]. Lymphocytes[47] and axon profiles[59] are also seen within the epithelium of normal airways. Epithelial cells are connected by tight junctions which are thought to control the permeability of the epithelium via the paracellular route[9]. The epithelium rests on a basement membrane which separates it from the next layer: the lamina propria.

2.4. Lamina propria

The lamina propria achieves its mechanical stability through longitudinally oriented elastic, collagenous and reticular fibers[8]. A capillary network is disposed among the elastic fibers just below the basement membrane[56]. The elastic fibers are in intimate relationship with two cell types: mast cells and fibroblast-type cells[19]. These mast cells are sometimes associated with nerve bundles within a few microns[19].

2.5. Smooth muscle

In the trachea, a layer of parallel oriented smooth muscle bundles which is about 30 cells thick is situated as a sheet that connects the arms of the horseshoe-shaped cartilaginous rings[108,109]. Occasionally mast cells, fibroblasts, blood vessels and nerve endings are seen in the interfascicular space[110]. The muscle cells are long and narrow (750 x 5 μm) and contain an oval or cigar-shaped nucleus[107,110]. Human tracheal smooth muscle cells are connected by gap junctions which are seen at a moderate frequency (2.7 $\pm$ 0.3 per 100 muscle cells)[18].

In the bronchi the muscle is incorporated into a connective tissue framework consisting of collagenous, elastic and reticular fibers[56]. In 4th to 7th generation human bronchi the muscle bundles are small. They run between the cartilage plates so that they are not so regularly oriented as in the trachea[18]. The cells contain no gap junctions and the innervation is about 10 times more dense than in the trachea[18], in addition more mast cells are found between the muscle cells compared to the trachea[18].

In non-cartilaginous bronchioles the lamina propria is surrounded by a loose network of smooth muscle bundles which are oriented spirally at a pitch of about 30°[25]. The relative thickness of the airway muscle increases toward the peripheral airways[27].

2.6. Submucosa and peribronchium

The submucosa of the central bronchi contains cartilage and glands[106]. The cartilage rings are C-shaped in the trachea, irregularly shaped in the main and large bronchi and become smaller when followed peripherally[114]. The smallest bronchi sometimes contain no cartilage at all[56].

The submucosal glands consist of: (1) acini containing mucous cells, serous cells and in some areas lymphocytes; (2) a tubule; and (3) an excretory duct[76,112].

The peribronchium is a connective tissue sleeve which envelopes the bronchi and bronchial arteries which also contains adipose tissue and aggregations of lymphoid tissue often containing dust-loaded histiocytic cells[56].

2.7. Blood supply and lymphatics

The bronchi require a nutrient blood supply from the bronchial arteries which derive from the thoracic aorta and, less frequently, from intercostal arteries[61,79]. Two to four divisions of these bronchial arteries, which enter the lung at the hilus, bifurcate alongside the bronchi[32] down to the bronchioles and supply the vascular bed inside the airway wall. This vascular bed forms one plexus internal and one plexus external to the airway muscle[88]. The two plexi are linked by transmuscular channels[3]. In the proximal tracheobronchial tree the capillaries

merge into venules which drain into bronchial veins. The plexi in the distal airways drain into the pulmonary veins[3].

Foci of lymphoid cells and glands lie internal to and between the plates of cartilage, and internal and external to the muscle layer. Lymphatics are also numerous in these regions of the airway wall[81].

2.8. Innervation

2.8.1. Anatomy

The parasympathetic cholinergic nerve supply stems from the vagus nerve which originates from the vagal nuclei in the brainstem[4,54]. The sympathetic supply originates from the upper 6 thoracic segments of the spinal cord and postganglionic fibers synapse in the middle and inferior cervical ganglia and the upper 4 thoracic ganglia[4]. Both enter the lung at the hilus[35,65]. The nerves, which run parallel to the bronchi and arteries, divide into a peribronchial and a perivascular plexus, with the peribronchial plexus dividing into an extrachondral and subchondral plexus[65]. In these plexi adrenergic and cholinergic fibers may be wrapped in the same Schwann cell, offering the possibility of direct interaction between the two pathways[34]. Both plexi contain myelinated and unmyelinated fibers[65]. Ganglion cells are scattered in the extra- and subchondral plexi up to the level of the smaller bronchi[104]. In the trachea and main bronchi ganglion cells are most commonly found in the posterior wall but in the intrapulmonary airways they are distributed more evenly in the bronchial wall[4]. The ganglia consist of up to 20 nerve bodies although single neuronal bodies are also seen[103].

2.8.2. Ultrastructure

The innervation of the human airway has been studied with histochemical techniques such as acetylcholinesterase staining for cholinergic nerves and fluorescence histochemistry for adrenergic nerves [17,74]. These techniques do however not allow evaluation of the ultrastructural relation between nerves and other airway structures. Electron microscopy allows a more quantitative approach [17]. Ultrastructurally three types of vesicles can be discerned in the nerve fibers (1) small agranular (clear) vesicles which are thought to contain acetylcholine, although this has not yet been shown directly[57,62,93], (2) small granular (dense core) vesicles[20], which are thought to be adrenergic[20,93] and, (3) large granular (dense core) vesicles which are known to contain peptides[13,62].

In addition to cell bodies and cholinergic nerves[4] ganglia may also contain adrenergic nerves[94,96]. Various neuropeptides have also been localized to airway ganglia[38,92]. Histochemical evidence has shown that although adrenergic nerves are present within the airway muscle of human airways, the majority of the nerve fibers are parasympathetic and contain acetylcholinesterase[62,83,85103]. In

human 4th to 7th order bronchi about 3% of the nerve varicosities contain adrenergic small granular vesicles[20]. Cholinergic nerves supply submucosal glands[78], but not bronchial vessels or epithelium[4] and adrenergic fibers have been found in close association with submucosal glands[83,85] and bronchial arteries[23,85]. The density of the innervation varies along the tracheobronchial tree. In the human trachea there are about 3 nerve profiles per 100 muscle cells and in 4th to 7th generation human bronchi this increases to some 36 nerve profiles per 100 muscle cells[18]. This number again decreases towards the bronchioles[85,103].

Since airways develop from the foregut and the gut contains a well-characterized non-adrenergic non-cholinergic peptidergic nervous system[13] the existence of a similar system in human airways has been postulated[4,62]. The ultrastructure of this third neural pathway has not yet been accurately described[62,95,97], but nerves containing cholinergic small agranular vesicles also contain some peptidergic large granular vesicles[17], raising the possibility that acetylcholine and peptide neurotransmitters are released from the same nerve endings. This association raises the question whether the non-adrenergic non-cholinergic system is really a third neural pathway or that it just forms part of the parasympathetic system. Vasoactive intestinal peptide (VIP) immunoreactivity has been localized to cholinergic fibers near to the airway muscle, the airway submucosal glands and, occasionally, to pulmonary and bronchial vessels and ganglia[21,58,105,113]. Neuropeptide Y shows a similar distribution, but most neuropeptide Y immunoreactivity is seen around the vessels, usually in conjunction with adrenergic fibers[105]. Peptide histidine isoleucine or its human analogue, peptide histidine methionine[44] also have distributions identical to that of VIP[70]. The density of this immunoreactivity decreases along the tracheobronchial tree[21].

Sensory fibers containing tachykinins such as substance P, neurokinin A, an eledoisin-like peptide and calcitonin gene related peptide[4,75,101] run in the mucosa of extrapulmonary airways and surround blood vessels and bronchial muscle bundles[10,53,71,105].

In human airways VIP and neuropeptide Y are more abundant than tachykinins[105], which are more frequently seen in rodent airways[58,59,75].

Morphologic information about afferent nerves in human airways is scanty[4]. Afferent myelinated nerves run in the vagus nerve to the nodose ganglia[119]. Slowly adapting stretch receptors, which mediate the Hering-Breuer reflex, are myelinated nerve endings localized mainly to the smooth muscle of the larger airways which have the ultrastructural appearance of mechanoreceptors[45]. Unmyelinated nerve endings have been found either close to the lumen or near the basement membrane in the human trachea and main bronchi[59]. These unmyelinated nerve endings may correspond to the non-myelinated C-fiber endings or to unmyelinated endings of the myelinated rapidly adapting (irritant) stretch receptors which have both been identified physiologically[4,55].

Pulmonary neuroendocrine cells which might have a receptor or a secretory function[67] are found in the airway epithelium along the basement membrane[50].

About 0.04% of the epithelial cells are neuroendocrine cells[37]. Sometimes these conical or spindle shaped cells[52] are found in clusters which are frequently innervated: the so-called neuroepithelial bodies[33,66,68]. In humans most of them are found in the smaller conductive airways[52]. Immunoreactivity to serotonin[67], gastrin releasing peptide (mammalian bombesin)[111], calcitonin[16], leu-enkephalin[16], and calcitonin-gene related peptide[51,84] has been demonstrated in the human lung some of which may be located to the neuroendocrine cells.

2.9. Morphological abnormalities in asthma and COPD

2.9.1. Asthma

Histological studies on airways from asthmatics who had died during an attack have shown occlusion of the small bronchi with plugs of consisting of mucus and an inflammatory exudate containing desquamated surface epithelial cells, lymphocytes and eosinophils[24]. In fatal asthma hyperplasia of the mucous glands and bronchial smooth muscle are seen and an intense submucosal inflammatory reaction with dilated capillaries, interstitial edema, thickening around the epithelial basement membrane and a cellular infiltrate containing large numbers of eosinophils[41]. It may therefore not be surprising that corkscrew-shaped twists of condensed sputum (Curshmann's spirals), clumps of surface airway epithelial cells (Creola bodies)[80] and aggregates of eosinophil cell membrane lysophospholipase (Charcot-Leyden cristals)[118] together with eosinophils and metachromatic cells, which morphologically resemble mast cells, are characteristically present in sputa from asthmatics[36]. Recent observations suggest that in asthma the airway muscle is hypertrophic[26] and not hyperplastic[39]. In some asthmatics smooth muscle hypertrophy is only seen in central airways and in some it is seen both in central and peripheral airways[28]. In asthmatics the airway wall is thickened. In airways $>$ 2mm in diameter from asthmatics there is a 1.5 to 2-fold increase in the percentage of the cross-sectional area of the airway that is occupied by airway wall tissue[46]. In biopsy specimens from patients with mild stable asthma shedding and damage of the airway epithelium is also seen[5,48,60], although not invariably[69]. A recent biopsy study comparing the epithelial tight junctions of asthmatic, normal and bronchitic subjects indicates that these junctions might well be damaged in asthma, which could cause fragility of the epithelium and changes in epithelial permeability[29]. In stable asthma thickening around the epithelial basement membrane is also a prominent feature[48], and it has recently been demonstrated that this thickening is due to subepithelial fibrosis as a result of myofibroblast activation[99]. Most of the inflammatory cells are found in the lamina propria[48], where increased numbers of (degranulated) mast cells[5,22,69], monocytes[5], activated eosinophils but not neutrophils[5,15,22] and lymphocytes[48,49] are found. These cells seemed to have migrated from the bronchial circulation into the lamina propria[5]. Increased numbers of mast cells have also

been reported within the epithelium of asthmatic airways[22,63]. It is unclear to what extent these pathological changes are related to asthma symptoms since degranulated mast cells and eosinophils (but not a thickened basement membrane) are also found in biopsies in non-asthmatic atopics[42]. Furthermore, in adults longterm anti-inflammatory treatment with steroids also reduces the cellular infiltrate (again without affecting thickening of the basement membrane), while this only has a limited effect on bronchial responsiveness[49,73]. It is not known whether steroids also reverse airway smooth muscle hypertrophy.

2.9.2. COPD

Histologically the mucus in the airways of patients with COPD lacks the eosinophils and epithelial cells typically seen in asthma. In chronic bronchitis inflammation of the cartilaginous airways consists of infiltration of predominantly mononuclear and also some polymorphonuclear cells in the mucosal surface, around the glands and gland ducts[77]. In sputa from patients with chronic bronchitis the predominant cell is the macrophage[36].

In airways from patients with chronic bronchitis there is a slight increase in the proportion of smooth muscle and cartilage in the airway wall[28,77]. These pathological changes are weakly correlated to changes in pulmonary function tests[77,103].

Peripheral airways disease is primarily a histological diagnosis, which refers to inflammatory changes as they are found in the terminal and respiratory bronchioles of smokers. These changes include: infiltration of the airway wall with neutrophils and mononuclear cells, smooth muscle hypertrophy, meta- and hyperplasia of goblet cells and of the epithelium, fibrosis, thickening of the basal membrane and an excess of airways $< 400\mu m$ in diameter[12,43]. In young smokers small pathologic changes in the peripheral airways are seen such as clusters of pigmented alveolar macrophages, increased numbers of mural inflammatory cells and denuded epithelium[82]. Recently it has been shown that in current and ex-smokers the peribronchiolar alveolar attachments are partly destructed[86,100,120]. All these changes seem to be related to clinical airflow obstruction[7,11,40,102].

The pathology of emphysema will not be discussed since it is largely a disease of the respiratory zone and not of the airways.

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Chapter 3

Airway Responsiveness *in Vivo* and *in Vitro*: General Concepts

3.1. Introduction

Any measure for airway responsiveness should quantify to what extent a certain stimulus alters airway caliber or any parameter directly related to airway caliber. Such a measure can be derived from the stimulus response characteristic which results if the response of the airway(s) to increasing stimulation is recorded. The classical way of studying dose response characteristics of airways *in vitro* is by stimulating an isolated airway preparation with increasing concentrations of an agonist (e.g. histamine) and measuring the development of force at a constant length (isometric), or shortening against a constant load (isotonic)[3,4,11]. The relation between the fraction of receptors on the airways occupied by the agonist and the concentration of the drug in the bath fluid is assumed to obey the mass action law, which predicts that the plot of the fractional response against the logarithm of the agonist concentration is a symmetrical sigmoid curve[1].

3.2. Airway responsiveness *in vivo* and *in vitro*

In vivo the dose-response characteristic to an inhaled agonist such as histamine is also a symmetrical sigmoid curve in normals and in asthmatics (only if a plateau can be reached)[14]. The *in vivo* and *in vitro* dose response characteristics can be accurately described by a logistic model, the general form of which can be expressed as[5]:

$$Y = \frac{a - d}{1 + (X/c)^b} + d$$

Where:
- Y   the response expressed as % of the maximal response;
- X   the concentration of the agonist;
- a   the response before addition of the agonist;
- b   the slope factor that determines the steepness of the curve;
- c   the concentration of the agonist that causes 50% of the maximal effect (EC_{50});
- d   the plateau response when the agonist concentration is "infinite".

In airway hyperresponsiveness *in vivo* (see *Figure* 1.1) the sigmoid stimulus response characteristic may be shifted to the left (i.e. hypersensitivity), have a steeper slope[2,8,10] and/or a higher maximum (i.e plateau) compared to normals[12,14].

The form and the position of the stimulus-response characteristic of human airways to any inhaled stimulus is primarily a function of the regulation and the properties of the airway smooth muscle and the geometry of the airway wall[9,12]. The pertinent *in vivo* variable relating an airway smooth muscle response to airway narrowing is airway muscle shortening[9]. *Figure* 3.1 shows a schematic representation of the events that connect stimulation of the airway to a change in its resistance. It is also shown how changes at different points in the process will alter the stimulus response characteristic. First of all stimulation will activate the smooth muscle either by interfering with its regulation or by stimulating it directly. Then, this will lead to activation of the contractile apparatus via various intracellular pathways. Next, activation of the contractile apparatus will cause smooth muscle shortening depending on the initial muscle length in relation to optimal length and on the load that the muscle has to overcome in relation to its strength[11]. Subsequently, smooth muscle shortening will alter airway caliber

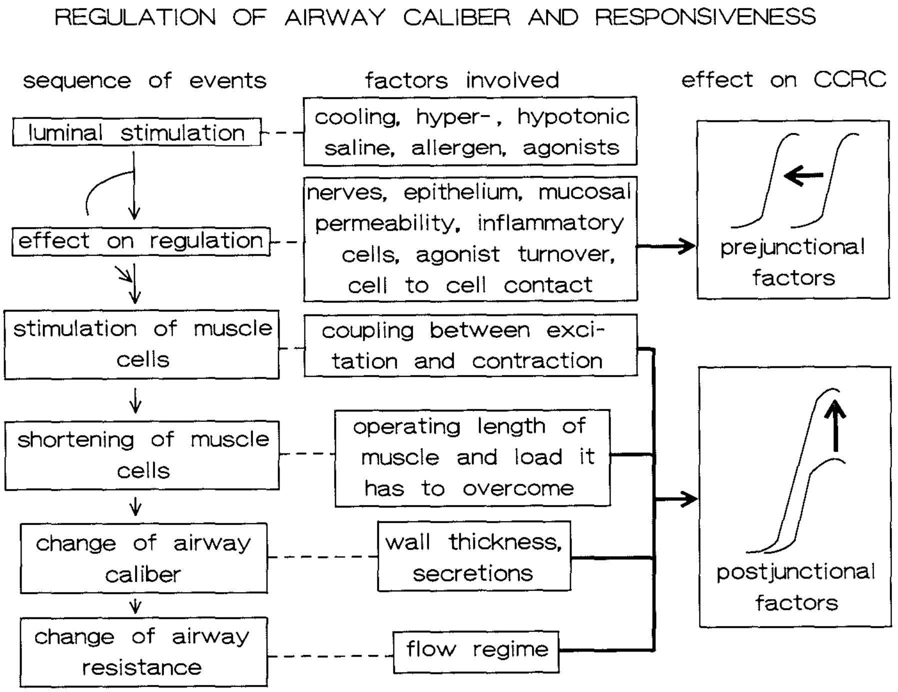

Figure 3.1. Schematic representation of events connecting airway stimulation to changes in airway resistance. Modified from[12]. *Abbreviations*: CCRC, cumulative concentration response curve.

depending on the geometry of the airway wall. Finally, changes in airway caliber will modify airway resistance depending on the flow regime.

Figure 3.1 also shows that changes in airway smooth muscle-regulating systems cause hyperresponsiveness which is characterized by an increase of $-\log EC_{50}$, i.e. a leftward shift of the dose-response curve. This type of hyperresponsiveness has been called prejunctional supersensitivity[6], type I[7], or deviation supersensitivity[13]. Prejunctional supersensitivity mainly reflects an increased availability of the agonist at the receptor site[7,13]. Changes beyond the receptor site may cause hyperresponsiveness characterized by an increase of the maximal effect. This type is called postjunctional supersensitivity[6], type II[7], or non-deviation supersensitivity[13]. Although in some instances the difference between prejunctional supersensitivity and postjunctional supersensitivity may not be clear the concept seems a useful one.

In *Chapters* 4 to 7 each step in the chain of events that separates stimulation of the airway from its narrowing will be discussed. Special emphasis will be on possible changes in asthma and chronic obstructive pulmonary disease.

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Chapter 4

Functional Aspects of Inflammatory Cells in the Airways

4.1. Introduction

Inflammation of the airways is probably of crucial importance in the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD) (see *Chapters* 1 and 2). The characteristic features of airway inflammation are brought about by inflammatory cells and the mediators they release. Many of these mediators are eicosanoids which are formed through activation of phospholipase A_2 which releases arachidonic acid from its intracellular stores. Free arachidonic acid may be metabolized to prostaglandins via the cyclooxygenase pathway, to leukotrienes and 5-hydroxy-eicosa-tetraenoic acid (5-HETE) via the 5-lipoxygenase pathway, to 15-HETE via the 15-lipoxygenase pathway, to 12-HETE via the 12-lipoxygenase pathway, or to platelet activating factor (PAF) via an acetyltransferase enzyme. *Figure* 4.1 summarizes the different eicosanoids that have been found in human airways and the pathways via which these are formed. The next paragraphs summarize the properties of different inflammatory cells which are seen in the airways in asthma and COPD. Emphasis is put on the possible effect of their products on other airway structures such as smooth muscle, vessels, glands, the epithelium, and other inflammatory cells.

4.2. Mast cells

The human mast cell (Ø 8 to 20 μm) is clearly implicated in the pathogenesis of asthma[46]. It has an eccentric nucleus and contains some 200 cytoplasmatic granules[98]. There is considerable heterogeneity to mast cells from different species[6,25] and to mast cells obtained from different locations within one species[2,96]. *Table* 4.1 summarizes the properties of human mast cells obtained from bronchoalveolar lavage fluid, from dispersed human lung tissue and from dispersed human foreskin. Differences in responsiveness to basic polyamines, neuropeptides, inhibitory drugs and hyperosmolarity have been described. The response also varies according to the stimulus. With calcium ionophore or anti-IgE not only histamine but also eicosanoids are released, whereas hyperosmolarity, morphine and peptides only release histamine[14].

The mediators released by purified human lung mast cells and lung fragments challenged with anti-immunoglobulin (Ig) E and their effect on airway smooth muscle, capillary blood flow, vascular permeability, mucus secretion and other inflammatory cells are indicated in *Table* 4.2. Lung fragments, in contrast to

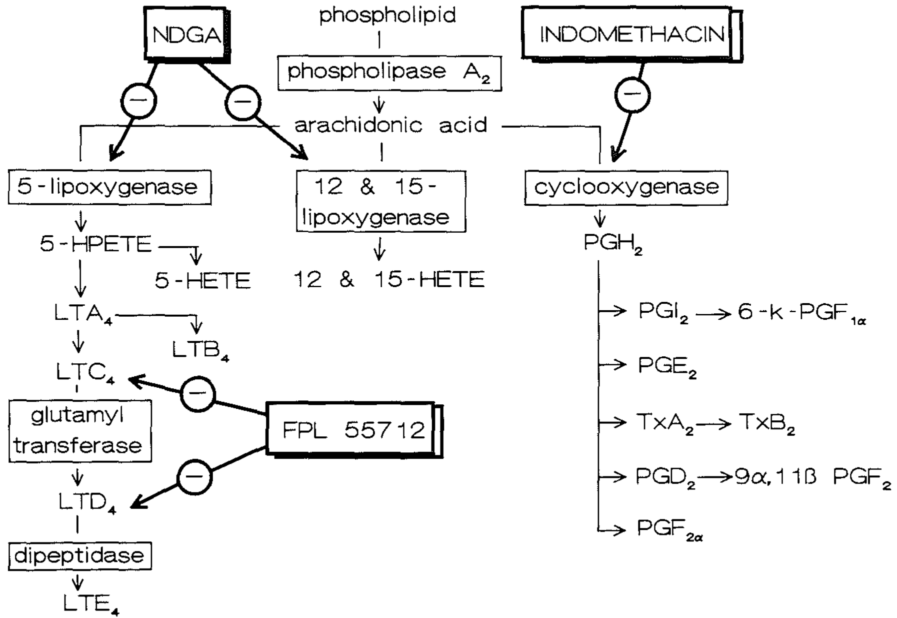

Figure 4.1. Metabolism of arachidonic acid and its pharmacological modulation. *Abbreviations*: PG, prostaglandin; LT, leukotriene; HETE, hydroxy-eicosa-tetraenoic acid; HPETE, hydroxyperoxy-eicosa-tetraenoic acid; Tx, thromboxane; NDGA, nordihydroguaiaretic acid.

Table 4.1. Properties of human mast cells from different sources

	BAL	dispersed lung	dispersed foreskin
sensitive to:			
anti-IgE[5,58]	+	+	+
calcium ionophore[5]	+	+	+
morphine[96]	–	–	+
compound 48/80[5]	–	–	+
VIP[14]	–	–	+
substance P[14]	–	–	+
hyperosmolarity[58]	+++	+	ns
inhibition by[a]:			
cromoglycate[59]	+	+/–	ns
nedocromil[59]	++	+	ns
salbutamol[14,74,97]	++++	+++	++

Abbreviations: BAL, bronchoalveolar lavage; Ig, immunoglobulin; VIP, vasoactive intestinal peptide; ns, not studied.

[a] As measured by inhibition of histamine release.

Table 4.2. Mediators released from lung parenchymal mast cells by anti-IgE and their effects on human airways[a]

	histamine	PGD_2	LTC_4	LTB_4	5-HETE	TxA_2	PGI_2	$PGF_{2\alpha}$	$PGE_{1,2}$	PAF
preparation										
lung fragments (ng/g)	1000-6000	20-160	10-90	2-15	10-35	60-250	10-50	7-50	0	
purified mast cells (ng/10^6 mast cells)	2000-5000	50-300	2-25	1-5	0-5	1-8	0-2	0-8	0-5	0
effect of mediator										
human airway muscle (-logEC_{50})	contr 6.0	contr 5.4	contr 8.2	no effect	contr ±6	contr 7.9	rel [b]	contr 4.9	rel/contr ±5.5	contr [c]
effect on tracheo-bronchial blood flow[d]	incr.	incr.	incr.					incr.	incr.	incr.
vascular permeability plasma extravasation[e]	+	±	+	−				±	±	+
mucus secretion (effective conc.)	+ μM	+ μM	+++ pM		++ nM		+ μM	+ μM	−	+ μM
chemotaxis[f]	−	−	+/−	++	+			−		+++

Abbreviations: PG, prostaglandin; LT, leukotriene; HETE, hydroxy-eicosa-tetraenoic acid; Tx, thromboxane; -logEC_{50}, effective concentration that causes 50% of the maximal effect.

[a] Based on references[1,10,17,24,26,33,35,37,39,40,44,45,55,58,60,62,73,75,76,77,78,79,93,100].

[b] Far more potent on vascular than on bronchial smooth muscle, effect on bronchi highly variable.

[c] Highly variable indirect contractile effect on isolated human airways, rapid tachyphylaxis.

[d] Based on animal studies.

[e] Based on animal studies (rodents).

[f] For granulocytes in Boyden chamber.

purified mast cells, release considerable quantities of prostaglandin (PG) I_2, PGE_2, and $PGF_{2\alpha}$. It seems therefore that during anaphylaxis these mediators may be released by cells other than mast cells. In view of the potency of each mediator in relation to the amount that is released (all mediators have a similar molecular weight) it seems likely that during anaphylaxis histamine, leukotriene (LT) C_4 and TxA_2 contribute to smooth muscle contraction. The effects of the prostanoids and thromboxane (Tx) A_2 on human airway muscle seems to be mediated by one type of receptor for which the different compounds have different affinities[15]. *In vitro* experiments suggest that only histamine and LTC_4 contribute to IgE-dependent bronchoconstriction[7], whereas *in vivo* the early asthmatic response after inhalation of allergen is substantially inhibited by a histamine (H_1) receptor antagonist[40], a lipoxygenase inhibitor[4] and, less prominently, by a TxA_2 receptor antagonist[65,92]. Data obtained from dogs suggest that histamine, LTC_4 and $PGF_{2\alpha}$ will all increase the submucosal blood flow, but that only histamine causes an appreciable, although small, increase in mucosal thickness[54]. LTC_4 is the strongest stimulant of mucus secretion, and this may be antagonized by PGE_2. Histamine and LTC_4 both increase the permeability of the mucosa. LTB_4 and 5-HETE can attract other inflammatory cells[66], although only very small amounts are released.

Table 4.3 gives an overview of the different proteins and proteoglycans located to and released by human pulmonary mast cells. Human mast cells can be divided in tryptase containing and tryptase + chymase containing cells. More than 95% of the epithelial mast cells and 75% of the subepithelial mast cells in human airways are of the tryptase-subtype[86]. The effects of these proteins on human airways have not yet been investigated, but results on canine airways suggest that they might alter the metabolism of neuropeptides, increase mucus secretion and increase the sensitivity of isolated airways to histamine, and therefore play a role in asthma and COPD.

4.3. Eosinophils

The mature human eosinophil (Ø 10 to 15 μm) has a characteristic bi-lobed nucleus and contains some 200 coarse eosinophilic granules in its cytoplasm[57]. Apart from its function in the killing of parasites the eosinophil also plays a key role in asthma, especially in the late phase asthmatic reaction[101]. Its most important properties are summarized in *Table* 4.4. Most data are based on animal studies.

Eosinophils may be activated through different receptors. The term activation refers to two distinct processes the first of which is priming and the second the release of products. Some, usually chemotactic, substances are known to increase the number of receptors on the cell surface (receptor enhancement)[48] and this leads to a greater production of LTC_4 upon challenge with a stimulus which acts on this receptor. Pre-incubation with PAF, for instance, enhances

Table 4.3. Proteins and proteoglycans located to or released by human pulmonary mast cells

name	comment
tryptase or TAME-esterase	degrades VIP[13] increases sensitivity of canine airways to histamine[71] cleaves C_3 into C_{3a}[84] anticoagulant[64,85]
chymase	degrades VIP and substance P[13,42] enhances mucus release by canine serous gland cells7[1,86] cleaves angiotensinogen[84]
cytokines	possibly GM-CSF, IL-3 and IL-5, which may recruite granulocytes into the airway wall[9,21,49]
heparin	inhibits complement activation[68]
kallikrein	cleaves kininogen into bradykinin and kallidin, which are both broncho-active[98]
β-exoglycosidases: β-hexosaminidase β-glucuronidase β-galactosidase	role in human disease unclear[103]

Abbreviations: VIP, vasoactive intestinal peptide; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; TAME, N-α-tosyl-L-arginine methyl ester, substrate used to measure tryptase activity

the release of LTC_4 by eosinophils[95]. Products from other cells such as neutrophils, T-cells or monocytes may also prime the eosinophil and enhance its production of LTC_4[19,29,53,90,91]. The term activation may also refer to the release of products, such as activated oxygen molecules, eicosanoids and granule proteins, which have a variety of actions on airway tissues such as listed in *Table* 4.4. The effect of granule proteins on human airways are unknown.

Several studies suggest that the density of eosinophils decreases if they are primed *in vitro*[52,81]. In peripheral blood of asthmatics the proportion of these so-called hypodense eosinophils is markedly increased compared to non-asthmatic controls[89] and after a late asthmatic reaction the proportion of hypodense eosinophils is even higher. In patients with a single early asthmatic reaction the proportion of hypodense eosinophils does not change[31]. Hypodense cells isolated from peripheral blood can generate more leukotriene C_4 than normodense eosinophils although in asthmatics this difference is not as pronounced as in non-asthmatics[38,47,83]. Several studies suggest that hypodense cells have retained their granules, and that the change in density is due to swelling of the cell and not to the release of granule proteins[32,47,89,94]. On the other hand the increased levels of eosinophil-derived proteins in peripheral blood of dual responders 4

Table 4.4. Some properties of the human eosinophil

property	comment
activation	
priming:	caused by activated neutrophils, GM-CSF, EAF, M-ECEF, IL-3, LTB_4, FMLP, 5-HETE, PAF, IL-5 which are chemotactic and/or cause receptor enhancement and/or alter the density of the cell and/or increase the capacity to generate LTC_4[8,19,29,32,48,50,53,72,81,95,90,91]
product release:	induced by, C3b and C5a fragments of complement (opsonized zymosan), Fc portion of IgG and IgE (low affinity), non-opsonized zymosan, IgA, FMLP and calcium ionophore[11,12,63,88,105]
products	
oxidative burst:	O_2^-, H_2O_2, HOCl, effect on human airways unclear
eicosanoids:	LTC_4. PAF[8,67], for effects see *Table* 4.2
proteins:	
granule core	MBP, stimulates epithelial chloride water secretion, exfoliates epithelium, impairs ciliary activity and increases sensitivity of isolated airways after prolonged incubation[30,36,43,70]
granule periphery	ECP, damages epithelium but is less potent than MBP
granule periphery	EDN, potent neurotoxin, does not damage epithelium
granule periphery	EPO, damages epithelium, potency similar to MBP
density	
normodense	density $>$ 1.072 to 1.095 g/ml
hypodense	density $<$ 1.072 to 1.095 g/ml, produces more LTC_4, greater cytotoxic activity, more surface receptors, more hypodense eosinophils especially after late asthmatic reaction[32,38,47,83]

Abbreviations: GM-CSF, granulocyte macrophage colony-stimulating factor; EAF, eosinophil-activating factor; M-ECEF, monocyte-eosinophil cytotoxicity-enhancing factor; IL, interleukin; FMLP, formyl-methionyl-isoleucyl-phenylalanine; LT, leukotriene; HETE, hydroxy-eicosa-tetraenoic acid; PAF, platelet activating factor; Ig, immunoglobulin; MBP, major basic protein; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EPO, eosinophil peroxidase.

to 24 hours after the inhalation of allergen[23], suggest that eosinophils do degranulate during the late asthmatic reaction.

Of all the inflammatory cells present in the airways in asthma the low density eosinophil is the one with the highest capacity to generate LTC_4 after stimulation with calcium ionophore. Per 10^6 cells low density eosinophils release 70 ng of LTC_4, high density eosinophils 40 ng, mast cells 25 ng, alveolar macrophages 10 ng, monocytes 30 ng, and neutrophils 7 ng[101].

4.4. Neutrophils

The human neutrophil (Ø 10 to 15 μm) forms part of the primary defence of the body against infection. The cell has a characteristic multilobed (polymorph) nucleus. During the late broncho-obstructive reaction in allergic asthmatic patients not only eosinophils[18] but also neutrophils are recruited into the airway wall[69]. However, during the late reaction eosinophils remain longer ($>$ 24 hours) in the airway wall than neutrophils ($<$ 4 hours)[28]. This, however, seems to depend on the stimulus used, because in late phase reactions induced by the occupational agent toluene diisocyanate neutrophils are more prominent than eosinophils[27]. In stable asthma the number of neutrophils in the airway wall seems to be normal[3]. Hence, the role of the neutrophil in asthma is probably limited to the late broncho-obstructive phase. In smoking-induced lung disease the neutrophil probably plays a central role (see *Chapter* 1)[41,50].

Neutrophils contain two types of granules: (1) the primary azurophilic granules which contain acid hydrolases (e.g. acid phosphatase, β-glucuronidase), neutral proteases (elastase) and myeloperoxidase, and (2) the specific granules which contain lysozyme, lactoferrin, cathepsin G and collagenase[104] and a protease which generates C_{5a} from C_5[102]. Like in eosinophils the oxidative burst in neutrophils also generates O_2- and related products[87]. The most important lipid mediators generated by activated neutrophils are LTB_4[22,82,99] and PAF[61]. Most substances that attract and/or activate eosinophils will also attract and/or activate neutrophils. In the presence of activated neutrophils eosinophils generate three times more LTC_4 than in their absence, indicating that important interactions may occur between these two types of cells[53].

4.5. Macrophages, monocytes and lymphocytes

The majority of the cells recovered from bronchoalveolar lavage fluid in normal as well as in asthmatics are macrophages[50]. There is now evidence that during the late asthmatic reaction after antigen challenge macrophages are activated[20,50,80]. Several studies suggest a role for T lymphocytes in asthma[16,34]. It seems that products derived from T-cells, monocytes and alveolar macrophages (cytokines) may modulate the function of other inflammatory cells such as granulocytes[51,56]. At the moment, however, little is known about these interactions, and whether or not these are important in the regulation of the inflammatory reaction in asthma and COPD[21].

4.6. Airway inflammation and bronchial hyperresponsiveness in asthma and COPD

In allergic asthma, the activation of mast cells inside the airway wall and the influx of granulocytes into the airway wall seem related to bronchoconstriction

and to changes in airway responsiveness (see *Chapter* 1.4.1). It seems likely that the relation between these events is not only temporal but also causal. One of the important questions is how these cells and their products cause airway narrowing or modulate airway responsiveness.

4.6.1. Asthma

From the data presented above (see 4.2) it seems reasonable to conclude that the most important mechanism contributing to the early asthmatic reaction is the release of histamine, LTC_4 and TxA_2 released by mast cells activated with IgE.

In contrast to the mechanism of the early reaction the mechanism of the late asthmatic bronchoconstriction after inhalation of allergen is unclear. The mechanism connecting airway inflammation in asthma to airway hyperresponsiveness to various provocative stimuli is also obscure. The reason for this is that it is not known how inflammatory cells in the airway wall, be it in symptom-free periods between attacks or during the late asthmatic reaction, interact with the regulation of airway caliber. The data summarized above show that mast cells and granulocytes (i.e. eosinophils and neutrophils) are capable of releasing different mediators and proteins which may affect the regulation of airway caliber (see *Chapters* 3, 5.6 and 6.4), and thereby lead to changes in airway caliber or airway responsiveness. However, studies on the effect of these cells (or even their products), on isolated human airways are sparse or, in the case of granulocytes, absent.

4.6.2. COPD

The predominant inflammatory cells in COPD are the macrophage and the neutrophil. The putative effects of these inflammatory changes on the regulation of airway caliber in COPD is discussed in *Chapters* 3, 5.6, 6.4 and 7.8. There is reason to believe that the effect of these cells on the mechanic properties of the airways and the parenchyma form an important basis for the narrowing of the airways and airway hyperresponsiveness in COPD.

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Chapter 5

Regulation of Airway Caliber: Autonomic Innervation*

5.1. Introduction

The autonomic innervation can influence airway caliber by acting on airway muscle, or by altering airway wall thickness via effects on vessels and glands in the bronchial wall. It is likely that abnormal activity of autonomic nerves contributes to airway narrowing in chronic obstructive pulmonary disease (COPD) and asthma, but the nature of this putative abnormality has been difficult to clarify.

Figure 5.1 provides a schematic representation of the afferent and efferent pathways of the autonomic innervation of human airways. Complex interactions occur between components of the parasympathetic and sympathetic system. A third class of neurotransmitters, the neuropeptides, has been demonstrated in human airways which may prove relevant to the pathogenesis of obstructive airways disease. Inflammatory reactions in the airway wall are clearly involved in airways obstruction and hyperresponsiveness, and interactions may occur between inflammation and autonomic control.

5.2. Cholinergic innervation

Table 5.1 and *Figure* 5.1 summarize the different muscarinic receptors and their localization in human airways. Parasympathetic nerves supply all airway generations but are most prominent in central airways. Short postganglionic cholinergic nerves supply bronchial and vascular muscle, mucus glands and, probably, mast cells. Acetylcholine stimulates muscarinic receptors on muscle cells and mucus glands which mediate contraction and mucus secretion. Several types of presynaptic receptors which inhibit cholinergic output have been identified over the years. These receptors include: muscarinic M_2, histamine H_3, adrenergic β_2 and probably also enkephalin receptors[18,40,55,63,74]. The M_2 receptors located on the presynaptic membrane may serve as a negative feedback.

* Adapted from: De Jongste JC, Jongejan RC, Kerrebijn KF. Control of airway caliber by autonomic nerves in asthma and COPD. Am Rev Respir Dis (In Press). Printed with permission of the American Review of Respiratory Disease.

AUTONOMIC INNERVATION OF HUMAN AIRWAYS

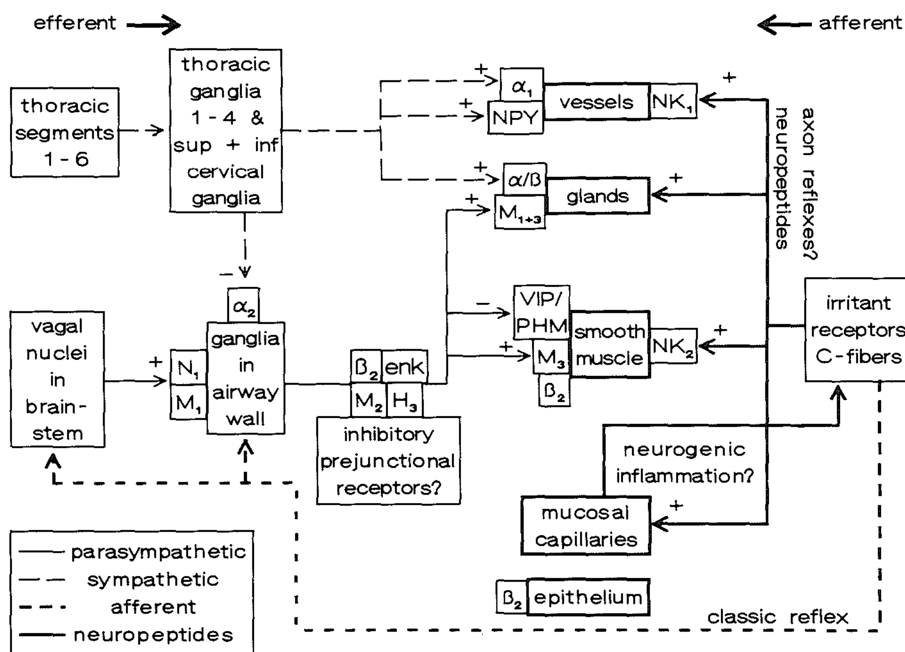

Figure 5.1. Schematic representation of the innervation of human airways. *Abbreviations*: enk, enkephalin; inf, inferior; M, muscarinic; N, nicotinic; NK, tachykinin receptor; NPY, neuropeptide Y; PHM, peptide histidine methionine; sup, superior; VIP, vasoactive intestinal peptide; ?, existence of pathway or mechanism in human airways uncertain.

5.3. Adrenergic innervation

The different adrenergic receptors and their localization in human airways are listed in *Table* 5.1. The sympathetic innervation of the human lung is sparse. Postganglionic fibers, containing noradrenaline and neuropeptide Y[54], supply ganglia, blood vessels and mucus glands in the bronchial wall[49]. Airway smooth muscle receives few sympathetic nerves[17,18,49,88], but has many relaxing β_2-receptors[87]. The existence and function of α-adrenoreceptors in human airways is controversial. Binding studies indicate that α_1-receptors are present[3] but autoradiography studies indicate that their number is almost too low to be detected[78] and the α_1-agonist phenylephrine does not alter the tone of isolated human bronchi. In parasympathetic ganglia, but not postganglionic cholinergic nerves[18], sympathetic nerves supply α_2-receptors which inhibit cholinergic activity[36]. Also, sympathetic fibers end on parasympathetic postganglionic nerves where they seem to inhibit cholinergic output via stimulation of prejunctional β_2-receptors[18].

Table 5.1. Muscarinic and adrenergic receptors in human airways

type	localization	function	specific agonist	specific antagonist
Muscarinic[5,8,15,29,50,57,58,63]				
M_1	ganglia	modulation of ganglionic transmission?	McN-A-343	pirenzepine
	glands	stimulation of secretion		
M_2	presynaptic	inhibition of Ach release	pilocarpine methocramine	gallamine AF-DX 116
M_3	bronchial muscle	contraction	—	4-DAMP hexahydrosiladifenidol
	glands	stimulation of secretion		
Adrenergic[3,6,13,14,36,72,74,78,88]				
α_1	vascular muscle	contracts pulmonary vessels	phenylephrine	prazosine
α_2	ganglia	inhibits Ach release?	clonidine	yohimbine
$\alpha_{1,2}$	glands	stimulate secretion		
β_1	glands	stimulates secretion		betaxolol
β_2	bronchial muscle	relaxation	salbutamol	butoxamine
	presynaptic	inhibits Ach release		
	epithelium	?		
	glands	stimulates secretion		

Abbreviations: Ach, acetylcholine

Pulmonary vessels are contracted by noradrenaline via an effect on α-receptors, and by neuropeptide Y[54,83]. Human lungs have many non-innervated β_2-receptors on epithelial cells; their function is not clear[13].

5.4. Non-adrenergic bronchodilation

It has been possible to evoke a non-adrenergic bronchodilatation in normal men in response to inhaled capsaicin after pretreatment with an anticholinergic drug and a β-blocker[39,50,62]. This could be inhibited by hexamethonium or lidocaine, suggesting a neurally mediated reflex non-adrenergic bronchodilata-

tion. In isolated human bronchi, non-adrenergic bronchodilatation appears after electric field stimulation *in vitro*[20,75]. This relaxation follows the cholinergic twitch, and is partly blocked by the sodium channel blocker tetrodotoxin. The nature of the tetrodotoxin-insensitive component of the non-adrenergic relaxation in human airways is unclear[43]; it may result from direct activation of neural varicosities or end plates, leading to release of a bronchodilator substance without nerve conduction. In guinea pigs there is clear evidence that relaxing neuropeptides such as vasoactive intestinal peptide (VIP) and the related peptide histidine methionine contribute to the non-cholinergic non-adrenergic bronchodilator response[25,26]. In humans this evidence is circumstantial[4]. Firstly, postganglionic parasympathetic nerves contain VIP and peptide histidine methionine[54], which may be coreleased together with acetylcholine[75]. Secondly, as shown in *Table* 5.2, both VIP and peptide histidine methionine have been shown to relax central human airways[69]. Thirdly, VIP seems to occur selectively in central airways, whereas VIP fibers, receptors and *in vitro* effects, just like the non-cholinergic non-adrenergic bronchodilator response are apparently absent in bronchioles[6,69].

5.5. Neuropeptides

Table 5.2 gives an overview of the effect and localization of various neuropeptides in human airways. Peptides in sensory nerves are probably released via axon reflexes after stimulation of C-fiber endings between epithelial cells[7,77]. These peptides may contract or relax bronchial muscle, dilate bronchial arteries, cause leakiness of postcapillary venules, increase mucus secretion[8], induce proliferation of airway epithelial cells[76], and may directly or indirectly recruit inflammatory cells into the airway mucosa[48]. In addition, neuropeptides seem to facilitate cholinergic neurotransmission in the airways in animals[37], but seem to have this action in human airways only during blockade of potassium channels, which may be unphysiological[12]. Furthermore, neuropeptides release mediators from rat mast cells[47], and initiate an inflammatory reaction in the bronchial wall, called neurogenic inflammation[7,27,46,61]. Vicious circles may occur because mediators can in turn augment nerve activity or enhance end organ responsiveness[12,44,47]. Neuropeptides are probably inactivated via proteases released from mast cells (chymase and tryptase), inflammatory cells and epithelium (neutral metallo-endopeptidase)[11,16,66]. It should be remembered that some of the above effects have been found in animals; the data on human tissue are scanty. The effects of stimuli that might be expected to release neuropeptides from human airways *in vitro* are somewhat disappointing. Electric field stimulation of isolated human bronchus only rarely seems to cause non-cholinergic contractions of neural origin (i.e. sensitive to tetrodotoxin)[6,20] and incubation of isolated human bronchus with capsaicin only rarely causes a contractile response (R.C. Jongejan, unpublished observation). Experiments with exogenously added tachykinins show that the receptors in human airways are of the NK_2 subtype[66]. Calcitonin gene

Table 5.2. Effects and localization of various neuropeptides in intact human airways

gonist	receptor	effect	-logEC_{50}	localization of immunoreactivity
vasoactive intestinal peptide[13,69,79]	?	relaxation of central airways	±7.9	glands, vessels, muscle, ganglia (abundant)
peptide histidine methionine[69]	?	relaxation of central airways	±7.7	idem
substance P[59,66]	NK_1	contraction	±4.9	more vessels, less bronchi
neurokinin A[59,66,79]	NK_2	contraction	±7.0	bronchi (sparse)
neurokinin B[56,66]	NK_3	inactive	–	not in airways
eledoisin[4,71]	?	contraction	±7.0	eledoisin-like in bronchi
physalaemin[4,71]	?	contraction	±6.2	?
calcitonin gene-related peptide[54,70]	?	contraction or inactive	±8.3	nerves, epithelium, vessels, ganglia (all sparsely)
cholecystokinin-octapeptide[81]	?	contraction, only central airways	±5.8	difficult to localize
neuropeptide Y[79]	?	contraction of vessels	?	muscle, glands, but mainly vessels

Abbreviations: -logEC_{50}, effective concentration that causes 50% of the maximal effect; NK, tachykinin receptor.

related peptide is a highly potent bronchoconstrictor in the hands of some, but inactive in those of other investigators[54,70].

5.6. Possible abnormalities in asthma and COPD

5.6.1. The cholinergic system

Cholinergic nerve activity contributes significantly to the bronchial narrowing both in asthma and COPD. This is obvious from the beneficial effect of anticholinergic drugs[35,38], which may even be first choice as a bronchodilator

in COPD[35]. Cholinergic bronchoconstriction could well be due to increased reflex activity of parasympathetic nerves[6]. Vagal reflexes are elicited in the airways after stimulation of afferent nerve endings by e.g. cigarette smoke, other nonspecific irritants or inflammatory mediators, and epithelial damage could enhance this process by exposing these nerve endings to the noxious stimuli. Apart from increased reflex activity of the cholinergic system, several factors may be responsible for the cholinergic component of airway narrowing, and three of those will be discussed in some detail.

5.6.2. Defective braking mechanisms

The different types of presynaptic receptors which may inhibit cholinergic output such M_2, H_3, β_2, and enkephalin receptors[18,40,55,63,74], together with ganglionic α_2-receptors may be regarded as brakes to reduce cholinergic excitation of airway tissues. Co-release of VIP from cholinergic nerves is another potential braking mechanism, underlining the functional importance of the cholinergic system. Defects in any of these brakes may lead to cholinergic bronchoconstriction. A recent study suggested that in asthma presynaptic M_2-receptors might be deficient because pilocarpin, in a concentration which selectively stimulated M_2-receptors, could inhibit sulphur dioxide-induced reflex bronchoconstriction only in normals but not in asthmatics[51]. There are no studies on M_2-receptor function in COPD, and results of *in vitro* experiments where human bronchi were stimulated with electric currents have suggested that airways from patients with COPD have similar responses to activation of postganglionic nerves as control airways and this argues against a defective presynaptic M_2 mechanism in COPD[19].

5.6.3. Inflammation

Inflammation may interact with cholinergic activity in several ways. There are some data indicating that prostaglandins and thromboxanes modulate cholinergic neurotransmission in experimental animals[41,65,68,85], and potentiation of cholinergic twitch contractions by exogenous prostaglandin $F_{2\alpha}$ has been found in human bronchus[1]. Also, a number of inflammatory mediators, including histamine and various prostanoids, have been found to enhance bronchial smooth muscle sensitivity to cholinergic stimulation[44]. This is probably relevant because these mediators are present in bronchoalveolar lavage fluid from asthmatics[34]. The nature of airway inflammation differs between asthma and COPD, and one could speculate that eosinophils in asthma and neutrophils in COPD airways may have different effects on autonomic regulation, according to their respective panels of secretory products. Whether this is indeed the case *in vivo* remains to be elucidated.

That mast cells are in close contact with cholinergic nerves[17] and that in the dog cholinergic stimulation enhances mast cell mediator release during antigen challenge[52] suggests that the cholinergic system can activate mast cells in the

airways. If this were true for humans, a vicious circle could result where mast cell mediators stimulate cholinergic reflex activity, which in turn enhances mast cell mediator release.

Postjunctional factors are discussed in *Chapter* 7.8.

5.6.4. Sympathetic nerves and adrenergic receptors

In asthma, but not in COPD and normals, β-blockers induce bronchoconstriction and this indicates abnormal β-receptor activity at some level. It is unlikely that in stable asthmatics circulating adrenalin prevents airway muscle contraction, because the basal adrenalin plasma level is too low to relax airway muscle. Furthermore, local release of noradrenaline from sympathetic nerves is probably not important because airway smooth muscle virtually lacks sympathetic innervation[17,18,49,88]. It has recently been postulated that β-blocker induced asthma is due to blockade of presynaptic β_2-receptors on cholinergic nerves, which normally inhibit acetylcholine release[9]. This would lead to exaggerated cholinergic bronchoconstriction especially if another brake on acetylcholine release, the presynaptic M_2-receptor, were also deficient[51]. Indeed, β-blocker induced asthma is due to increased cholinergic activity because it responds to anticholinergic drugs. Beta$_2$ receptors on airway smooth muscle are probably intrinsically normal in asthmatics. De Jongste *et al.* measured the responses of isolated airways from three stable asthmatics who underwent surgery for coin lesions and found normal β-adrenergic relaxations[23]. In contrast, a variable deficiency of β_2-receptor function has been found in human isolated bronchus from asthmatics who died from a severe attack[33], suggesting that impairment of β_2-receptor function may be present only during severe asthma attacks.

In COPD, β_2-receptors on airway muscle are probably normal, as similar responses to isoproterenol were found in isolated airways from COPD patients and normal controls[24]. Also, β_2-adrenergic relaxations in COPD airways *in vitro* are not related to the sensitivity and maximal bronchoconstriction to methacholine *in vivo*[22,82]. Van Koppen demonstrated normal β_2-receptor numbers, function and affinities in COPD airway muscle[84].

As far as the airway muscle is concerned the role of α-adrenergic receptors in asthma and COPD is probably limited, although insufficient information is present on ganglionic α-receptor function in humans[10,32]. Airway muscle from humans seems to lack α-receptors[32] as α-adrenergic bronchoconstriction cannot be elicited in isolated human bronchus from non-asthmatic subjects[32,42].

5.6.5. Non-adrenergic bronchodilation

Data on non-adrenergic bronchodilatation in asthma are scanty. De Jongste *et al.* observed a reduced relaxation to electric field stimulation in asthmatic human bronchus *in vitro*[21], with lack of the tetrodotoxin-insensitive component of this relaxation. Recently, the airways from 3 other asthmatics were studied,

two of whom failed to relax to electric field stimulation, and one showing a normal non-adrenergic dilatation (R.C. Jongejan, unpublished observations). Bai and Prasad[2] found no evidence of abnormal nonadrenergic bronchodilatory responses on tracheal smooth muscle obtained from patients with fatal asthma.

Non-adrenergic bronchodilatation may result from the release of VIP from parasympathetic nerves[6,69]. Ollerenshaw and coworkers reported a complete absence of VIP immunoreactivity in airways from 5 asthmatics, 3 of whom died from asthmatic attacks[67]. It seems unlikely, however, that non-adrenergic bronchodilating nerves are intrinsically absent in asthma[21]. In non-asthmatics inhalation of capsaicin after prior bronchoconstriction causes bronchodilatation[39,50,62] which is unaltered by anticholinergics or β-blockade, suggesting that it is non-adrenergic. Preliminary data suggest that asthmatics also bronchodilate after vagal reflex activation and pretreatment with propranolol and anticholinergics[62]. Therefore, the available data suggest that non-adrenergic inhibitory nerve activity is not entirely absent in asthma. More detailed studies are needed to see if it is reduced to a variable degree. Furthermore, the physiological significance of the non-adrenergic inhibitory system remains to be determined.

In COPD airways the net non-adrenergic bronchodilatation *in vitro* is similar to that in control preparations from subjects without COPD indicating that non-adrenergic bronchodilation is normal in COPD[19]. Taylor and colleagues found that in smokers with various degrees of airway hyperresponsiveness no relation was present between non-adrenergic bronchodilatation *in vitro* and the degree of hyperresponsiveness *in vivo*[82].

5.6.6. Sensory nerves and neuropeptides

Peptidergic sensory nerves are present in human airways[54,59], but it has not yet been possible to prove a role for these substances in human asthma. *In vivo*, inhalation or infusion of substance P or VIP has no effect on airway caliber, possibly due to rapid degradation and difficult access to receptors. Inhalation of neurokinin A, however, does cause bronchoconstriction and asthmatics are more sensitive than non-asthmatics[45]. The same group that reported VIP deficiency in asthmatic airways also found an abundance of substance P fibers in asthma compared to COPD[67]. This supports a possible role of substance P and neurogenic inflammation in asthmatic airways. On the other hand, stimulation of sensory nerve endings by inhalation of capsaicin causes bronchoconstriction to a similar extent in asthmatics and non-asthmatics[28], suggesting that asthmatics are not hyperresponsive to stimulation of afferents.

Although there are no data to support a role of peptides in sensory nerves in COPD, it can be speculated that increased reflex activation of C-fiber endings may occur during cigarette smoking[54]. This might lead to airway inflammation via neurogenic reflexes.

5.6.7. Effects of airway denervation

The role of abnormal autonomic nerve activity is difficult to establish as long as we are largely unaware of the importance of a normal autonomic airway innervation. Insight in this could result from studies in lung transplant recipients, who have permanent complete vagal and sympathetic denervation. Several months after transplantation these patients often exhibit bronchial hyperresponsiveness to inhaled methacholine and histamine[30,31,60,64]. Preliminary reports suggest that airway hyperresponsiveness after lung transplantation is independent of baseline airway caliber, muscle hyperplasia or airway wall inflammation, and is not due to upregulation of cholinergic receptors secondary to denervation[30,60,64,80,86]. Therefore, hyperresponsiveness in denervated lungs may indeed be the result of autonomic denervation. This is supported by the finding that bronchial hyperresponsiveness also occurs in diabetics with severe neuropathy[73]. These data suggest that the autonomic innervation normally prevents bronchial hyperresponsiveness. However, diabetics and transplant recipients have no symptoms of asthma or COPD, and therefore the mechanisms of hyperresponsiveness in denervated lungs may well differ from those in asthma or COPD.

5.7. References

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Chapter 6

Regulation of Airway Caliber: the Mucosa

6.1. The airway mucosa

The airway mucosa may interact with smooth muscle function in several ways. It may produce inhibitory factors or act as a diffusion barrier. In addition it may act as a metabolic site for bronchoconstrictor or bronchodilator substances and it may be an important determinant of the thickness of the airway wall. The different functions are summarized in *Figure* 6.1.

6.2. Epithelium

6.2.1. Epithelium derived relaxing factor(s)

Removal of the epithelium increases the sensitivity of isolated bovine[3], porcine[54], canine[17] and guinea-pig[24,20] airways to histamine and cholinergic stimulation by a factor 2 to 5. In the absence of epithelium the response of isolated animal airways to prolonged electrical stimulation[17,54,59] fades significantly less than in airways with epithelium. Epithelial denudation also increases the *in vitro* sensitivity of sensitized guinea-pig trachea to antigen[22], and to leukotriene (LT) C_4/D_4, but not to serotonin, the thromboxane A_2 mimetic U46609, or LTE_4[23]. In contrast, in the absence of epithelium the contractile responses to depolarization with KCl are either normal or reduced in the airways of several animal species[3,20,24,54].

Results in human airways are similar. In isolated human trachea epithelial denudation causes a more than two-fold increase in sensitivity to methacholine[49] and in human cartilaginous bronchi it induces a 2 to 3 fold increase in sensitivity to acetylcholine, histamine and prostaglandin (PG) $F_{2\alpha}$[1,41]. The effect of epithelial denudation in human bronchioles has not yet been investigated.

In the absence of the epithelium the relaxant responses to isoprenaline after precontraction with a cholinergic agonist are reduced in several species[3,17,20,54] including man[1]. Taken together these data suggest that the inhibitory effect of the epithelium is rather non-specific for receptor-activating agonists, and that the inhibitory effect is only absent if the airways are stimulated with KCl.

This raises the question whether these effects are not simply due to the activity of the epithelium as a diffusion barrier (non-specific mechanism). This very issue has been addressed in two animal studies, where the effect of removal of the epithelium on the sensitivity of luminally perfused guinea-pig[40] or rat trachea[42] to cholinergic stimulation from the serosal side was evaluated. In one study[40]

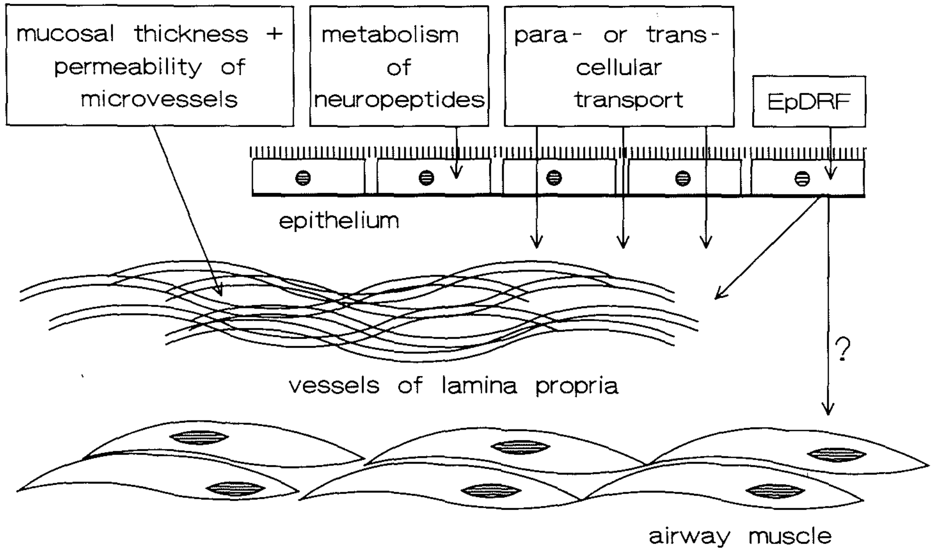

Figure 6.1. The regulatory function of the airway mucosa.
Abbreviations: EpDRF, epithelium derived relaxing factor(s).

no difference was found between tracheas with and without epithelium whereas in the other study the removal of epithelium was found to cause a 4-fold increase in sensitivity to cholinergic stimulation from the serosal side[42] suggesting that the effect of removal of the epithelium was due to release of a relaxing factor. In several species it seems that a prostanoid, probably PGE_2, acts as an epithelium derived relaxing factor[7,14,53,57,59]. In human airways a role for PGE_2 seems doubtful for three reasons. First, human epithelial cells predominantly generate 15-lipoxygenase products[29] and only a minor amount of PGE_2 in response to acetylcholine and no metabolites of arachidonic acid are released in response to mast cell mediators or anti-IgE[51]. Secondly, indomethacin does not alter the basal tone of isolated human airways nor does it influence their responsiveness to histamine[10,25]. Thirdly, PGE_2 is a weak relaxant with low potency in isolated human airways[19].

Bioassay experiments have now confirmed that guinea-pig and human airways superfused with acetylcholine or histamine release a vascular smooth muscle relaxing factor which is not a prostanoid[15,31], but direct evidence for the production of an airway smooth muscle relaxing epithelium derived relaxing factor by human epithelial cells is still lacking.

6.2.2. *Epithelium as a metabolic site for neuropeptides*

Another important feature of the airway epithelium is that it contains neutral metalloendopeptidase[34], which rapidly degrades endogenously released and exogenously added neuropeptides. Epithelium-denuded guinea-pig airways are 5 to 150 times more sensitive to substance P[16,58], neurokinin A and B[12,18], endothelin[21], neurotensin[13] and vasoactive intestinal peptide[50] than airways with intact epithelium. In human airways a neutral metalloendopeptidase inhibitor and removal of the epithelium also increase the sensitivity to substance P, neurokinin A, eledoisin and physalaemin by 7 to 63 fold[5,41].

6.2.3. *Epithelial permeability*

Airway epithelium is quite impermeable[26,27], and is therefore potentially important in regulating the access of luminal stimuli such as cooling, changes in osmolarity of the airway lining fluid, or inhaled agonists and allergens to the airway smooth muscle. Small molecules may cross the epithelial layer by diffusion between cells, i.e. the paracellular route, which is controlled by tight junctions[9]. Macromolecules such as proteins are transported across the cell, and this is a slow process[11]. Munakata *et al.* have shown that luminally perfused guinea-pig trachea is 35 to 115 fold more sensitive to stimulation with histamine or acetylcholine from the serosal side than from the luminal side, and this difference disappears after epithelial denudation[40]. In another study using similar techniques no such difference in sensitivity was found but it was observed that the time course of the response was much slower when stimulating the preparation from the mucosal side[42]. This may account for the effect observed in the study by Munakata *et al.* Studies with human airways are lacking. Thus, the epithelium may not only release inhibitory factors, but also act as a diffusion barrier which could modulate airway responsiveness.

6.3. Lamina propria

6.3.1. *Regulation of the capillary blood flow*

The capillary network in the lamina propria may be important in several ways. Firstly, just like the epithelium, it might regulate the access of luminal stimuli to the smooth muscle. Secondly, changes in capillary flow may lead to changes in airway wall thickness.

Animal studies show that the tracheobronchial and therefore probably also the capillary blood flow is regulated by autonomic agonists, inflammatory mediators, and neuropeptides. In animal models α-agonists reduce the tracheobronchial blood flow[2,35,36], whereas β-agonists, histamine, bradykinin, substance P, vasoactive intestinal peptide, $PGF_{2\alpha}$, PGE, peptide histidine isoleucine,

calcitonin gene related peptide, neurokinin A and B, and capsaicin all cause vasodilation[2,35,36,38,39]. Cholinergic agonists have been found to increase the tracheobronchial bloodflow[36] or leave it unchanged[2]. Thus, it seems that in animals the pharmacologic regulation of bronchial flow resembles that of the general circulation. Whether the same is true for humans is unknown.

Whether vasodilatation contributes to thickening of the airway wall is controversial. On the one hand an inhaled α_1-adrenergic agonist which constricts bronchial vessels prevented bronchial obstruction in patients with impaired left ventricular function[8]. On the other hand vasodilatory drugs have little effect on the mucosal thickness of the dog trachea[35], suggesting that the effect of inhaled α_1-adrenergic agonists may not be mediated by an effect on wall thickness.

6.3.2. Capillary permeability

An increase in capillary permeability is accompanied by widening of gaps between endothelial cells in post-capillary venules. Through these gaps protein-rich plasma is thought to seep through the basement membrane to reach the lamina propria and the airway lumen[44]. This leads to edema which causes thickening of the airway wall. Plasma extravasation may also lead to activation of complement, which is strongly chemotactic [48] and therefore may lead to the influx of inflammatory cells in the airway wall. In animals venular permeability increases in the presence of allergen, histamine, LTC_4/D_4 but not LTB_4, substance P, neurokinin A and B, eledoisin, capsaicin and platelet activating factor[6,33,37,46,52]. In rodents the effect of mediator- and neuropeptide-induced vascular leakage can be antagonized with sodium cromoglycate[45], budesonide[55], α- and β-agonsists[6], high doses of xanthine derivates, lidocaine[46], indomethacin[33], histamine H_3-receptor antagonists[30] but not histamine H_1-receptor antagonists, anticholinergics or bradykinin antagonists[33]. The effects of β-agonists and xanthine derivates are however not undisputed[6].

6.4. The airway mucosa and bronchial hyperresponsiveness in asthma and COPD

From the above it follows that the airway mucosa may contribute to bronchial hyperresponsiveness in several ways.

Firstly, if the epithelium is damaged such as it may be in asthma (but not COPD) this may contribute to bronchial hyperresponsiveness via a diminished production of putative epithelium derived relaxing factor(s) and a reduction in the degradation of endogenous constrictive neuropeptides.

Secondly, increased mucosal permeability may contribute to bronchial hyperresponsiveness. Although it has long been thought that mucosal permeability in asthma is normal recent evidence suggests that it is increased[32]. In COPD mucosal permeability to macromolecules also seems increased[28] and the mucosal permeability of the airways of young smokers is indeed increased but not related

to the level of bronchial responsiveness[56]. The mechanism of such increases is unclear since the regulation of mucosal permeability in humans is poorly understood.

Thirdly, in asthma dilatation of postcapillary venules in the lamina propria and plasma exudation may occur[43,44,47]. This may contribute to bronchial hyperresponsiveness since they may increase the thickness of the airway wall (see *Chapter* 7) through the formation of edema, an increased volume of the periciliary fluid and the formation of viscous complexes from proteins. Whether vasodilatation actually contributes to thickening of the airway wall remains to be proven.

Finally, the postcapillary venules also act as a supply route for inflammatory cells[4], which may migrate into the airway wall from this network and may release bronchoactive mediators.

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Chapter 7

Regulation of Airway Caliber: Smooth Muscle and Airway Mechanics

7.1. Cell-to-cell contact

Smooth muscle can be divided in single-unit and multi-unit or intermediate type smooth muscle[16]. Classically, single unit smooth muscle is sparsely innervated, i.e. under myogenic control, and there is a good electric coupling between the muscle cells by low impedance cell-to-cell communications such as gap-junctions[57], which allow the tissue to respond as a single unit[20,116]. Single unit muscle such as intestinal, uteral and urethral smooth muscle, exhibits spontaneous activity which is associated with oscillations and action potential activity of the membrane potential. Multi-unit smooth muscle is densely innervated, has no spontaneous activity and the electric coupling between the cells is less well developed. Human tracheal smooth muscle is sparsely innervated, has a moderate frequency of gap junctions and has spontaneous oscillations of the membrane potential[46] and therefore seems to be of the single unit type[21]. Bronchial smooth muscle is more densely innervated, has no gap junctions and is electrically quiescent at baseline[3]. Although the absence of gap junctions in human bronchial smooth muscle does not prove the absence of cell-to-cell coupling[22], the available data suggest that human bronchial smooth muscle is of the multi-unit type[21]. It is not clear to what type human bronchiolar smooth muscle pertains[117].

7.2. Coupling of excitation and contraction in airway smooth muscle cells

7.2.1. Calcium (Ca^{2+})

Since data on the coupling between excitation and contraction in human airway muscle are scanty much of the following relates to experiments on smooth muscle from other organs and/or species. The findings are summarized in *Figure* 7.1. In the steady state the intracellular calcium concentration is around 10^{-7} M[13,32,83] and the external Ca^{2+} concentration is around 10^{-3} M. During activation the intracellular Ca^{2+} concentration transiently rises to 10^{-6} M or more[12,76,119,131,132], this is called the phasic component of the response. During the tonic phase of the response, when steady-state stress development occurs, the Ca^{2+} influx rate remains high[63] but the intracellular Ca^{2+} concentration returns close to its basal value[4,85]. In human airway muscle the calcium is recruited from both intra- and/or extracellular pools[11,18,58,59,64,88], depending on the stimulus used.

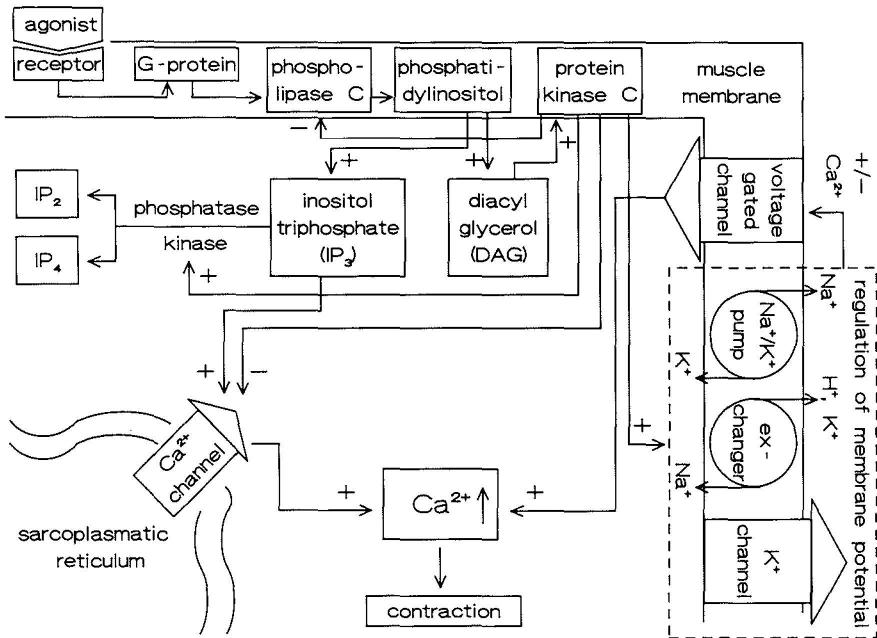

Figure 7.1. Coupling of excitation and contraction in airway smooth muscle.

When the intracellular Ca^{2+} concentration surpasses a critical level it binds to calmodulin and the Ca_4^{2+}-calmodulin initiates the contraction as described in 7.4 and shown in *Figure* 7.4.

7.2.2. Ca^{2+} stores

Of the several putative calcium stores in the airway smooth muscle cell the most important one is probably the sarcoplasmic reticulum[34,81]. The sarcoplasmic reticulum releases Ca^{2+} upon stimulation with caffeine[77,108,119] and the second messenger inositol 1,4,5-triphosphate (IP_3)[127]. There seems to be little evidence for the existence of Ca^{2+}-stores bound to the cell membrane (caveolae) and mitochondria do not absorb Ca^{2+} with physiological Ca^{2+} concentrations[127].

7.2.3. Regulation of the intracellular Ca^{2+} concentration

Two integrated membrane systems are involved in the control of the intracellular Ca^{2+} concentration: (1) the cell membrane (sarcolemma) and (2) the sarcoplasmic reticulum[127]. The influx of Ca^{2+} is regulated by voltage-gated Ca^{2+} channels in the sarcolemma and by receptors which are coupled to a second messenger

system such as the phospholipase C or the adenylate cyclase system (see 7.2.6 and 7.3.2)[12,28,66]. The efflux of Ca^{2+} is regulated by a Na^{+}-Ca^{2+} translocator and ATP dependent Ca^{2+} efflux pumps[1,94] (see *Figure* 7.2).

7.2.4. Influx of Ca^{2+} via voltage-gated Ca^{2+} channels

The existence of voltage-gated Ca^{2+} channels in isolated human airway smooth muscle cells has recently been demonstrated[65]. The membrane potential, which oscillates between -60 and -50mV in human airway muscle[3], depends on ionic concentration gradients and is regulated close to the K^{+} equilibrium potential by (1) the opening or closing of voltage- or calcium-activated K^{+} channels[65], (2) by the activity of the ouabain-sensitive Na^{+}-K^{+} pump[12,109], (3) by amiloride-sensitive Na^{+}-H^{+} exchange and by furosemide-sensitive Na^{+}-K^{+} exchange[87,110] (see *Figure* 7.1). Ca^{2+}-influx starts if the membrane potential rises above -25 mV and is maximal around +18 mV[65]. Electrical depolarization originates from adjacent cells through gap junctions or from activation by agonists of relatively nonspecific cation channels that depolarize the cell membrane[127]. Voltage-gated Ca^{2+} channels are sensitive to blockade with verapamil, diltiazem and dihydropyridines such as nifedipine and to activation with other dihydropyridines such as Bay K 8644[65,123].

7.2.5. Influx of Ca^{2+} after receptor occupation

The evidence for a separate group of receptor-operated channels in human airway smooth muscle has become rather slim[63,95]. The binding of any agonist to its receptor is coupled to the activation of a second messenger system by so-called G-proteins, of which some 15 different types have now been identified[10]. In airway smooth muscle cells most receptors seem to be coupled to the activation of phospholipase C or adenylate cyclase[28]. In mammalian airway muscle muscarinic agonists[67], histamine[42], tachykinins[37] and leukotriene C_4/D_4[19,36] all induce the formation of IP_3. Except for muscarinic agonists[68], a similar effect of these agonists in human airway smooth muscle remains to be demonstrated[17].

7.2.6. Phospholipase C

Activated phospholipase C hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol[9]. IP_3, which is inactivated by a 3'-kinase and a 5'-phosphatase, is capable of releasing Ca^{2+} from non-mitochondrial intracellular stores[17,43,47,125]. IP_3 loses its Ca^{2+}-releasing properties through metabolization into IP_2 and IP_4[47]. The IP_3-induced Ca^{2+}-release may be amplified if the myoplasmic Ca^{2+} concentration rises above $\pm$ 3μM since this activates large cation channels in the sarcoplasmic reticulum to release extra Ca^{2+}[122]. However, the existence of this so-called calcium-induced calcium release in smooth muscle is not certain[96].

Metabolites of inositol phosphate may also be important for the transport of Ca^{2+} across the sarcolemma and/or between intracellular stores[47,49]. The mechanism of this process is still unclear although several hypotheses have been put forward[49,86].

Diacylglycerol is known to activate protein kinase C[80]. The effects of protein kinase C activation have been studied with phorbol esters such as phorbol myristate acetate, which selectively activate protein kinase C. Activation of protein kinase C has been shown to inhibit the release of intracellular Ca^{2+} [60], to inhibit the activation of phospholipase C[14], to promote the inactivation of IP_3 by activating 5'-phosphatase[71], and to indirectly promote the influx of Ca^{2+} via voltage gated Ca^{2+} channels[112]. In animal tracheal smooth muscle activation of protein kinase C causes a triphasic response: an initial contraction followed by a relaxation[101,111] and a sustained contraction[110]. The contractile phases are accompanied by the influx of Ca^{2+} over the cellular membrane and by Na^+/H^+ (amiloride-sensitive) or Na^+/K^+ (furosemide-sensitive) cotransport over the sarcolemma[101,110]. The relaxation phase is accompanied by activation of the Na^+/K^+pump, which will hyperpolarize the sarcolemma[101,110]. There is indirect evidence that activation of protein kinase C plays a key role in the tonic phase of smooth muscle contraction[63,91,92].

7.3. Uncoupling of excitation-contraction and relaxation

Figure 7.2 is a schematic representation of the coupling between excitation and relaxation of airway muscle[91,92]. Although the exact mechanism of smooth muscle relaxation has not yet been elucidated, it is clear that airway smooth muscle relaxation is associated with the activation of adenylate cyclase and with lowering of the intracellular Ca^{2+} concentration.

7.3.1. Adenylate cyclase

Activated adenylate cyclase catalyzes the transformation of adenosine triphosphate (ATP) into adenosine-3',5'-monophosphate (c-AMP), which may be hydrolyzed into inactive 5'-AMP by phosphodiesterases[61]. c-AMP may uncouple excitation and contraction in two ways. Firstly, it activates a c-AMP-dependent protein kinase which phosphorylates myosin light chain kinase which then becomes less sensitive to activation by Ca_4^{2+}-calmodulin[82,111]. Secondly, c-AMP seems to antagonize the activation of phospholipase C and vice versa[42,53,62,97,121]. Whether these effects of c-AMP contribute to relaxation is uncertain[69]. c-AMP-dependent protein kinase relaxes contracted smooth fibers much slower[111] than c-AMP elevation does, suggesting that c-AMP contributes to relaxation via other pathways like Ca^{2+} withdrawal[91,92].

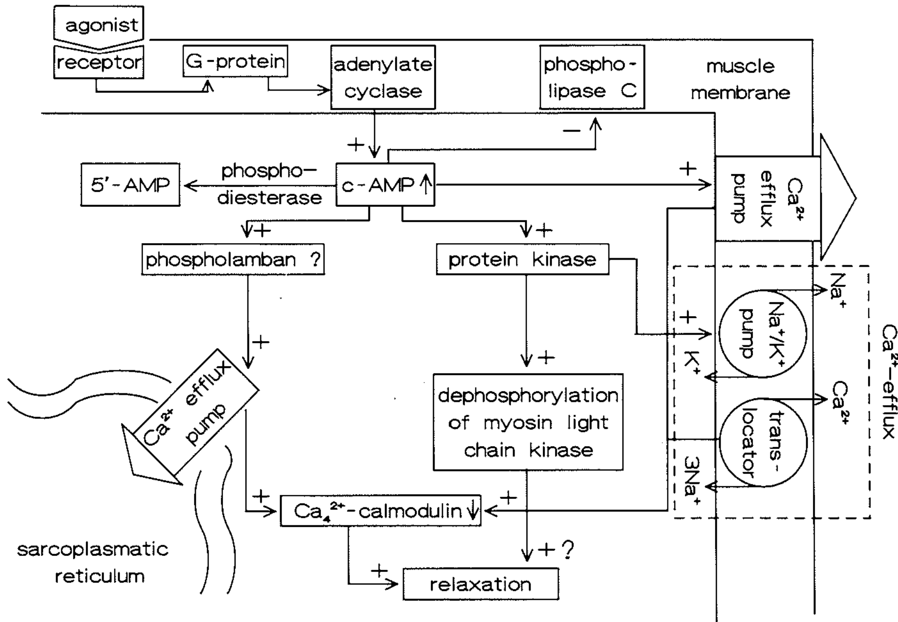

Figure 7.2. Coupling of excitation and relaxation in airway smooth muscle. *Abbreviations*: c-AMP, cyclic adenosine monophosphate; 5'-AMP, 5-adenosine monophosphate.

7.3.2. Lowering of the intracellular Ca^{2+} concentration

The efflux of calcium through the sarcolemma is not only regulated by translocators but also by pumps. These pumps are Ca^{2+}-calmodulin[45,128] and c-AMP-sensitive Ca-Mg ATPases[126]. In vascular smooth muscle calcium efflux from the myoplasma into the sarcoplasmic reticulum is regulated by Ca-Mg ATPase located in the sarcoplasmic reticulum which is activated through Ca^{2+}-dependent phosphorylation[118] which in turn is regulated through c-AMP-mediated phosphorylation of the protein phospholamban[89]. Whether a similar system is present in airway smooth muscle remains to be established. Ca-Mg ATPases exchange $2H^+$ against Ca^{2+} [79].

Stimulation of the Na^+/K^+ pump by c-AMP-dependent protein kinase will increase the influx of Na^+ and so contribute to the efflux of Ca^{2+}[94] via the Na^+-Ca^{2+} exchanger[15,93,94]. During each transport cycle 3 Na^+ are transported for each Ca^{2+} [15,38].

7.4. Biochemistry of contractile proteins

7.4.1. Cross bridges

Smooth muscle of any type is thought to contract through interdigitation of thick and thin filaments inside the smooth muscle cell[2,94]. This process is shown schematically in *Figure* 7.3.

The thick filaments consist of myosin, which is composed of 6 subunits: 2 high molecular weight subunits (200.000 D), or heavy chains, and 2 each of 2 types of low molecular weight subunits (20.000 D and 17.000 D), or light chains[56]. Myosin is configured as an intertwined, coiled-tail molecule embedded in the thick filament, with 2 globular head regions that protrude from the thick filament at regular intervals[56].

The thin filaments are made up of actin which is composed of two linear polymers of a 42.000 D globular protein wrapped in the form of a helix[94].

The proximity of actin turns the actin-binding domain on the globular heads of the thick filaments into catalytic sites for ATP hydrolysis, the so-called actin-activated myosin Mg ATPase, which provides the energy for the cycling of crossbridges[94]. Cycling of crossbridges refers to the cycle of attachment of the actin-binding-domain on the globular head of myosin to the actin filament, its detachment and its re-attachment further down the actin filament. Each actin-

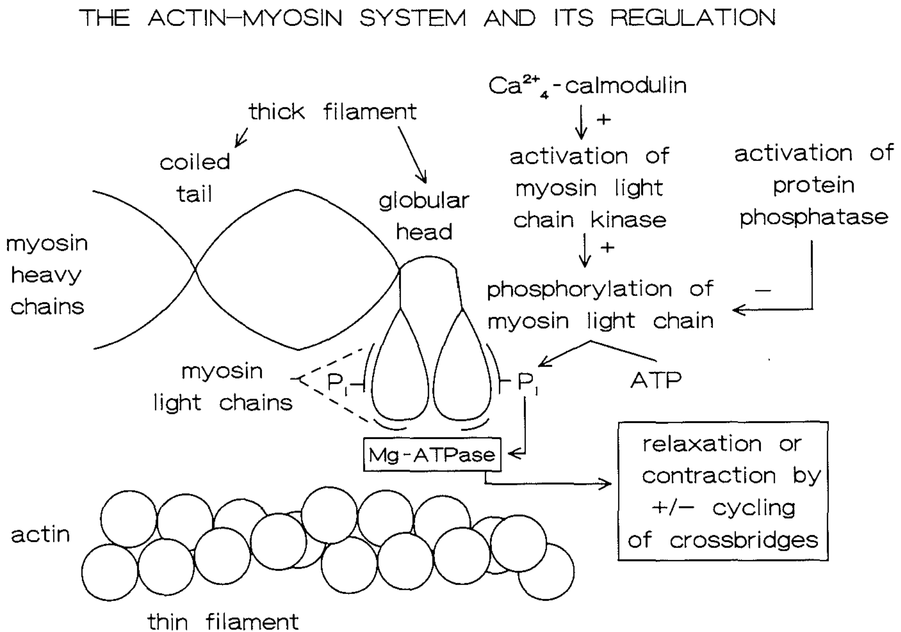

Figure 7.3. Schematic representation of the actin-myosin contractile system.

binding-domain on the thick filament is associated with 2 light chain subunits[56]. Phosphorylation of myosin light chain kinase by Ca_4^{2+}-calmodulin activates this enzyme to catalyze the transfer of a phosphate moiety from ATP to a specific residue (serine 19) of the 20.000-dalton light chain subunit of myosin[2,54]. This results in an increase in activity of actin-activated Mg ATPase[54,105] which is reversed upon dephosphorylation by a protein phosphatase[54,107,124]. This concept of a sliding filament-crossbridge mechanism as the basis for force development in smooth muscle is also supported by recent imaging techniques[5,33].

7.4.2. Latch bridges

Several authors observed that although smooth muscle contraction can be explained by the cycling of cross bridges sometimes parameters associated with crossbridge turnover such as the shortening velocity at zero-load or the ATP consumption are lower than predicted by the parameters reflecting the number of attached crossbridges such as steady-state force, stress, stiffness, or load bearing capacity[27,77]. Subsequent studies on vascular and airway smooth muscle showed that the initial phase of isotonic shortening is caused by the rapidly cycling phosphorylated cross bridges but that tonic stress, is at least partially, maintained by a second class of slowly cycling dephosphorylated bridges called "latch bridges"[27,55,115]. In canine airway muscle latch bridges start cycling some 2.5 s after activation[116,117]. They seem to cycle at about one-quarter of the rate of the normal cycle[104]. The functional importance of latch-bridges lies in that they allow the smooth muscle to maintain sustained contractions at a very low energy consumption which seems an important property for muscles in the hollow organs[77]. Several classes of explications have been invoked to explain latch[29,40,48,78,90,106]. A detailed review of these explanations is beyond the scope of this introduction.

7.5. Effect of initial muscle length and tension on smooth muscle function

Stephens *et al.* investigated the isometric length-tension characteristics of canine airway smooth muscle *in vitro*[114]. *Figure* 7.4 shows the resting tension due to stretching of the muscle and the total tension generated during electric field stimulation, plotted against the length of the muscle. The curve describing the difference between total and resting tension, i.e. the active tension, is also shown. This characteristic indicates that active tension increases with length until an optimal length (L_{max}) is reached. Stretching the muscle beyond L_{max} decreases the active tension that can be achieved. The passive tension at L_{max} is only 5 to 10% of the active tension. Unlike cardiac or striated smooth muscle airway smooth muscle develops tension at lengths as short as 10 to 20% L_{max}. Isotonic shortening is also maximal around L_{max}, and decreases rapidly as the muscle is stretched beyond L_{max}.

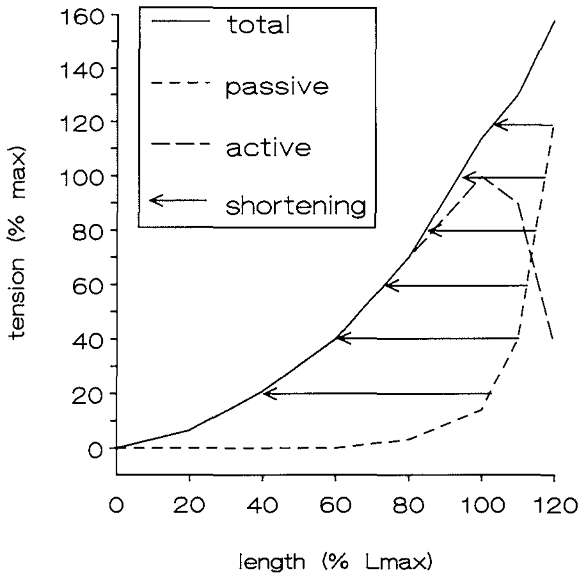

Figure 7.4. Airway smooth muscle isotonic length tension curve. The arrows indicate isotonic muscle shortening at different lengths. The isometric active, passive and total length tension curves are also shown. Modified from[73].

In vivo the airway muscle is embedded in a matrix of connective tissue which provides part of the preload that stretches it. The operating length of human airway muscle *in vivo* is not known but animal studies indicate that at functional residual capacity *in vivo* the tracheal smooth muscle is stretched to around L_{max}[75]. The transmural pressure and tracheal cartilage each provide about 50% of the preload needed to stretch the muscle to L_{max}[50,75]. The transpulmonary pressure needed to bring the airway muscle to L_{max} increases towards the lung periphery[102], probably because of less cartilage in the periphery. Since the mechanical properties of the airway smooth muscle seem to be constant along the tracheobronchial tree[44,103,113], it seems possible that at functional residual capacity (i.e. a static situation where the transmural pressure is similar in the different airway generations) the airway muscle in the bronchi is below L_{max}. However, there is also evidence suggesting that *in vivo* bronchial smooth muscle operates near L_{max} at functional residual capacity[41]. Whether bronchiolar smooth muscle operates near L_{max} *in vivo* is not known.

7.6. Load or resistance opposing smooth muscle shortening

Several animal studies show that maximal airway muscle shortening *in vivo* is far less than the 80% of L_{max} observed in isolated muscle *in vitro*. This suggests that maximal airway muscle shortening *in vivo* is limited by the load it has to overcome[74,84]. This so-called afterload is elastic by nature: it increases as the muscle shortens. Several factors contribute to the afterload. Firstly, in the large airways the cartilage acts as an important afterload[39,50,98]. In rabbits softening of the cartilage with papain increases the maximal airway response to acetylcholine[72]. Secondly, the transmural pressure[98], reflecting the elastic recoil pressure of the parenchyma which exerts axial traction on the airway wall, will also contribute to the afterload. Factors which affect lung elastic recoil such as pulmonary emphysema[8] or fibrosis might therefore alter the afterload on airway smooth muscle in intrapulmonary airways and this may influence maximal smooth muscle shortening and airway narrowing. Furthermore factors which alter the longitudinal traction on the airway may also influence maximal airway narrowing because in medium-sized bronchi and bronchioles the helicoidal orientation of the muscle may become more or less longitudinal during breathing and airway narrowing and this will strongly influence the force available for narrowing of the airways[7].

It should be kept in mind that when measuring the narrowing of bronchial rings against a constant load (i.e. isotonically) *in vitro*, only part of the elastic afterload which opposes smooth muscle shortening *in vivo* is present[70].

7.7. From muscle shortening to an increase in airway resistance

The increase in resistance (R_{aw}) of one isolated airway segment that would occur with a given degree of smooth muscle shortening can be computed with the equation derived by Moreno *et al.*[73]:

$$R_{aw} = \left(\frac{\sqrt{(1-(PMS \times PMP))^2 - P_w}}{\sqrt{1 - P_w}} \right)^{-4}$$

In this equation the airway is considered as a cylinder. The outermost layer of airway muscle is the outer limit of the cylinder wall and the secretions in the lumen are the inner limit of the cylinder wall. PMP represents the proportion of the circumference of the cylinder occupied by smooth muscle. In lungs of subjects without pulmonary disease this proportion is lowest in the trachea and maximal in the bronchioles[30]. P_w represents the proportion that the cylinder wall occupies in the total cross-sectional area. PMS represents the proportion of muscle shortening i.e. the ratio of the length of the muscle in the presence

of an agonist and the length of the muscle in the fully relaxed airway. Results generated with the above-mentioned equation cannot be simply extrapolated to the *in vivo* situation since the equation only refers to the resistance of a single airway. However, a computer model is being developed which might overcome this limitation[130].

7.8. Abnormalities of smooth muscle and airway mechanics in asthma and COPD

Airway smooth muscle from asthmatics is difficult to obtain, and a relatively small number of studies has shown either exaggerated, normal or decreased isometric contraction of cholinergic stimulation on isolated asthmatic airways[6,23,35,99,100,129]. Different results between various studies are probably due to differences in patient selection and tissue processing. *In vitro* hyperresponsiveness of asthmatic airway muscle may well depend on smooth muscle hypertrophy[23]. It seems likely that the degree of muscle hypertrophy as it is commonly seen in asthma[31] will increase the constriction which results from a given level of cholinergic stimulation. This is clearly different from chronic obstructive pulmonary disease (COPD), where muscle hypertrophy is much less prominent than in asthma especially in central airways[31,52].

Smooth muscle hypertrophy will not only increase the force of the airway muscle, but it will also contribute to thickening of the airway wall[51] (see *Chapter* 2.9). According to Moreno's equation this will greatly amplify the effect of smooth muscle shortening on airway resistance and this might be an important factor in asthmatic airway hyperresponsiveness.

Little is known about changes in the elastic properties of the airway wall and the parenchyma (i.e. pre- and afterload) in asthma.

There is conclusive evidence that *in vitro* patients with COPD have a normal airway smooth muscle responsiveness to cholinergic stimulation[24-26,120]. *In vitro* concentration-response curves to methacholine are similar in airways from patients with and without COPD, and in patients with COPD there is no relation between the maximal bronchoconstriction *in vivo* and that *in vitro*[25]. As pointed out above this discrepancy may be caused by differences in afterload on the smooth muscle which may exist between patients with and without COPD *in vivo* and which are not mimicked *in vitro*.

7.9. References

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Chapter 8

Aims of the Studies

8.1. Introduction

From the foregoing chapters it seems clear that airway hyperresponsiveness is due to a defective regulation of airway caliber. *Figure* 8.1 shows how inflammation of the airway wall, which probably causes the pathological changes in asthmatic airways, could lead to hyperresponsiveness of the airways to different stimuli. To establish that any one of these pathological changes actually contributes to hyperresponsiveness of the airways in asthma it is necessary to demonstrate that such a change, on its own or in combination with other changes, increases the responsiveness of the airways. This requires a valid model for airway responsiveness in humans. The development of such a model has proven difficult. Animal models may provide a reasonable approach but the interpretation of

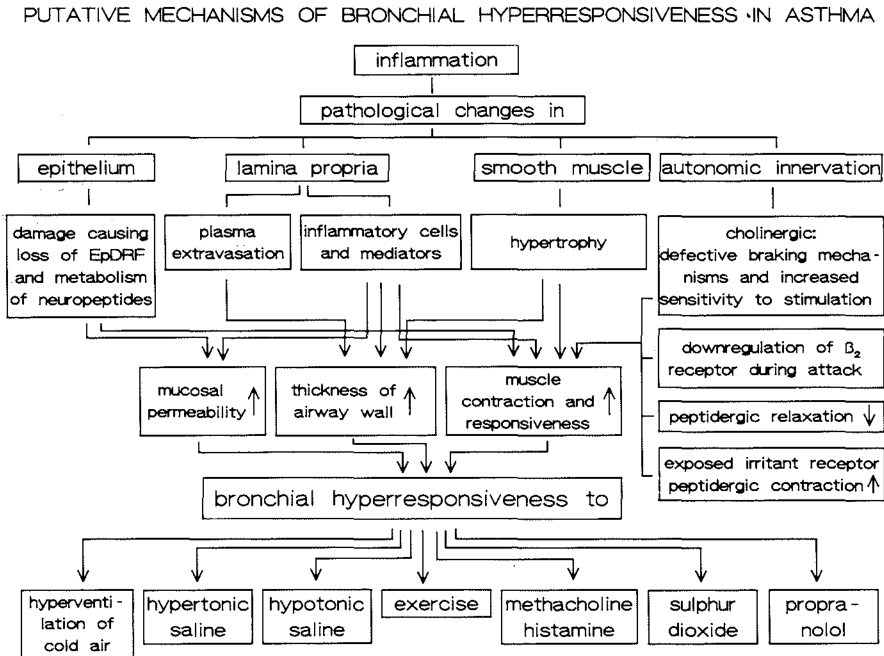

Figure 8.1. Mechanisms that may be involved in pathogenesis of bronchial hyperresponsiveness in asthma. *Abbreviations*: EpDRF, epithelium derived relaxing factor(s).

the results is problematic. Firstly, because asthma does not occur in animals and secondly because of important differences between species[33,35]. Another approach, which does not have these disadvantages, is to study isolated human airways. In the following paragraphs the validity of such an *in vitro* model of human airway responsiveness is discussed and the questions to be answered with this model are further developed.

8.2. Validity of the *in vitro* model

To investigate possible intrinsic abnormalities of the airway smooth muscle in bronchial hyperresponsiveness De Jongste *et al.* have previously validated an *in vitro* method to measure human airway smooth muscle function[15]. They found that the responsiveness of strips of human airways could be measured accurately and reproducibly, both isometrically and isotonically[9-11]. In addition, they found that electric field stimulation selectively stimulates intramural postganglionic nerve endings in isolated human airways[12]. This allows for the *in vitro* evaluation of factors that may interact with the regulation of airway muscle by these nerve endings.

Using the above methods De Jongste *et al.* failed to find differences between the *in vitro* responses to methacholine and electric field stimulation of airways from subjects with and without chronic obstructive pulmonary disease (COPD)[13,17]. This indicates that in COPD airway hyperresponsiveness to cholinergic stimulation of the airways is not the consequence of abnormalities in airway smooth muscle function or its regulation and that airway hyperresponsiveness in COPD is primarily due to factors which apparently do not influence the function of isolated bronchi in the organ bath such as mechanic (decreased elastic recoil) or geometric (changes in wall thickness) factors[14]. The apparent absence of abnormalities in airway smooth muscle function or its regulation in COPD suggests that it is permissible to use mixed populations of airways from both groups for *in vitro* studies when investigating factors that may alter airway smooth muscle function or its regulation.

8.3. Improving the *in vitro* model

In many studies on isolated human airways strips of human airways have been used[9]. This has several disadvantages. Firstly, the use of airway strips is inefficient. Spiralizing an airway strip requires an intact airway tube of considerable length and this limits the number of preparations that can be obtained from a given tissue specimen. Secondly, when spiralizing intact airways into strips the structure in which the airway muscle is embedded and which forms a pre- and an afterload for the airway muscle (see *Chapters* 7.5 and 7.6) is disrupted. Thirdly, it is not known how contraction of airway strips relates to airway narrowing, due to

the helicoidal orientation of the muscle in the airway wall[4,26]. It could be reasoned therefore, that the use of bronchial rings instead of strips may improve the *in vitro* model. With bronchial rings less tissue is needed, the structure in which the airway muscle is embedded is less damaged and the responses that are measured directly relate to airway narrowing.

In order to make a valid comparison between responses from different isolated airways it is necessary to know if the airway muscle in the different airway preparations is stretched to similar extent. For this to happen the preload against which the airways contract should stretch the airway muscle to or near its optimal length (see *Figure* 7.4)[34]. However, studies into the optimal preload for isolated human airways are lacking.

8.4. Mechanisms of airway hyperresponsiveness in asthma

Using the *in vitro* model De Jongste *et al.* have searched for abnormalities in the airway smooth muscle from patients with asthma. It proved very difficult to find such abnormalities since airways from asthmatics are only rarely available for *in vitro* experiments. In the one asthmatic specimen that de Jongste *et al.* investigated they found increased maximal (isometric) responses to contractile stimuli[16]. This finding has recently been confirmed on several other tissue specimens from asthmatics by Bai *et al.*[3]. The most likely explanation for this finding seems to be that the smooth muscle hypertrophy that is commonly seen in asthmatic airways increases the maximal force that the airway can generate. The stronger muscle will more easily overcome the elastic recoil forces that oppose bronchoconstriction, and this may allow excessive narrowing of the airways to occur[14,18].

If smooth muscle hypertrophy were to be the only abnormality involved in asthmatic bronchial hyperresponsiveness we would expect bronchial hyperresponsiveness to be invariable over short time periods, since smooth muscle growth will take considerable time. However, there are several stimuli that cause short term changes in bronchial hyperresponsiveness in asthmatics. Bronchial hyperresponsiveness increases shortly before or at the onset of the late asthmatic reaction[28] or after inhalation of hypotonic saline[6,32]. During exercise the responsiveness to methacholine decreases[23]. Therefore it seems likely that other factors than muscle hypertrophy are also involved in the pathogenesis of bronchial hyperresponsiveness in asthma (see *Figure* 8.1).

8.5. Inflammation

As described in *Chapter* 2.9.1 and summarized in *Figure* 8.1 there is evidence for inflammation in the airways of stable atopic asthmatics. In these patients inhalation of aeroallergens may lead to an early and late fall in FEV_1. The

onset of the late asthmatic reaction is associated with an acute increase in bronchial hyperresponsiveness and an influx of inflammatory cells, predominantly granulocytes, into the airway wall[8,19-21,25,30]. Furthermore in biopsy studies and studies with bronchoalveolar lavage (BAL) fluid from stable asthmatics several interesting correlations have been found (see *Figure* 8.2). The severity of bronchial hyperresponsiveness to inhaled methacholine is related to the number of epithelial cells and mast cells and to the amount of histamine, PGD_2, $PGF_{2\alpha}$ and TxB_2 in the BAL fluid[22,24,36]. Inhalation of aeroallergens leads to the release of the aforementioned mediators[37,38] whereas prolonged allergen avoidance leads to a decrease of bronchial hyperresponsiveness[29]. The above correlations suggest a causal relationship between bronchoconstriction or changes in bronchial hyperresponsiveness on the one side and the release of inflammatory mediators by mast cells, damage of the airway epithelium and the influx of granulocytes on the other side. Little is known, however, about the effect of inflammatory mediators, epithelial damage, mast cell activation and granulocytes on human airway muscle function or its regulation.

8.6. Physical stimuli

An important feature of airway hyperresponsiveness in asthma is its nonspecificity[1]. There are many stimuli which only seem to cause airway narrowing

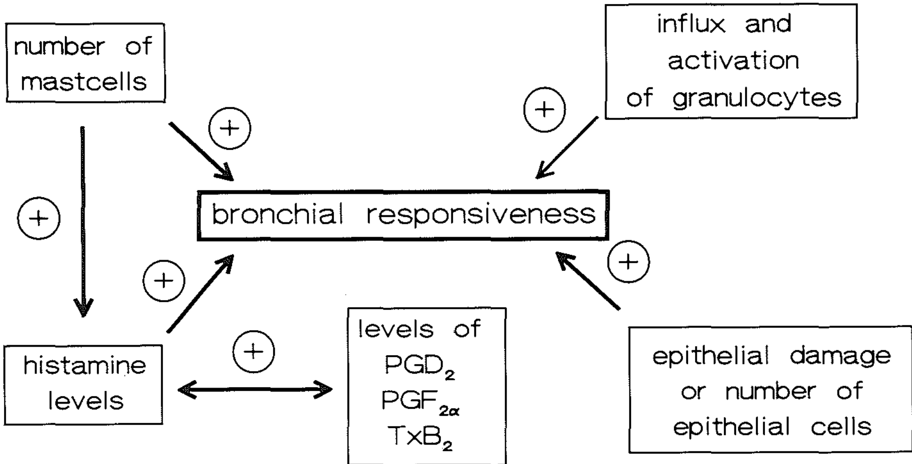

Figure 8.2. Correlations observed in studies on biopsies or bronchoalveolar lavage fluid from stable asthmatics[5,22,24,36]. *Abbreviations*: BAL, bronchoalveolar lavage fluid; PG, prostaglandin; Tx, thromboxane.

in asthmatics but not normals. Examples are cooling of the airways through isocapnic hyperventilation of cold dry air and inhalation of hypo- or hypertonic saline[2,7,27,31]. In view of the smooth muscle hypertrophy that is seen in asthma it seems reasonable to assume that if these stimuli lead to smooth muscle stimulation, asthmatics will be hyperresponsive to these stimuli. The problem is that it is not known how these stimuli interact with human airway muscle function or its regulation.

8.7. Aims of the studies

From the above we can conclude that:

1. There are several disadvantages to the use of bronchial strips for *in vitro* studies. With bronchial rings less tissue is needed and the *in vitro* situation might be closer to the *in vivo* one. However, studies that compare these types of preparations and studies into the optimal preload for such preparations are lacking.

2. The association between the onset of the late asthmatic reaction (i.e. bronchoconstriction), the influx of granulocytes into the airway wall, and an increase in bronchial responsiveness suggests that granulocytes might contribute to the late asthmatic reaction and the concomitant increase in bronchial responsiveness, but the effects of activated human granulocytes on human airways are not known.

3. In stable asthmatics the degree of bronchial responsiveness is related to the number of epithelial cells and mast cells as well as to the concentrations of inflammatory mediators in the BAL fluid (see *Figure* 8.2). Therefore it could be that inflammatory mediators, epithelial damage and mast cell activation alone or in combination alter the responsiveness of human airway muscle.

4. It is poorly understood if and how physical stimuli such as cooling or inhalation of hypo- or hyperosmolar saline interact with human airway muscle function or its regulation. Characterization of the effect of these stimuli on human airways might improve the understanding of why asthmatics are hyperresponsive to these stimuli.

The studies in this thesis address the following questions:

1. Is it possible to measure airway sensitivity and contractility accurately and reproducibly in segments of human airways? How do the responses of these

segments compare to those of strips? What is the optimal preload for these preparations?

2. What is the effect of activated human granulocytes on isolated human airways? Do they alter the cholinergic responsiveness of the airways? Do they interfere with the function of the autonomic innervation? Do they alter airway caliber, and if so, by what mechanism(s)?

3. Do mast cell-derived inflammatory mediators increase the cholinergic responsiveness human airways? Considering the epithelial damage that occurs in asthma, is the effect of these mediators similar in airways with and without epithelium? Does the activation of mast cells also increase the responsiveness of isolated human airways and is the effect similar in airways with and without epithelium?

4. If inflammatory mediators increase the cholinergic responsiveness of human airway muscle how would such a change in airway responsiveness *in vitro* translate into a change in airway responsiveness *in vivo*?

5. What is the effect of cooling, hypo- and hyperosmolarity on isolated central and peripheral human airways? Do these stimuli interfere with cholinergic responsiveness of the airways and with the function of the autonomic innervation and, if so, by what mechanism(s)?

The aspects of bronchial hyperreponsiveness that were studied in this thesis are schematically summarized in *Figure* 8.3 which is based on the schematic representation given in *Figure* 8.2

8.8. Note on pulmonary tissue used for the studies

The aim of this study was to gain more insight in the pathogenesis of various aspects of bronchial hyperresponsiveness in asthma. For this purpose it would have been optimal to use airways from asthmatics. However, pulmonary tissue from asthmatics is only rarely available for *in vitro* studies. The pulmonary tissue used for the studies described here was usually obtained from (ex) smokers who were operated for bronchial carcinoma. According to the criteria of the American Thoracic Society some 50% of the tissues that we studied was from patients with COPD. Naturally, this may have consequences for the interpretation of the results and this is discussed in *Chapter* 17. In order to evaluate the possible consequences of the use of airways from smokers the introduction has not only described the structure of human airways and their regulation in normals and asthmatics but also in patients with COPD.

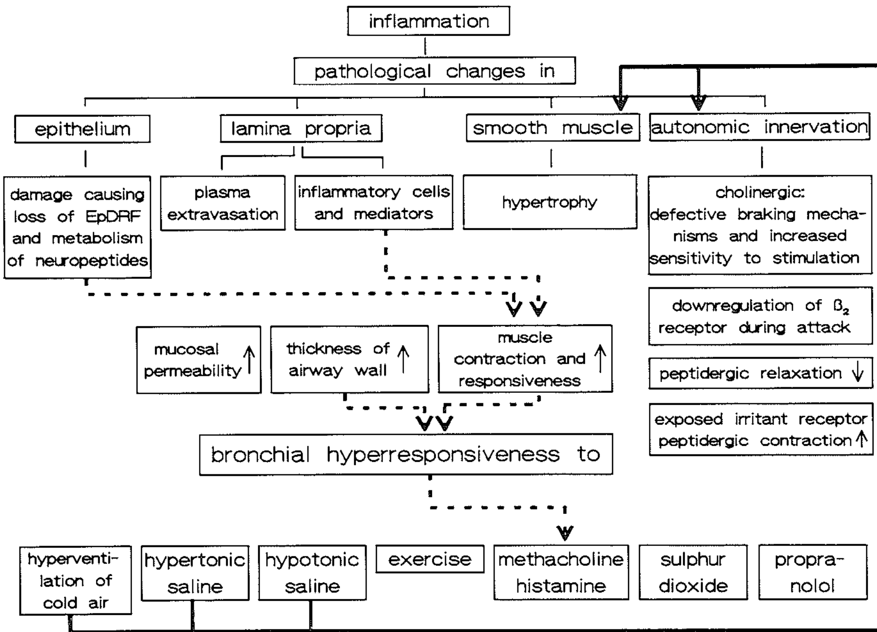

Figure 8.3. Schematic representation of aspects of bronchial hyperresponsiveness that were studied in this thesis. The dotted lines refer to the studies of *Part* III and the continuous lines to the studies of *Part* IV. *Abbreviations*: EpDRF, epithelium derived relaxing factor(s).

8.9. References

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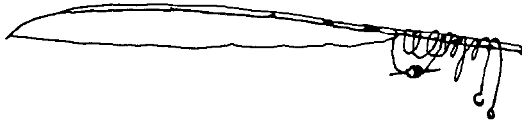
Part II

Methods



Chapter 9

Measurement of Human Small Airway Smooth Muscle Function *in Vitro*: Comparison of Bronchiolar Strips and Segments*

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9.1. Summary

We have compared isotonic responses to methacholine of human bronchiolar segments and spiral strips. Both types of preparations contracted dose-dependently to methacholine, and had a stable intrinsic contractile activity, which was significantly higher in segments ($P<0.001$). ANOVA indicated that the total variation in responses of both strips and segments was similar, and was mainly due to a significant between-preparations/within-patients variation. There was a small but significant trend towards a decrease of sensitivity (EC_{50}) in time for both segments and strips. Net contraction, i.e. the difference between resting length and the length at maximal contraction, did not change in time. Limited length-active shortening experiments indicated that 250 mg was a suitable load for both strips and segments. We concluded that, although human bronchiolar strips and segments are functionally comparable, bronchiolar segments are preferable because of their practical and theoretical advantages over bronchiolar strips.

Key words: airway smooth muscle, human bronchiolar strip, human bronchiolar segment, methacholine.

9.2. Introduction

Different types of human airway smooth muscle preparations have been used

* Jongejan RC, de Jongste JC, van Strik R, Raatgeep HC, Bonta IL, Kerrebijn KF. Measurement of human small airway smooth muscle function in vitro, comparison of bronchiolar strips and segments. J Pharmacol Meth 1988; 20:135-142. Printed with permission of Elesevier Science Company.

for *in vitro* research over the years: slices of lung tissue[9], bronchial tubes and spirals[13], strips of lung parenchyma[7] and bronchiolar strips[3]. These preparations serve as a model to gain insight in the factors that determine bronchial smooth muscle tone *in vivo*. Such a model should yield results that are reproducible and discriminative.

We have previously reported on the reproducibility of *in vitro* contractile responses from human bronchiolar strip preparations and found a considerable within-subject variability of maximal responses, which was attributed, amongst others, to the spiral cutting of the bronchioles[4].

Recently we have further developed another preparation, the bronchiolar segment[8] which, apart from practical and theoretical advantages over bronchiolar strips, does not require spiral cutting and might therefore give better results.

In the present study we have compared the reproducibility and variability of various parameters of functioning of bronchiolar segments and strips, obtained from the same specimens of lung tissue.

9.3. Methods

Human lung tissue was obtained from patients who had undergone a thoracotomy because of bronchial carcinoma. Within 30 minutes after surgical resection, a macroscopically normal part of the resected tissue was immersed in ice-cold Krebs-Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 5.55), which had been aerated with carbogen (95% O_2, 5% CO_2) to produce a pH 7.35, a Pco_2 of 4.66 kPa and a Po_2 of 71.98 kPa.

After prompt transportation of the lung tissue to the laboratory, bronchioles with a diameter of 1 to 2 mm were identified and dissected as described in detail previously[5]. The bronchiole was either cut spirally in order to obtain a strip of around 15 mm length or was cut into segments with a diameter of 1 to 2 mm. Thin surgical silk threads (6/0) were tied to the ends of the strip. Segments were mounted between two small polished stainless steel hooks (diameter 0.3 mm), modified from[10].

Three strips and 3 segments from each of 7 lung-tissue specimens were mounted in double jacketed organ baths containing aerated Krebs-Henseleit buffer at 37°C (see *Figure* 9.1). One thread was attached to a glass hook at the bottom of the bath and the other was attached to the arm of a high precision isotonic angular position transducer (Penny and Giles, 3810/60, Great Britain)[11] (see *Figure* 9.1) which was connected to a digital voltmeter (Fluke 73 multimeter, USA) and a pen-recorder (Kipp BD 40, The Netherlands). The preparations were stored overnight at 4°C. The next day, preparations were rewarmed to 37°C and stimulated twice with methacholine (10^{-5} M, 10^{-4} M) against an isotonic

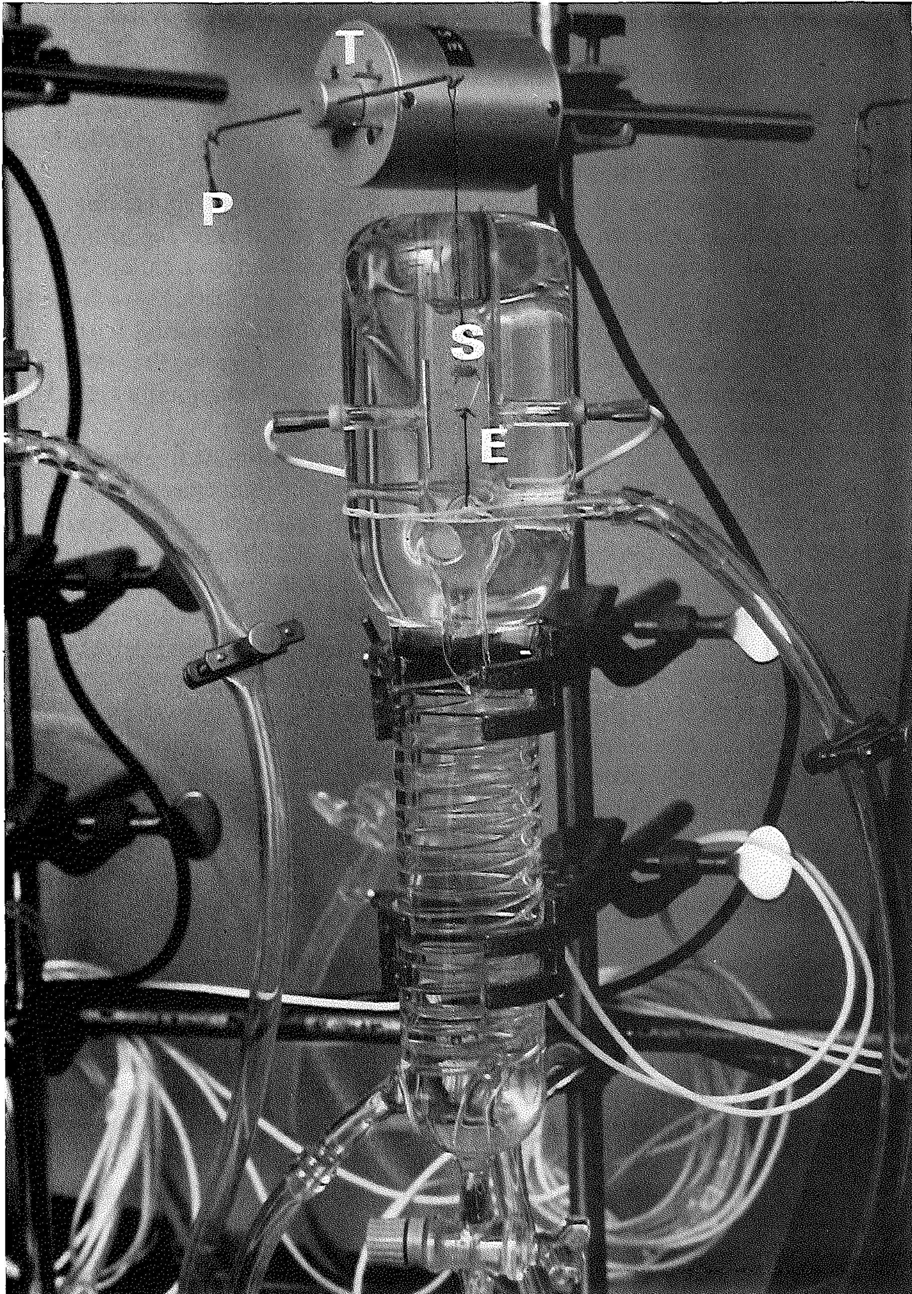

Figure 9.1. Bronchiolar segment (S) mounted in an organ bath. The segment is attached to an angular position transducer (T), which is loaded with a preload (P). The platinum plate electrodes for electric field stimulation (E) (see *Chapters* 13.3.7, 14.3.3 and 16.3.4) are also shown.

load of 250 mg, followed by wash-out, in order to obtain a stable function for the rest of the day[5].

After re-establishment of resting length, 3 consecutive Cumulative Concentration Response Curves (CCRC) (10^{-8} M to 10^{-4} M) to methacholine were made on each preparation, with an interval of 1.5 hour, to asses the reproducibility in time of the contractile responses of strips and segments.

To measure the effect of different loads on the maximal shortening (S_{max}) of each strip and segment, a limited lengthactive shortening experiment was done after each series of CCRC. To this purpose the load was changed to 125 mg and, after stabilization, preparations were contracted maximally with methacholine (10^{-5} M, 10^{-4} M). After washing and stabilization of resting length, this procedure was repeated with loads of 250 and 500 mg. Finally, the initial load of 250 mg was re-established and the preparations were maximally relaxed by adding L-isoprenaline (10^{-4} M) and EDTA (4×10^{-3} M).

From each CCRC the following parameters were obtained: $-\log EC_{50}$: the negative logarithm of the methacholine concentration that caused 50% of the maximal contraction (EC_{50}); S_{max}: the maximal response of a preparation expressed in mm shortening; the net contraction: the difference between the length at S_{max} and the resting length in mm and the spontaneous intrinsic contractile activity of a preparation: the difference in mm between resting length and maximal relaxation in the presence of L-isoprenaline and EDTA. The responses to 10^{-4} M methacholine during length-shortening experiments were expressed as a % of the highest response at any load ($S_{max\%}$). To examine whether the spontaneous resting length had changed during the experiments all S_{max} values and the resting length were related to the maximal relaxation in the presence of EDTA and L-isoprenaline.

9.4. Statistical methods

CCRC were constructed using the BMDP software module for non-linear curvefitting[2]. The curves were fitted to a four parameter logistic function[6]. The distributions of S_{max}, $-\log EC_{50}$, resting length and net contraction values were close to normal. For each individual specimen the residual variances of strip and segment parameters were computed using two-way analysis of variance (ANOVA)[14]. For all specimens, these variances were compared using Wilcoxon's signed rank test (two-tailed, $\alpha = 0.05$). This revealed that the residual variances of the parameters for strips and segments within a given specimen were comparable. The contributions of different factors to the variation in the data were then analyzed via a three-way ANOVA. This ANOVA was interpreted according to the model specified in *Table* 9.1. The variance components were calculated for between patients, between-preparations/within-patients, time (= order of measurement), time x patients and residual variability. A similar procedure was followed for the analysis of the influence of different loads on

Table 9.1. Sources of variation

	source of variation	degrees of freedom
1	between-patients	6
2	between-preparations/within-patients	2
3	time = order of measurement	2
4	time x patients	12
5	time x between-preparations/within-patients	4
6	between-preparations/within-patients x patients	12
7	residual	24
8	total	62

Statistical model of possible sources of variation. Time was considered as fixed, patients and preparations were considered as random. For analysis of the influence of different loads a similar model was used with the factor time replacing the factor load.

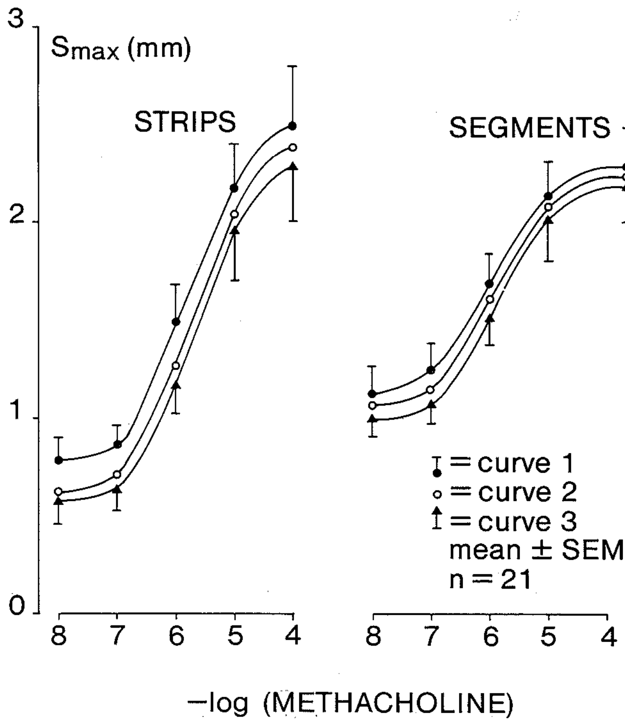

Figure 9.2. *In vitro* CCRC of human bronchiolar strips (n = 21) and segments (n = 21), measured in triplicate with intervals of 1.5 hours. The vertical axis depicts the shortening in mm from the point of maximal relaxation, in the presence of L-isoprenaline (10^{-4} M) and EDTA (4×10^{-3} M), obtained at the end of each experiment.

$S_{max\%}$. The statistical significance of the mean squares was evaluated according to *Table* 9.1 using the F-test at $\alpha = 0.05$. This means that item 1 was compared to (2 + 6), 2 to (5 + 7), 3 to (4 + 6) and 4 to (7 + 5). From these F-ratios, 95% confidence intervals for the net variation coefficients were calculated. Net coefficients of variation are expressed as a percentage of the overall mean of a given parameter.

9.5. Results

Seven lung tissue specimens were obtained from 7 patients, 6 male and 1 female, with a mean age of 62 years (range 36 to 75 years). None had a history of asthma; 5 were smokers, 3 had chronic obstructive pulmonary disease[1]. Pre-operative lung function: Vital Capacity (VC), 4075 ± 370 ml; forced expiratory volume in 1 second as a percentage of VC, 64 ± 4.2% (mean ± SEM). Medication during anesthesia was the same for all patients: atropine, thiopental, fentanyl, O_2/N_2O, halothane and pancuronium.

All strips and segments contracted dose-dependently to methacholine. Mean curves are presented in *Figure* 9.2. The outcomes of the ANOVA are given

Table 9.2. Comparison of strips and segments (ANOVA)

		net variation coefficients and their 95% confidence intervals[a]			
		$-\log EC_{50}$		S_{max}	
		strips	segments	strips	segments
between-patients	co var	3.1**	3.8**	0	0
	95% CI	2.0 – 5.2	1.5 – 9.8	0 – 58.7	0 – 45.2
between-preparations/ within-patients	co var	5.3**	3.1**	56.6**	46.0**
	95% CI	2.7 – 12.4	1.6 – 5.7	26.4 – 91.7	29.6 – 74.8
time	co var	2.7**	1.7**	6.3**	2.1**
	95% CI	–	–	–	–
time x patients	co var	0	0	0	0
	95% CI	0 – 1.6	0	0	0 – 1.9
residual	co var	1.4	3.1	3.0	2.9
	95% CI	1.2 – 1.6	2.7 – 3.6	2.7 – 3.6	2.5 – 3.4

Abbreviations: 95% CI, 95% confidence limits; co var, coefficients of variation; $-\log EC_{50}$, the negative logarithm of the methacholine concentration that caused 50% of the maximal contraction (EC_{50}); S_{max}, the maximal response of a preparation expressed in mm shortening; ANOVA, analysis of variance.

** $P<0.01$.

[a] Net coefficients of variation and interval limits are expressed as percentage of the overall mean.

in *Table* 9.2 as the net variation coefficients and their 95% confidence intervals. It can be seen that the variability in the responses of strips and segments is fully comparable, because the confidence intervals largely overlap. For both types of preparations the between-preparations/within-patients component is responsible for a major part of the total variation. In this small series, no significant between-patient variation was present for S_{max}. In *Figure* 9.2 the effect of time on the mean CCRC of strips and segments is shown. The $-logEC_{50}$ to methacholine decreased slightly but significantly in time both for strips and segments (see *Table* 9.2). S_{max} decreased and the resting length increased significantly in time (see *Table* 9.2). The net contraction was stable in time. Segments had a significantly higher spontaneous intrinsic contractile activity than strips (t-test, $P < 0.001$).

In *Figure* 9.3 the mean $S_{max\%}$ values of strips and segments under different loads are depicted. ANOVA revealed that for strips there was a significant decrease in response with increasing load for all subjects, although the strength, but not the direction, of this relation differed significantly between and within subjects.

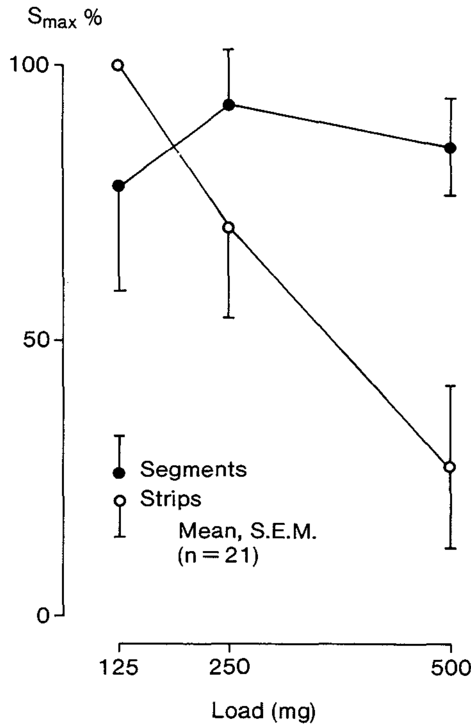

Figure 9.3. Maximal responses of human bronchiolar strips (n = 21) and segments (n = 21) with increasing isotonic loads. The same preparation was consecutively submitted to three different loads (horizontal axis). The vertical axis depicts mean maximal responses, expressed as % of the highest response at any of the three loads ($S_{max\%}$)

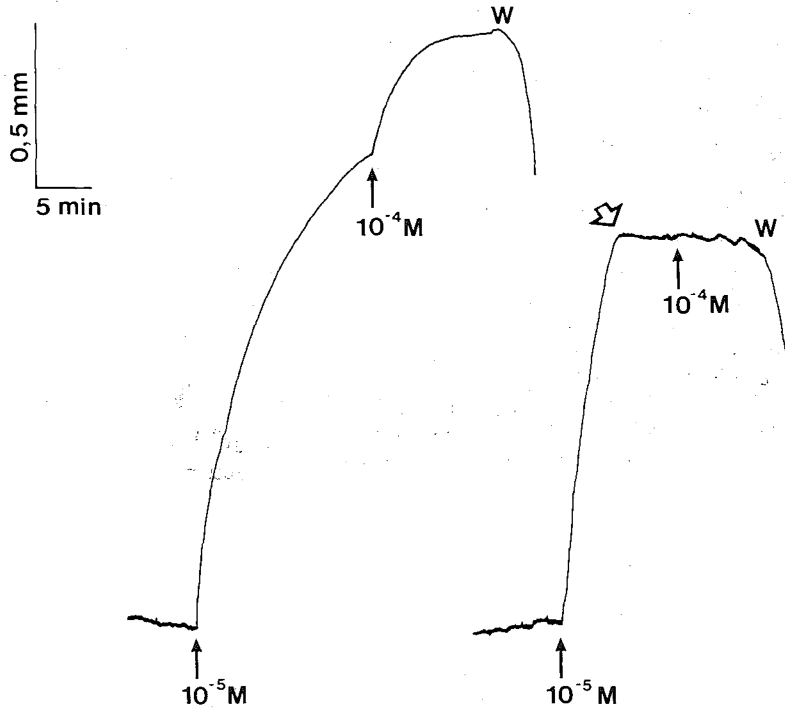

Figure 9.4. Example of a normal recording of a contracting segment (left) and a recording (right) of a segment where 10^{-5} M methacholine caused luminal narrowing to such a degree that at the point indicated by the open arrow, the two hooks in the lumen made contact and impaired further narrowing. Small arrows indicate where methacholine (10^{-4} M and 10^{-5} M) was added.

For segments, the results of the ANOVA indicated that the relation between load and response differed significantly between subjects.

In 3 of the 21 segments, the highest methacholine doses caused luminal narrowing to such a degree that the two hooks made contact, and impaired further contraction. This only occurred with a load of 125 mg during the limited length-shortening experiment, and produced a typical contraction pattern, as shown in *Figure* 9.4.

9.6. Discussion

We found that responses to methacholine of human bronchiolar segment and strip preparations were similar. $-\log EC_{50}$ and S_{max} showed a small but statistically significant trend towards a decrease in time for both types of preparations. These trends could reach statistical significance because of the very low residual, that is unexplained, variability. Because net contractions did not change in time the

decrease of S_{max} in time is probably due to an increase in resting length in time (see *Figure* 9.2). This suggests that some stretching of elastic components might have occurred, while smooth muscle contractility remained stable. Segments had a higher intrinsic contractile activity than strips. It seems possible that the different dissection procedures cause the differences in spontaneous contractile activity.

The variability of the responses of isolated airways is largely dependent on the quality of the dissection procedure and the properties of the lung before dissection. The first factor is most likely to increase the between-preparations/within-patients variability. The second factor will mainly influence the between-patients variability. The between-preparations/within-patients variability was the major determinant of the total variation in the responses of both segments and strips. This indicates that the spiral cutting of strips was not responsible for the between-strips/within-patients variation of strips.

The maximum isotonic shortening (S_{max}) of the preparation depends on the load applied. As the length-shortening curve is a parabola with one maximum[15], the optimal load seems to be about 250 mg for segments and may even be below 125 mg for strips (see *Figure* 9.3). In our system a 250 mg load, which is considerably lower than the loads commonly used, seems a good compromise between optimal load and mechanical stability for both strips and segments.

ANOVA revealed significant between-subject differences both for strips and segments in their length-shortening characteristics. This deserves more detailed study, because it has been suggested that such differences might be of importance in the pathogenesis of asthma and COPD[12].

The aim of an *in vitro* research model is to mimic the *in vivo* situation as closely as possible. In this sense bronchiolar segments have theoretical advantages over bronchiolar strips because their contraction relates directly to luminal narrowing. In addition, the segment method does not interfere with the configuration of the smooth muscle bundles in the bronchioles.

A practical advantage of the segment method is, that less tissue is needed. Furthermore, a smaller size of bronchioles can be used because spiral cutting is not necessary. A disadvantage of segments is that the hooks in the lumen may impair muscle contraction. However, we only found this with a load of 125 mg. The phenomenon was always easily recognizable (see *Figure* 9.4).

In *summary* we have found that human bronchiolar segments and strips have a fully comparable variation pattern in their isotonic responses *in vitro*. However, segmental preparations have several theoretical and practical advantages over strips, and may therefore be preferable for the study of human airway smooth muscle function *in vitro*.

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Part III

Inflammation

Chapter 10

Effects of Inflammatory Mediators on the Responsiveness of Isolated Human Airways to Methacholine*

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10.1. Summary

Several studies have suggested that in asthmatics the quantities of inflammatory mediators such as histamine, thromboxane A_2 (TxA_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and leukotriene C_4 (LTC_4) which are present in the airway lumen are related to the degree of bronchial responsiveness to inhaled methacholine (Mch). Therefore we studied the effect of these mediators on the cholinergic responsiveness of isolated human airway segments. Lung tissue collected at thoracotomy from 30 patients was studied. Dose-response curves to Mch were obtained from bronchial segments before, during and after incubation with either a subthreshold or a threshold concentration of histamine (10^{-10} M or 10^{-8} M), the stable TxA_2 analogue U46619 (10^{-11} M or 10^{-9} M), PGD_2 (5 x 10^{-9} M or 5 x 10^{-7} M), $PGF_{2\alpha}$ (10^{-9} M or 10^{-7} M) or LTC_4 (10^{-11} M or 10^{-9} M). With the exception of LTC_4, the presence of any of these mediators at either concentration, increased the sensitivity to Mch by a factor 1.1 to 2 ($P<0.05$, ANOVA). This increase did not depend on the dose of the mediator ($P>0.05$, ANOVA). These data indicate that mediator-induced muscle hypersensitivity can explain a small part of the leftward shift of the dose response curve to inhaled methacholine as observed in asthma.

Key words: human airway muscle, bronchial hyperresponsiveness, inflammatory mediators

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* Jongejan RC, de Jongste JC, Raatgeep HC, Stijnen T, Bonta IL, Kerrebijn KF. Effects of inflammatory mediators on the responsiveness of isolated human airways to methacholine. Am Rev Respir Dis 1990; 142: 1129-1132. Printed with permission of the American Review of Respiratory Disease.

10.2. Introduction

In allergic asthmatics the increase in bronchial responsiveness following allergen inhalation relates to an inflammatory reaction in the airway wall[20]. Several studies suggest that the quantities of inflammatory mediators such as histamine, thromboxane A_2 (TxA_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and leukotriene C_4 (LTC_4) present in the airway lumen are related to the degree of bronchial responsiveness[6,12,18]. Most of these relations were found by analyzing bronchoalveolar lavage fluid obtained from asthmatics during symptom-free periods[6,12]. This means that, if inflammatory mediators cause an increased airway responsiveness to inhaled methacholine (Mch), they do so in low concentrations which do not lead to bronchoconstriction.

The effect of low concentrations of inflammatory mediators on the responsiveness of isolated airways has been investigated in several species. Potentiation of the response to low concentrations of acetylcholine by a threshold concentration of histamine has been reported in canine trachealis[13]. In the same species others found that a threshold concentration of PGD_2 did not alter the sensitivity of the airway muscle to exogenous acetylcholine[23]. In the dog, the stable TxA_2 analogue U46619 does not affect the responses to acetylcholine[21], but in isolated rabbit bronchi this mediator potentiates the contractile responses to acetylcholine[3]. $PGF_{2\alpha}$ potentiates the contractile response to exogenous acetylcholine in isolated segments of the rabbit trachea[4]. Trachealis from mongrel dogs does not contract to LTC_4/D_4[21], and no interactions were found between these mediators and acetylcholine. In contrast guinea pig isolated airway smooth muscle does contract to LTC_4/D_4, and LTD_4 (10^{-8} M and 10^{-7} M) significantly enhanced contractions induced by acetylcholine but only if the calcium concentration in the organ bath was reduced from 2.5 mM to 0.1 mM[7]. The physiological relevance of this finding is therefore not clear. As these animal studies have shown potentially important interactions, but also marked differences between species, it seemed relevant to examine the interaction between mediators and a cholinergic agonist using human airway tissue. Therefore we have investigated whether low concentrations of histamine, U46619, PGD_2, $PGF_{2\alpha}$ and LTC_4 increase the responsiveness of isolated human bronchi to Mch.

10.3. Methods

10.3.1. Patients, airway preparations and experimental protocol

Human lung tissue was obtained from patients who underwent a thoracotomy because of a pulmonary malignancy. Within 30 minutes after surgical resection a macroscopically normal part of the resected tissue was immersed in Krebs-Henseleit buffer of room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 5.55), which had

been aerated with carbogen (95% O_2, 5% CO_2) to produce a pH of 7.35, a Pco_2 of 4.7 kPa and a Po_2 of 71.8 kPa. The tissue remained in fresh aerated buffer throughout the dissection procedure and the experiments. On the cut surface, non-cartilaginous airways with a diameter of 2 to 3 mm after full relaxation (6th to 11th generation)[25] were identified, cannulated, taken out and dissected free from parenchyma and vessels under a stereo microscope (20x magnification) using iris-scissors and forceps. The cleaned airway was cut into segments 3 to 4 mm long which were stored overnight in a slow flow of aerated buffer of 4°C, containing penicillin (3 x 10^{-5} g/l) and tobramycin (5 x 10^{-3} g/l). We formerly demonstrated that this storage procedure does not affect the contractility of the preparations to Mch[8]. The next day the segments were mounted between two small polished stainless steel hooks (diameter 0.3 mm), and placed between a glass hook at the bottom of a double jacketed 10 ml organ bath and a high precision isotonic angular position transducer (Penny and Giles, 3810/60, Great Britain). The transducer was connected to a digital voltmeter (Fluke 73 multimeter, USA) and a pen-recorder (Kipp BD 40, The Netherlands). This method has been described in detail previously[8,16]. The preparations contracted against an isotonic load of 250 mg which has been shown to be optimal for human bronchial segments[16].

During an equilibration period of 2 hours at 37°C, the preparations were contracted twice with Mch (10^{-5} M, 10^{-4} M), to assess their contractile function. Next, three consecutive cumulative concentration response curves (CCRC) to Mch (10^{-8} M to 10^{-4} M) were obtained from each preparation: one before addition of a mediator, one in the presence of the mediator 15 minutes after its addition, and one after washout of the mediator. As the effect of PGD_2 seemed difficult to wash out, we did 5 additional experiments with prolonged washing (8 times during 60 extra minutes) after exposure to PGD_2, before generating the third curve.

The concentrations of mediators that we used were determined from our previous studies[26]: the threshold concentration (i.e. the concentration in the organ bath at which the preparation just started to contract), and a 100-fold lower concentration. For histamine these two concentrations were 10^{-10} M and 10^{-8} M; for U46619: 10^{-11} M and 10^{-9} M, for PGD_2: 5 x 10^{-9} M and 5 x 10^{-7} M; for $PGF_{2\alpha}$: 10^{-9} M and 10^{-7} M and for LTC_4: 10^{-11} M and 10^{-9} M.

Control experiments were done where three consecutive CCRC's to Mch were obtained without addition of a mediator.

To evaluate whether the slight precontraction caused by the threshold concentration of a mediator per se altered the sensitivity to Mch, we did a separate set of experiments where the second CCRC was made after precontraction with a threshold concentration of Mch (5 x 10^{-7} M).

10.3.2. Data analysis

The contractile state of the preparations was expressed on a scale which defines the maximal active contractile range (MACR)[19]. The second response to a bolus dose of Mch 10^{-4} M, obtained before the first CCRC to Mch, was called 100% shortening. Maximal relaxation after 10^{-4} M isoprenaline and 4×10^{-3} M EDTA, determined at the end of each experiment, was called 0% shortening. On this scale the baseline contractile state (BCS) was defined as the spontaneous position of a preparation on the MACR scale under a 250 mg load. The potency of Mch was expressed as the negative logarithm of the Mch concentration that caused 50% of the maximal muscle shortening ($-\log EC_{50}$) starting from BCS.

The $-\log EC_{50}$ was calculated using the BMDP software module for non-linear regression[5]. The curves were fitted to a four parameter logistic function[10]. Values of $-\log EC_{50}$ and BCS were analyzed with a three-way analysis of variance (ANOVA) using the MANOVA program of SPSSx[22]. The dose of a mediator and the number of the curve (first, second or third curve) were taken as factors and different lung-tissues were taken as random factors. The relation between BCS in the absence of a mediator and $-\log EC_{50}$ was analyzed with a standard analysis of variance with lung tissues as groups and BCS as a covariable using the data from all the first CCRC, obtained before addition of a mediator. P values < 0.05 (twosided) were considered significant. All data are presented as mean $\pm$ SEM.

10.3.3. Drugs

Methacholine hydrobromide, histamine hydrochloride (Janssen Chimica, Belgium), EDTA (Sigma, USA) and LTC_4 (a gift from Merck Frosst Laboratories, Canada) were dissolved in saline. The stable TxA_2 analogue U46619 (9.11-dideoxy-$11_{\alpha},9_{\alpha}$-epoxymethano-prostaglandin $F_{2\alpha}$)(a gift from Upjohn, USA) was dissolved in absolute ethanol, and PGD_2 and $PGF_{2\alpha}$ (Sigma, USA) in 70% ethanol. L-isoproterenol (Janssen Chimica, Belgium) was dissolved in water containing ascorbic acid (88 mg/l). Preliminary experiments showed that ascorbate and ethanol in these concentrations have no effect on airway smooth muscle function. U46619, PGD_2, $PGF_{2\alpha}$ and LTC_4 were kept in −70°C and thawed just before use.

10.4. Results

Lung tissue specimens were obtained from 30 patients, 25 male and 5 female, with a mean age of 63.5 years (range 50 to 76 years). Preoperative lung function showed a mean value for the FEV_1 as a percentage of the IVC of 63.5 $\pm$ 3.5%. Of these patients 18 had chronic obstructive pulmonary disease (COPD) according to the criteria of the American Thoracic Society[1], but none had characteristics

of asthma. Medication during anesthesia was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane and pancuronium. 15 Patients received steroids and/or theophylline before and during the operation.

All preparations contracted dose-dependently to Mch. Addition of 10^{-9} M U46619, 5 x 10^{-7} M PGD_2, 10^{-7} M $PGF_{2\alpha}$, 10^{-9} M LTC_4 or 5 x 10^{-7} M Mch to the organ baths resulted in a similar significant increase of BCS of 9.4 ± 6.4%,

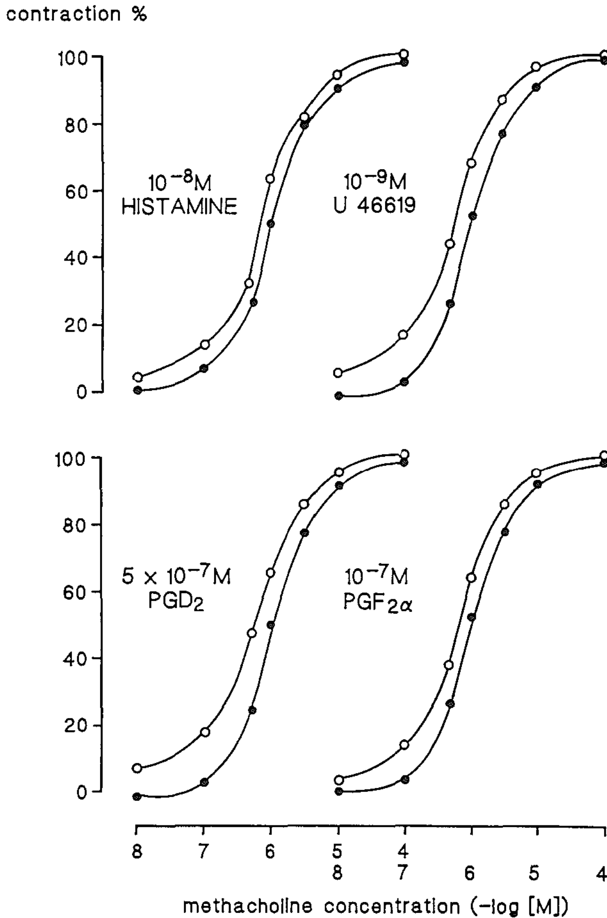

Figure 10.1. Mean CCRC to Mch of human airway segments during exposure to inflammatory mediators (open circles). The closed circles represent the second curve of control preparations in the presence of 5 x 10^{-7} M Mch. For PGD_2, the mean CCRC is shown of the preparations in which a long washing procedure was performed between the second and the third CCRC (see 10.3.1). The horizontal axis depicts the negative log of the Mch concentration in the organ bath, the vertical axis represents the contractile state of the preparations which is expressed as a % of the difference between the maximal contraction to Mch (10^{-4} M) and baseline contractile state before starting the Mch CCRC. For reasons of clarity SEM bars were omitted. The SEM varied from 2 to 7%. (n = 5 to 7).

10.2 ± 2.1%, 5.5 ± 2.3%, 7.7 ± 3.1% and 13.6 ± 2.9% MACR respectively. With 10^{-8} M histamine mean BCS increased 0.4 ± 1.7% (NS). The CCRC's in the presence of histamine (10^{-8} M), U46619 (10^{-9} M), PGD_2 (5 x 10^{-7} M), and $PGF_{2\alpha}$ (10^{-7} M) and controls are shown in *Figure* 10.1. *Table* 10.1 shows the mean values for $-logEC_{50}$ to Mch before, during and after exposure to the two concentrations of mediators together with those of controls. The presence of histamine, U46619, PGD_2 or $PGF_{2\alpha}$ at either concentration, caused a significant increase of $-logEC_{50}$ (*Figure* 10.1 and *Table* 10.1) (ANOVA, $P < 0.05$). These increases were not significantly different for the subthreshold or threshold concentration of the mediators (ANOVA, $P > 0.05$). The presence of LTC_4 did not significantly alter the $-logEC_{50}$ to Mch. In the mediator-treated preparations the increase of $-logEC_{50}$ in the presence of the mediators varied from 0.03 to 0.16 of a logstep. In contrast, in control preparations there was a decrease of the mean $-logEC_{50}$ values of the second curve compared to the first of 0.08 to 0.10 of a logstep (NS). This indicates that there is no spontaneous increase in sensitivity to Mch with time and that a small increase in BCS induced by precontraction with Mch (5 x 10^{-7} M) does not induce hypersensitivity. The maximal response to 10^{-4} M Mch did not increase significantly in the presence

Table 10.1. $-LogEC_{50}$ values of methacholine in the presence and absence of inflammatory mediators

mediator added	concentration	n	first CCRC[a]	second CCRC[a]	third CCRC[a]
none		6	6.06 ± 0.09	5.96 ± 0.07	6.05 ± 0.07
methacholine	5 x 10^{-7} M	6	6.03 ± 0.09	5.95 ± 0.03	6.07 ± 0.05
histamine	10^{-10} M	7	5.87 ± 0.13	6.01 ± 0.12*	5.89 ± 0.10
histamine	10^{-8} M	7	6.03 ± 0.10	6.12 ± 0.11*	6.08 ± 0.09
U46619	10^{-11} M	6	6.04 ± 0.08	6.18 ± 0.09**	6.11 ± 0.07
U46619	10^{-9} M	6	6.13 ± 0.12	6.25 ± 0.13**	6.08 ± 0.09
PGD_2	5 x 10^{-9} M	7	5.95 ± 0.09	6.02 ± 0.13*	6.08 ± 0.09[b]
PGD_2	5 x 10^{-7} M	7	6.04 ± 0.05	6.16 ± 0.06*	6.13 ± 0.06[b]
PGD_2[c]	5 x 10^{-7} M	5	6.10 ± 0.12	6.26 ± 0.11*	6.09 ± 0.14
$PGF_{2\alpha}$	10^{-9} M	7	6.03 ± 0.10	6.06 ± 0.12*	6.04 ± 0.10
$PGF_{2\alpha}$	10^{-7} M	7	6.03 ± 0.10	6.17 ± 0.09*	6.08 ± 0.07
LTC_4	10^{-11} M	6	6.16 ± 0.08	6.13 ± 0.11	6.17 ± 0.08
LTC_4	10^{-9} M	6	6.14 ± 0.11	6.20 ± 0.10	6.11 ± 0.10

* $P < 0.05$; ** $P < 0.01$, the presence of a mediator is significant factor in the ANOVA. The magnitude of the increase in $-logEC_{50}$ was not related to the dose of the mediator (ANOVA, $P > 0.05$).

[a] First CCRC's were obtained before addition of a mediator, second CCRC's in the presence of a certain mediator and third CCRC's after washout.

[b] Significantly higher ($P < 0.01$) than the $-logEC_{50}$ of the first CCRC.

[c] These preparations were washed longer (60 minutes) between the second and the third curve (see 10.3.1).

of any of the mediators. Thus, in the presence of histamine, U46619, PGD_2 and $PGF_{2\alpha}$ there was a small significant leftward shift of the CCRC to Mch. *Table* 10.1 shows that the third CCRC's, obtained after washing out the mediators, were not significantly different from the initial CCRC's in controls, in preparations exposed to histamine, U46619, $PGF_{2\alpha}$, LTC_4, and in the preparations exposed to PGD_2 (5 x 10^{-7} M) with prolonged washing between the second and the third curves. In the preparations treated with PGD_2 (5 x 10^{-7} M or 5 x 10^{-9} M) where a standard washing procedure had been executed, the sensitivity to Mch during the third curve was significantly higher than during the first ($P < 0.01$).

There was no relation between BCS before the first CCRC and the $-logEC_{50}$ of the first CCRC (regression coefficient for covariate B = -0.0017, P = 0.31), indicating that $-logEC_{50}$ does not dependent BCS.

10.5. Discussion

Our results indicate that low concentrations of histamine, U46619, PGD_2 and $PGF_{2\alpha}$, but not LTC_4, significantly increase of the sensitivity of isolated non-asthmatic human airways to Mch. This potentiation does not seem to depend on the dose of the mediator and is reversed after washing out the mediator.

The results may be influenced by differences in BCS between CCRC obtained in the absence and in the presence of a mediator but this seems unlikely for two reasons. Firstly, because raising BCS with methacholine itself did not alter $-logEC_{50}$. Secondly, because there was no correlation between BCS prior to first CCRC and the $-logEC_{50}$ of the first CCRC. In view of our results this lack of correlation may be somewhat unexpected assuming that BCS is caused by the presence of inflammatory mediators in the milieu of the muscle. However, BCS in isolated human airways is not reduced by a cyclooxygenase inhibitor[15,17] indicating that contractile prostaglandins do not contribute to BCS. A histamine (H_1) receptor antagonist causes a small decrease of BCS in isolated human airways[17], indicating that the contribution to BCS of endogenously released histamine is limited.

We have no reason to believe that our results would have been different if we had measured $-logEC_{50}$ isometrically. Although we have previously shown that $-logEC_{50}$ values obtained by isometric and isotonic measurement are slightly different, this difference was of similar magnitude and direction in all the preparations studied[9]. In addition, data obtained by isometric or isotonic measurement are probably equally relevant for the *in vivo* situation. *In vivo*, smooth muscle shortening is neither isometric nor isotonic since the load that the muscle has to overcome increases as it shortens.

Van den Brink[24] has predicted that the type of interaction that we describe occurs if two agonists occupy different receptors which are coupled to the formation of the same second messenger, the level of which has to surpass a certain threshold concentration in order to lead to an effect[2]. Although we did

not study the mechanism of the interaction that we have found, this seems a plausible explanation for its mechanism.

Fuller *et al.* found that prior inhalation of a small doses of PGD_2 caused a twofold increase in the responsiveness to methacholine of mild asthmatic subjects, which is similar to the effect of PGD_2 in our *in vitro* experiments [11]. Studies on the effect of histamine, TxA_2, $PGF_{2\alpha}$ or LTC_4 on the Mch responsiveness in humans *in vivo* have, to our knowledge, not been published.

Our results only partially support the concept that in asthma inflammatory mediators increase the airway responsiveness to inhaled Mch by causing a dose-dependent increase in the sensitivity of the airway muscle to cholinergic stimulation since the magnitude of the mediator-induced increase in muscle sensitivity did not depend on the dose of the mediator. That we did not find such a dose-dependent increase may be due to the limited dose range and number of observations. However, if the contribution of mediator-induced muscle hypersensitivity to the increased airway responsiveness in asthma is small, other factors, which may also be related to inflammatory changes in the airway wall such as epithelial damage or airway wall-thickening, are needed to explain the big leftward shift of the dose response curve to methacholine which is observed in many asthmatics[14].

In *conclusion* our data demonstrate that the presence of low concentrations of histamine, TxA_2, PGD_2 and $PGF_{2\alpha}$ leads to a twofold increase in the sensitivity of isolated non-asthmatic human airways to Mch, probably via a direct effect on airway smooth muscle. This indicates that mediator-induced muscle hypersensitivity can explain a small part of the leftward shift of the dose response curve to inhaled methacholine in asthma.

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Chapter 11

The Increased Responsiveness to Inhaled Methacholine in Asthma: Combination of Causative Factors*

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11.1. Summary

In asthma the dose response curve to inhaled methacholine is shifted leftwards and shows an increased slope and maximum. Two factors might contribute to the different form and position of this curve: (1) mediator-induced airway muscle hypersensitivity and (2) thickening of the airway wall. We attempt to estimate the effect of a combination of these two factors on the increase in airway resistance of a single isolated human airway to increasing concentrations of methacholine. The result suggests that mediator-induced hypersensitivity to cholinergic stimuli may explain a small part of the leftward shift of the asthmatic dose response curve to metacholine especially at lower levels of bronchoconstriction and that increased thickness of the airway wall may be more important for the increased slope and maximum of the asthmatic dose response curve.

11.2. Combination of causative factors

It is generally agreed that inhaled methacholine (Mch) causes bronchial obstruction by a direct action on airway smooth muscle (ASM). In asthmatics the dose response curve to inhaled Mch shows a leftward shift, corresponding to a 10 to 1000 fold increase in sensitivity, together with an increased slope and maximum[3]. There are several factors which could explain the altered form and position of this curve in asthma. Two of these are ASM hypersensitivity and amplification of the effect of ASM shortening on airway caliber. In this paper an attempt is made to predict the combined effect of these two factors

* Jongejan RC, de Jongste JC, Kerrebijn KF. The increased responsiveness to inhaled methacholine in asthma: combination of causative factors Am Rev Resp Dis (In Press). Printed with permission of the American Review of Respiratory Disease.

on the increase in airway resistance (R_{aw}) of one isolated airway to increasing concentrations of Mch.

Studies into the cellular contents and the mediator levels of bronchoalveolar lavage (BAL) fluids from symptom-free asthmatics with bronchial hyperresponsiveness have shown that the histamine level in BAL fluid is related to both the levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2) and thromboxane B_2 (TxB_2) and to the number of mast cells in the same BAL fluid specimen[2,9]. The number of mast cells and the histamine levels were both found to be strongly related to the $PC_{20}FEV_1$ of methacholine[2,7], suggesting that an ongoing production of inflammatory mediators by mast cells may be related to the sensitivity of the airways to inhaled Mch. Therefore, we recently investigated if low levels of mast cell-derived mediators such as histamine, PGD_2, $PGF_{2\alpha}$ or the stable TxA_2 analogue U46619 increased the cholinergic responsiveness of isolated bronchial rings dissected from human lung tissue specimens obtained at thoracotomy[6] (see *Chapter* 10). In these bronchial rings we measured isotonic ASM shortening to increasing concentrations of Mch before, during and after addition of a low dose of a mediator that gave little or no contraction. Each of these mediators caused a small (up to twofold) increase of ASM sensitivity to Mch[6]. The mediators did not alter the maximal response to Mch. To evaluate the functional implications of our measurements we approximated the resistance (R_{aw}) of one isolated airway segment that would occur with a given degree of smooth muscle shortening by using the equation derived by Moreno *et al.*[8]:

$$R_{aw} = \left(\frac{\sqrt{(1-(PMS \times PMP))^2 - P_w}}{\sqrt{1 - P_w}} \right)^{-4}$$

In this equation PMP represents the proportion of the airway perimeter occupied by smooth muscle[8]. P_w represents the proportion that the airway wall occupies in the total area of the circle formed by the outermost layer of ASM. PMS represents the proportion of muscle shortening i.e. the ratio of the length of the muscle in the presence of an agonist and the length of the fully relaxed muscle[8]. In the type of bronchi that we used (non-cartilaginous airways of 2 to 3 mm in diameter) PMP equals 1 and P_w 0.15[5]. In our experiments[6] we determined PMS as follows. After maximal relaxation the bronchial rings were fixed in a buffered formaldehyde solution (3.6%, pH 7.0) under a 250 mg load. Our own preliminary experiments and results from others[4] showed that this fixation procedure does not change the contractile state of the airway. Next, the distance between the hooks (d) in the airway lumen was measured with a vernier caliper. This is illustrated in *Figure* 11.1. We estimated the length of the muscle in the fully relaxed airway to equal 2 x d (see *Figure* 11.1) because histological examination showed that the thickness of the mucosa was negligible where it was in contact with the hooks. Since we used isotonic transducers it was possible to calculate the difference between airway muscle length in the

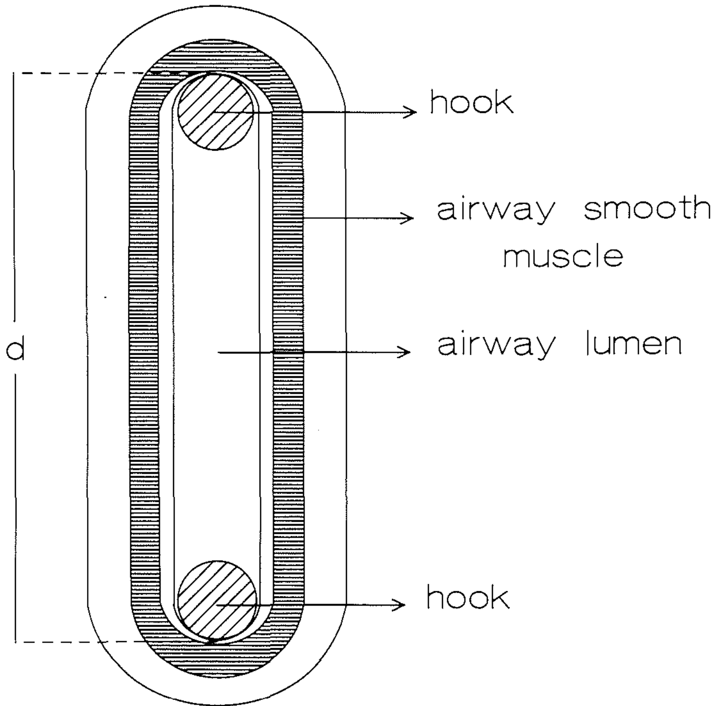

Figure 11.1. Schematic representation of a section through a fully relaxed airway segment with two hooks in its lumen. The length of the airway muscle in the segment is approximated by 2 x d, where d is the distance between the hooks.

presence of a given concentration of methacholine and airway muscle length after maximal relaxation. From this, PMS was calculated according to the above definition, allowing us to predict R_{aw} of an airway segment at any given PMS.

As an example, changes in R_{aw} after addition of methacholine in the presence and absence of U44619 (10^{-9} M) were calculated as follows. The preparations had a spontaneous intrinsic contractile activity which is called the baseline contractile state (BCS). R_{aw} at BCS was arbitrarily set at 1. PMS at BCS was 44.3 ± 3.6% for controls and 40.1 ± 2.3% for preparations treated with U46619 (NS). The predicted relative increase in R_{aw} induced by the addition of Mch in the absence and presence U46619 (10^{-9} M) was plotted against the Mch concentration. The maximal increase in PMS induced by Mch (departing from PMS at BCS) was 33.2 ± 2.3% for control preparations and 34.1 ± 5.3% in preparations exposed to U46619 (10^{-9} M) (NS). *Figure* 11.2 shows the results of our calculations for the Mch dose response curve in the absence of U46619 (controls, line number 1) and in the presence of 10^{-9} M U46619 (line number 2).

James *et al.* have recently found that in airways from asthmatics the airway wall is thickened ($P_w' = 0.25$) and they predicted that airway wall thickening

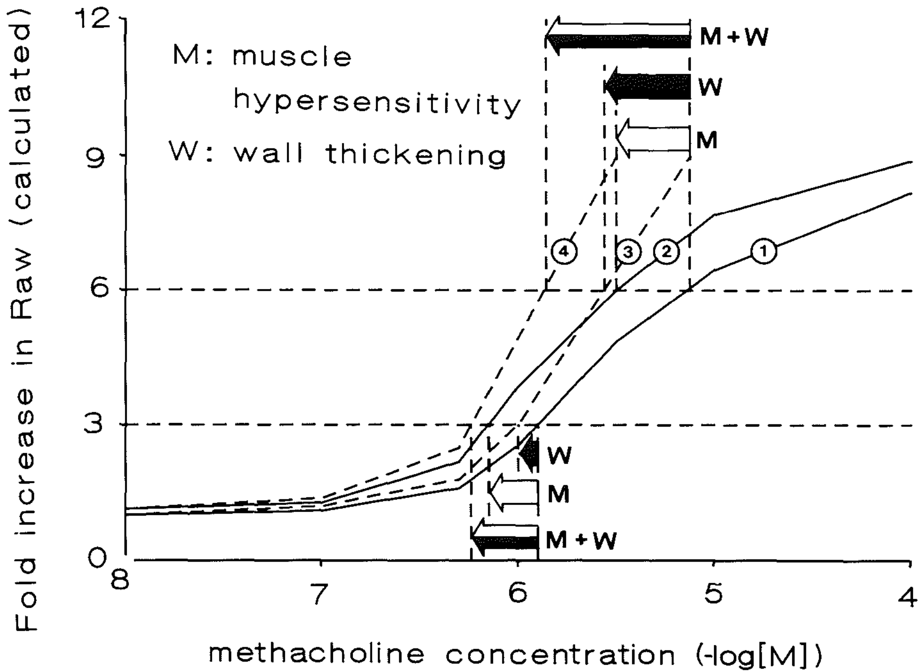

Figure 11.2. Predicted mean increase in resistance of a single airway (R_{aw}) by different concentrations of methacholine (Mch). The horizontal axis depicts the negative logarithm of the Mch concentration, the vertical axis the calculated relative increase in R_{aw} if the R_{aw} at BCS (see text) is set at 1. Curve 1 shows the dose response curve for control preparations ("normal airways"). Curve 2 illustrates the effect of U46619 (10^{-9} M) on the dose response curve to Mch. Curve 3 shows the dose response curve which would result from an increase in wall thickness (P_w) from 0.15 to 0.25. Curve 4 illustrates the combined effect of increased smooth muscle responsiveness and airway wall thickening on the dose response curve to Mch. The broken horizontal lines indicate a threefold (lower line) or sixfold (upper line) increase in R_{aw}. The arrows indicate, for curves 2,3 and 4, the differences from controls in the concentration of Mch needed to induce a 3-fold (lower arrows) or 6-fold (upper arrows) increase in R_{aw}. The closed arrows indicate the magnitude of the leftward shift of the dose response curve caused by thickening of the airway wall at a level of bronchial obstruction which results in a 3-fold (lower closed arrow) or a 6-fold (upper closed arrow) increase in R_{aw}. The open arrows indicate the magnitude of the leftward shift due to increased muscle responsiveness by U46619. The half open arrows indicate the magnitude of the shift caused by the combination of increased muscle responsiveness and thickening of the airway wall.

would amplify the effect of muscle shortening on airway resistance. *Figure* 11.2 shows how wall thickening would affect the mean increase in R_{aw} in response to Mch in control preparations (line number 3) and in preparations where 10^{-9} M U46619 was present (line number 4). From each of the curves shown in *Figure* 11.2 the concentration of Mch required to cause a threefold (PC_{3R}) and a sixfold (PC_{6R}) increase in R_{aw} was determined. These indices were derived from *in vivo* studies in humans[10,1] which showed that inhalation of the $PC_{20}FEV_1$ of histamine will lead to a 3 to 6 fold increase in airway or lung resistance. In addition,

Table 11.1. Calculated effects of U46619 and wall thickening on airway responsiveness and maximal increase in R_{aw}

	normal	+ U46619 (10^{-9} M)	thickening of airway wall[a]	thickening of airway wall[a] + U46619 (10^{-9} M)
PC_{3R}	1.6×10^{-6} M	0.8×10^{-6} M	1.3×10^{-6} M	0.6×10^{-6} M
PC_{6R}	8.5×10^{-6} M	4.1×10^{-6} M	3.7×10^{-6} M	1.8×10^{-6} M
MAX_{Raw}	8.2	8.9	4.6	16.3

Abbreviations: PC_{3R}, the concentration of Mch that causes a threefold increase in R_{aw}; PC_{6R}, the concentration of Mch that causes a sixfold increase in R_{aw}; MAX_{Raw}, calculated maximal increase in R_{aw} at maximal airway narrowing in the presence of Mch (10^{-4} M).

[a] These calculations were made with P_w 0.25 (see 11.2).

the maximal increase in Raw in the presence of Mch (10^{-4} M) was calculated. The results are shown in *Table* 11.1. Comparison of the lower open and closed arrows in *Figure* 11.2 shows that at a 3-fold increase in R_{aw} the increased sensitivity of the airway muscle due to the presence of a mediator causes a bigger leftward shift of the dose response curve to Mch than thickening of the airway wall does. However, when R_{aw} increases further, comparison of the upper open and closed arrows shows that the contribution of airway wall thickening becomes relatively more important. The half-open arrows indicate the combined effect of mediator-induced muscle hypersensitivity and increased wall thickness.

So, using the equation of Moreno *et al.*[8], our calculations suggest that at a level of bronchial obstruction which roughly corresponds to a 20% decrease of FEV_1, i.e. a 3-fold increase in R_{aw}, the increased sensitivity of airway muscle to a cholinergic stimulus caused by mediators contributes more to the leftward shift of the dose response curve than thickening of the airway wall does. As appears from *Figure* 11.2 at higher levels of bronchial obstruction thickening of the airway wall seems to become more important, because this will increase the slope and maximum of the dose response curve. When interpreting these results several assumptions should be kept in mind. Firstly, we have calculated the effects on the resistance of a single isolated airway and we do not know to what extent such calculations can be extrapolated to the resistance of the whole bronchial tree. Secondly, we have assumed a baseline R_{aw} of 1 both in the airway with a thickened wall and in the normal one. This is correct in case of centrifugal wall thickening. If wall thickening were centripetal, as might be the case in patients with severe asthma, this would lead to an increase in the baseline R_{aw}. Considering that provocation tests are usually performed in patients with moderate asthma in whom baseline FEV_1 is not lower than 70% of predicted and that marked hyperresponsiveness is often present in asthmatics with a normal baseline lung function it seems reasonable to depart from the same baseline R_{aw}. Thirdly, we have looked at shortening of the smooth muscle against a constant load whereas *in vivo* it contracts against an elastic load. It

is not known, however, to what extent this elastic load impairs bronchial smooth muscle contraction in normal and asthmatic airways *in vivo*. Conceivably, an increasing load will limit bronchoconstriction, and will make the resulting airway obstruction less severe.

In *conclusion*, if the *in vitro* data are relevant to the *in vivo* situation our analysis suggests that mediator-induced hypersensitivity to cholinergic stimuli may explain a small part of the leftward shift of the asthmatic dose response curve to Mch especially at lower levels of bronchoconstriction, and that increased thickness of the airway wall may be more important for the increased slope and maximum.

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Chapter 12

Effect of Epithelial Denudation, Inflammatory Mediators and Mast Cell Activation on the Sensitivity of Isolated Human Airways to Methacholine*

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12.1. Summary

1. Since in asthmatics both the continuous release of mast cell-derived inflammatory mediators and damage of the airway epithelium may be related to the degree of bronchial responsiveness we evaluated the effect of inflammatory mediators and mast cell activation on the cholinergic responsiveness of strips of human airways with and without epithelium.
2. Cumulative concentration response curves (CCRC) to methacholine were generated from strips with or without epithelium before, during and after incubation with threshold doses of either methacholine (3×10^{-7} M, controls), histamine (3×10^{-7} M), the thromboxane A_2 analogue U46619 (10^{-9} M), prostaglandin (PG) D_2 (3×10^{-7} M), $PGF_{2\alpha}$ (3×10^{-7} M), leukotriene (LT) C_4 (10^{-9} M), or anti-human IgE (24.4 ± 4.0 μg/ml).
3. Strips without epithelium were 1.6 times more sensitive to methacholine than strips with epithelium ($-\log EC_{50}$: 5.76 ± 0.04 versus 5.97 ± 0.04, $P < 0.0001$). The average contraction to identical doses of anti-IgE in strips without epithelium was 3 times bigger than the contraction in strips with epithelium ($P < 0.05$).
4. Both in strips with or without epithelium threshold concentrations of histamine, U44619 and PGD_2 caused a similar non-parallel leftward shift of the CCRC to methacholine ($P < 0.05$). When taken together epithelial

* Jongejan RC, de Jongste JC, Raatgeep HC, Stijnen T, Bonta IL, Kerrebijn KF. Effect of epithelial denudation, inflammatory mediators and mast cell activation on the sensitivity of isolated human airways to methacholine. (Submitted).

denudation and low levels of mediators caused a 4.0 to 9.1-fold increase in sensitivity based on the $-\log EC_{10}$ and a 1.8 to 3.0-fold increase in sensitivity based on the $-\log EC_{50}$.
5. It is concluded that removal of the epithelium and the presence of inflammatory mediators additively increase the cholinergic sensitivity of peripheral human airways especially to low concentrations of a cholinergic agonist and that the epithelium may be an important determinant of the magnitude of the response to inhaled allergens.

Key words: human airway muscle, bronchial hyperresponsiveness, inflammatory mediators, epithelium, mast cell activation, anti-human IgE.

12.2. Introduction

Epithelial damage has been observed in bronchial biopsies from clinically stable allergic asthmatics[4,11,16]. In this type of patients both the number of epithelial cells and the concentration of inflammatory mediators such as histamine, thromboxane (Tx) A_2, prostaglandin (PG) D_2 and $PGF_{2\alpha}$ in the bronchoalveolar lavage fluid seem to be related to the responsiveness to inhaled methacholine[4,5,9]. The strong concordance between the concentrations of histamine, TxA_2, PGD_2 and $PGF_{2\alpha}$ in the bronchoalveolar lavage fluid is compatible with the possibility that these inflammatory mediators originate from one source, presumably the mast cell[9]. Considering these findings, it seems conceivable that in bronchial asthma damage of the airway epithelium and the continuous release of inflammatory mediators by mast cells contribute to bronchial hyperresponsiveness to methacholine. There is some *in vitro* evidence in support of this concept. Firstly, epithelial denudation has been found to increase the responsiveness of isolated cartilaginous non-asthmatic human airways to acetylcholine, histamine and $PGF_{2\alpha}$[1,22]. Secondly, we have previously shown that threshold concentrations of histamine, PGD_2, $PGF_{2\alpha}$ and the stable TxA_2 analogue U46619 cause a twofold increase in sensitivity of isolated human airways to methacholine[15]. The combined effect of epithelial denudation and inflammatory mediators or mast cell activation on the cholinergic responsiveness of human airway muscle is unknown. It seems possible that damage or removal of the epithelium modulates the effect of an inflammatory mediator on the sensitivity of the airway muscle. The aim of the present study was threefold. First of all, to clarify if epithelial denudation, apart from increasing the *in vitro* sensitivity of central human airways to methacholine, also increases the sensitivity of peripheral human airways to this agonist. Secondly, to investigate the possibility that damage of the epithelium potentiates mediator-induced muscle hypersensitivity. Thirdly, to evaluate if low-grade activation of mast cells has a similar effect in strips with and without epithelium and if this alters the *in vitro* responsiveness to methacholine.

12.3. Methods

12.3.1. Patients, airway preparations and experimental protocol

Macroscopically normal human lung tissue was obtained from 17 male patients, with a mean age of 67.0 years (range 54 to 80 years) who were operated for bronchial carcinoma. Preoperative lung function showed a mean value for the forced expiratory volume in 1 s as a percentage of the inspiratory vital capacity of 56.4 $\pm$ 2.0%. Of these patients 11 had chronic obstructive pulmonary disease according to the criteria of the American Thoracic Society[2], but none had characteristics of asthma. Medication during anesthesia was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane and pancuronium. Eight patients received steroids and/or theophylline before and during the operation, none received cromoglycate or β_2-agonists. Thirty to sixty minutes after surgical resection a macroscopically normal part of the resected tissue was immersed in Krebs-Henseleit buffer of room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.55), which had been gassed with carbogen (95% O_2, 5% CO_2) to produce a pH of 7.35, a Pco_2 of 4.7 kPa and a Po_2 of 71.8 kPa. The tissue remained in fresh aerated buffer throughout the dissection procedure and the experiments. On the cut surface, airways with a diameter of 2 to 3 mm (6th to 11th generation)[25] were identified, cannulated, taken out and dissected free from parenchyma and vessels under a stereo microscope (20x magnification) using iris-scissors and forceps. From each cleaned airway two adjacent segments of 3 to 4mm length were cut off and then cut open into strips. From each pair of strips one was gently rubbed with a wet gauze to remove the epithelium. Surgical silk threads (6/0) were tied to the ends of each strip. The preparations were stored overnight in a slow flow of aerated buffer of 4°C, containing penicillin (3 x 10^{-5} g/l) and tobramycin (5 x 10^{-3} g/l). We formerly demonstrated that this storage procedure does not affect the contractility of the preparations to methacholine[6]. The next day the strips were mounted between a glass hook at the bottom of a double jacketed 10 ml organ bath and a high precision isotonic angular position transducer (Penny and Giles, 3810/60, Christchurch, Dorset, Great Britain)[21]. The signal of the transducer was monitored with a digital voltmeter (Fluke 73 multimeter, Everett, WA, USA) and a pen-recorder (Kipp BD 40, Delft, The Netherlands). This method has been described in detail previously[6,13]. As in previous studies, the preparations contracted against an isotonic load of 250 mg[13-15].

During an equilibration period of 2 hours at 37°C, each pair of strips was contracted with methacholine (10^{-5} M, 10^{-4} M) twice, to assess the contractile function. Then, a first cumulative concentration response curve (CCRC) to methacholine (10^{-8} M to 10^{-4} M) was obtained, followed by washing and stabilization of resting length. Next, a threshold concentration of a stimulant was added to each pair of strips and after stabilization a second CCRC to methacholine

was obtained in the presence of this stimulant. "Threshold concentration" is defined as a concentration that causes a contraction of 5 to 10% of the maximal active contractile range (see 12.3.2). Threshold concentrations were determined in preliminary experiments. A third CCRC to methacholine was made after washout and stabilization of resting length. The following inflammatory mediators and concentrations were used: histamine (3 x 10^{-7} M), U46619 (10^{-9} M), PGD_2 (3 x 10^{-7} M), $PGF_{2\alpha}$ (3 x 10^{-7} M) and LTC_4 (10^{-9} M). To evaluate the effect of low grade mast cell activation on the responsiveness to methacholine anti-IgE was used. This stimulus has previously been shown to release mediators from human pulmonary mast cells recovered by bronchoalveolar lavage[18] and from mast cells in isolated human airways[12]. Due to the variability in responsiveness to anti-IgE between preparations from different tissues it was necessary to assess the threshold concentration of anti-IgE separately for each pair of strips. To this purpose increasing doses of anti-IgE were added to the strips until the plateau of a threshold response was observed in both strips, after which the methacholine dose response curve was started. Thus, in each pair of strips the same concentration of anti-IgE was taken for the strip with and the strip without epithelium.

Parallel control experiments were done on separate pairs of strips in which the second of the three consecutive CCRC was made after precontraction with a threshold concentration of methacholine (3 x 10^{-7} M).

To quantify the effect of luminal rubbing on the epithelium all strips were examined histologically and the percentage of the basement membrane covered with epithelium was estimated by an observer who was unaware of the previous treatment of the preparations. The integrity of the basement membrane was also examined.

12.3.2. Data analysis

The contractile state of the preparations was expressed on a scale which defines the maximal active contractile range (MACR)[14,15,19]. The second response to methacholine 10^{-4} M, obtained at the beginning of each experiment, was called 100% shortening. Maximal relaxation after 10^{-4} M isoprenaline and 4 x 10^{-3} M EDTA, determined at the end of each experiment, was called 0% shortening. After maximal relaxation the preparations were fixed in a buffered formaldehyde solution (3.6%, pH 7.0) under a 250 mg load and the length of the strips was measured with a vernier caliper. Our own preliminary experiments and results from others[10] showed that this fixation procedure does not change the contractile state of the airway. To compare smooth muscle function in strips with and without epithelium the MACR expressed as percentage of the length of the strip was determined in both groups. The baseline contractile state (BCS) was defined as the spontaneous position of a preparation on the MACR scale.

The sensitivity to methacholine was expressed as the negative logarithm of the methacholine concentration that caused 10, 20 or 50% of the maximal muscle

shortening ($-\log EC_{10}$, $-\log EC_{20}$, $-\log EC_{50}$) starting from BCS. These parameters were calculated by linear interpolation between the two concentrations on either side of the EC_{10}, EC_{20} or EC_{50}. To evaluate if BCS was a determinant of $-\log EC_{50}$ the correlation between BCS and $-\log EC_{50}$ was calculated using Spearman's rank correlation coefficient (r_s). Values of $-\log EC_{10}$, $-\log EC_{20}$ or $-\log EC_{50}$ were analyzed with a three way analysis of variance (ANOVA) using the MANOVA program of SPSS^x [23]. The absence or presence of epithelium and the number of the CCRC (first, before mediator; second, in the presence of the mediator; third, after washout of the mediator) were taken as factors and the different lung tissues as random factors. The influence of the absence of epithelium was also evaluated for all 54 pairs of strips from the 17 lung tissue specimens with a two way analysis of variance (ANOVA) using the ANOVA program of SPSS^x [23]. The absence of epithelium and the different lung tissues were taken as factors. All other parameters were compared with Wilcoxon's matched pairs signed rank test. P values <0.05 (twosided) were considered significant. All data are presented as mean $\pm$ SEM.

12.3.3. Drugs

Methacholine hydrobromide, histamine hydrochloride (Janssen Chimica, Belgium), EDTA (Sigma, USA) and LTC_4 (a gift from Merck Frosst Laboratories, Canada) were dissolved in saline. The TxA_2 analogue U46619 (9.11-dideoxy-11_α,9_α-epoxymethano-prostaglandin $F_{2\alpha}$, a gift from Upjohn, USA) was dissolved in absolute ethanol, and PGD_2 and $PGF_{2\alpha}$ (Sigma, USA) in 70% ethanol. Goat ϵ-chain specific anti-human IgE (Sigma, USA) was dissolved in saline. L-isoproterenol (Janssen Chimica, Belgium) was dissolved in water containing ascorbic acid (88 mg/l). Preliminary experiments showed that ascorbate and ethanol in these concentrations have no effect on airway smooth muscle function. Fresh drug solutions were prepared daily and kept on ice during the experiments. U46619, PGD_2, $PGF_{2\alpha}$ and LTC_4 were kept in -70°C.

12.4. Results

In rubbed strips 2.5 $\pm$ 1.3% of the basement membrane was covered with epithelium whereas in unrubbed strips this was 96 $\pm$ 5.2% ($P < 0.0001$). In most rubbed strips the basement membrane was found to be intact, indicating that damage to the lamina propria had been minimal. The MACR expressed as percentage of the length of the strips was similar in strips with and without epithelium (38.6 $\pm$ 3.9% versus 37.4 $\pm$ 3.1%, NS), indicating that the maximal smooth muscle shortening was similar in strips with and without epithelium and that removal of the epithelium had not damaged the airway muscle.

The mean difference between the sensitivity to methacholine of strips with and without epithelium based on the $-\log EC_{10}$ was 0.25 $\pm$ 0.07 of a logstep,

based on the $-\log EC_{20}$ the difference was 0.26 ± 0.06 and based on the $-\log EC_{50}$ it was 0.21 ± 0.04 (54 pairs of strips from 17 lung tissue specimens, all $P < 0.001$). This indicates that the absence of epithelium caused a parallel leftward shift of the CCRC equivalent to a 1.6 to 1.8-fold increase in sensitivity to methacholine. This is illustrated in *Figure* 12.1A. The BCS in strips with and without epithelium was similar (43.0 ± 2.6 versus 45.9 ± 2.6 %MACR, NS). BCS (range 2.8 to

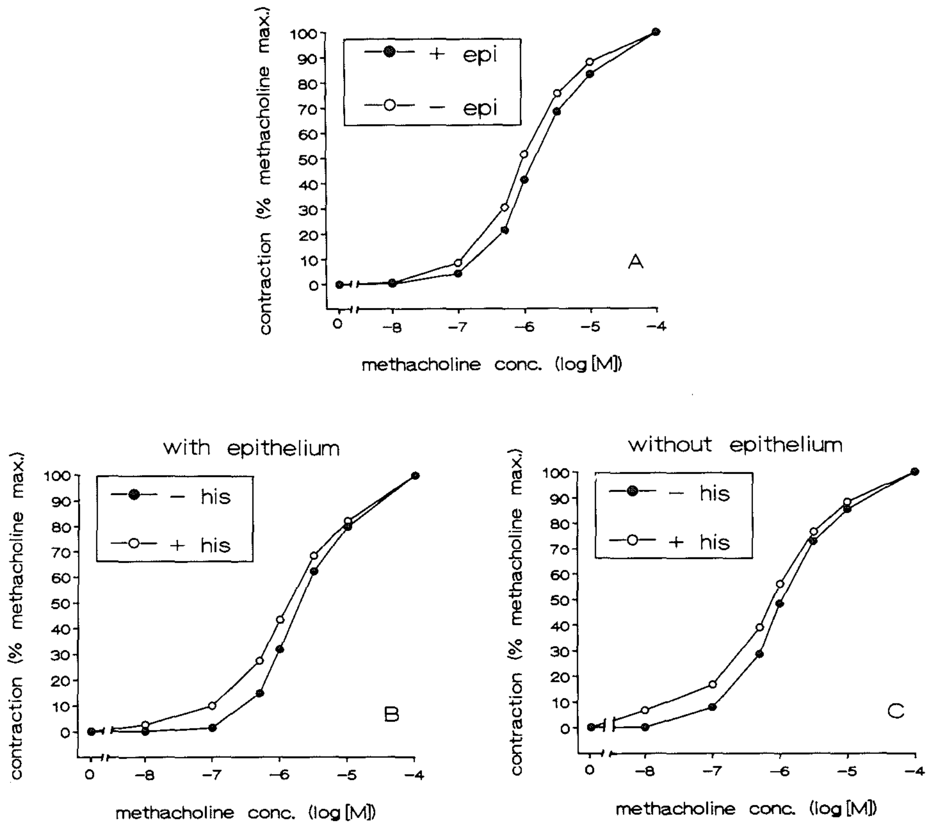

Figure 12.1. Panel A shows mean CCRC to methacholine of strips of human airways **with** epithelium (closed symbols) and **without** epithelium (open symbols). Panel B shows mean CCRC to methacholine of strips of human airways **with** epithelium in the absence (closed symbols) and presence (open symbols) of histamine (3×10^{-7} M). Panel C shows mean CCRC to methacholine of strips of human airways **without** epithelium in the absence (closed symbols) and presence (open symbols) of histamine (3×10^{-7} M). The horizontal axes depict the logarithm of the methacholine concentration in the organ bath, the vertical axis represents the contractile state of the preparations which is expressed as a % of the difference between the maximal contraction to methacholine (10^{-4} M) and baseline contractile state before starting the CCRC to methacholine. For reasons of clarity SEM bars were omitted. The SEM was < 7%. For statistical analysis see *Tables* 12.1, 12.2 and 12.3 and section 12.4. Panel A: 54 preparations from 17 lung tissue specimens. Panels B and C: 6 preparations from 6 different tissue specimens.

76.8 %MACR) was not related to $-logEC_{50}$ in strips with or without epithelium ($-0.09 < r_s < -0.11$, P>0.21).

Tables 12.1, 12.2 and 12.3 show the $-logEC_{10}$, $-logEC_{20}$ and $-logEC_{50}$ values to methacholine in the absence (first CCRC) and presence (second CCRC) of the different agonists together with those of the controls. For reasons of clarity the data of all control experiments have been pooled. Both in strips with and without epithelium histamine, U44619 and PGD_2 caused a significant ($P < 0.05$) increase of the $-logEC_{10}$, $-logEC_{20}$ and $-logEC_{50}$ to methacholine, corresponding to a 1.5 to 4.0-fold increase in sensitivity for the $-logEC_{10}$, a 1.5 to 2.5-fold increase for the $-logEC_{20}$ and a 1.3 to 1.7-fold increase for the $-logEC_{50}$. In controls there was no increase in sensitivity. In the presence of LTC_4 the increase of the $-logEC_{10}$ was significant ($P < 0.05$). With $PGF_{2\alpha}$ or anti-IgE there was no significant increase of either measure of sensitivity. Thus the presence of histamine, PGD_2 and U46619 caused a non-parallel leftward shift of the CCRC both in strips with and without epithelium. For histamine this is illustrated in *Figure* 12.1B and 12.1C.

Table 12.1. $-LogEC_{10}$ values of methacholine in the presence and absence of several inflammatory mediators in pairs of strips with and without epithelium

mediator added	+/− epithelium	n	CCRC first[a]	second[a]	third[a]
controls					
methacholine[b]	+	17	6.80 ± 0.15	6.90 ± 0.09	6.95 ± 0.09
3×10^{-7} M	−		6.97 ± 0.09	7.05 ± 0.10	6.89 ± 0.10
treated					
histamine[b]	+	6	6.44 ± 0.15	7.04 ± 0.25**	6.38 ± 0.15
3×10^{-7} M	−		6.88 ± 0.20	7.40 ± 0.25**	6.95 ± 0.18
U46619[b]	+	6	6.94 ± 0.13	7.16 ± 0.15*	6.90 ± 0.23
10^{-9} M	−		7.35 ± 0.28	7.54 ± 0.21*	7.11 ± 0.26
PGD_2	+	5	6.82 ± 0.18	7.42 ± 0.27**	6.99 ± 0.27
3×10^{-7} M	−		7.09 ± 0.08	7.48 ± 0.15**	7.21 ± 0.20
$PGF_{2\alpha}$	+	6	6.93 ± 0.12	7.01 ± 0.17	6.91 ± 0.21
3×10^{-7} M	−		7.03 ± 0.16	7.26 ± 0.19	6.80 ± 0.17
LTC_4	+	5	6.62 ± 0.10	7.10 ± 0.13*	6.84 ± 0.09
10^{-9} M	−		6.88 ± 0.18	7.67 ± 0.20*	6.65 ± 0.12
Anti-IgE[b]	+	9	6.53 ± 0.08	6.76 ± 0.12	6.34 ± 0.13
24.4 ± 4.0 μg/ml	−		6.88 ± 0.15	7.11 ± 0.20	6.81 ± 0.13

Abbreviations: n, number of preparations of different lungs.

* $P < 0.05$; ** $P < 0.01$, mediator is significant factor in ANOVA.

[a] First CCRC's were obtained before addition of an inflammatory mediator, second CCRC's in the presence of a certain inflammatory mediator and third CCRC's after washout.

[b] $P < 0.05$, epithelium is significant factor in ANOVA.

Table 12.2. $-LogEC_{20}$ values of methacholine in the presence and absence of several inflammatory mediators in pairs of strips with and without epithelium

mediator added	+/– epithelium	n	CCRC first[a]	second[a]	third[a]
controls					
methacholine[b]	+	17	6.32 ± 0.09	6.36 ± 0.07	6.48 ± 0.08
3 x 10^{-7} M	–		6.60 ± 0.08	6.61 ± 0.08	6.56 ± 0.09
treated					
histamine[b]	+	6	6.20 ± 0.13	6.54 ± 0.19*	6.38 ± 0.12
3 x 10^{-7} M	–		6.52 ± 0.18	6.92 ± 0.23*	6.71 ± 0.11
U46619[b]	+	6	6.43 ± 0.13	6.64 ± 0.14*	6.49 ± 0.18
10^{-9} M	–		6.85 ± 0.24	7.06 ± 0.20*	6.67 ± 0.19
PGD_2	+	5	6.47 ± 0.10	6.60 ± 0.10**	6.55 ± 0.13
3 x 10^{-7} M	–		6.68 ± 0.10	6.87 ± 0.09**	6.74 ± 0.14
$PGF_{2\alpha}$	+	6	6.55 ± 0.10	6.60 ± 0.10	6.55 ± 0.12
3 x 10^{-7} M	–		6.62 ± 0.10	6.72 ± 0.08	6.46 ± 0.11
LTC_4	+	5	6.30 ± 0.06	6.59 ± 0.06	6.33 ± 0.04
10^{-9} M	–		6.54 ± 0.06	6.90 ± 0.08	6.40 ± 0.10
Anti-IgE[b]	+	9	6.23 ± 0.07	6.41 ± 0.10	6.04 ± 0.13
24.4 ± 4.0 μg/ml	–		6.55 ± 0.13	6.67 ± 0.18	6.49 ± 0.10

Abbreviations: n, number of preparations of different lungs.
* $P < 0.05$; ** $P < 0.01$, mediator is significant factor in ANOVA.
[a] First CCRC's were obtained before addition of an inflammatory mediator, second CCRC's in the presence of a certain inflammatory mediator and third CCRC's after washout.
[b] $P < 0.05$, epithelium is significant factor in ANOVA.

Figure 12.2 illustrates the combined effect of removal of the epithelium and the presence of histamine on the CCRC to methacholine. It can be seen that the biggest leftward shift occurs at low concentrations of methacholine. Based on the $-logEC_{10}$, the $-logEC_{20}$ and the $-logEC_{50}$, the sensitivity between the two series of preparations differs by a factor 9.1, 5.2 and 3.0 respectively. Results for U46619 and PGD_2 showed a similar pattern although the differences were less pronounced. None of the inflammatory mediators altered the maximal effect of methacholine. The effect of removal of the epithelium on the sensitivity to methacholine was significant in the groups treated with histamine, U46619 and anti-IgE but not in the groups treated with LTC_4, PGD_2 or $PGF_{2\alpha}$.

Figure 12.3 shows the effect of threshold concentrations of methacholine, histamine, U46619, PGD_2, $PGF_{2\alpha}$, LTC_4 or anti-IgE on the BCS of strips with and without epithelium. Anti-IgE was used in a mean concentration of 24.4 ± 4.0 μg/ml. It can be seen that for the strips with epithelium all these agonists caused a similar contraction (range 7 to 12 %MACR). For all agonists except

Table 12.3. –LogEC_{50} values of methacholine in the presence and absence of several inflammatory mediators in pairs of strips with and without epithelium

mediator added	+/– epithelium	n	CCRC first[a]	second[a]	third[a]
controls					
methacholine[b]	+	17	5.78 ± 0.09	5.79 ± 0.08	5.77 ± 0.08
3 x 10^{-7} M	–		5.98 ± 0.06	5.95 ± 0.06	5.97 ± 0.07
treated					
histamine[b]	+	6	5.61 ± 0.14	5.82 ± 0.16*	5.66 ± 0.12
3 x 10^{-7} M	–		5.95 ± 0.18	6.08 ± 0.17*	5.98 ± 0.15
U46619[b]	+	6	5.71 ± 0.14	5.81 ± 0.15*	5.72 ± 0.14
10^{-9} M	–		6.02 ± 0.16	6.17 ± 0.12*	5.96 ± 0.10
PGD_2	+	5	5.86 ± 0.12	6.02 ± 0.15*	5.89 ± 0.13
3 x 10^{-7} M	–		6.01 ± 0.12	6.11 ± 0.09*	6.04 ± 0.12
$PGF_{2\alpha}$	+	6	5.98 ± 0.10	5.94 ± 0.08	5.93 ± 0.06
3 x 10^{-7} M	–		5.94 ± 0.05	6.02 ± 0.06	5.86 ± 0.07
LTC_4	+	5	5.78 ± 0.07	5.94 ± 0.06	5.82 ± 0.05
10^{-9} M	–		5.93 ± 0.10	6.04 ± 0.07	5.85 ± 0.07
Anti-IgE[b]	+	9	5.67 ± 0.07	5.74 ± 0.09	5.54 ± 0.08
24.4 ± 4.0 μg/ml	–		5.98 ± 0.10	5.92 ± 0.12	5.93 ± 0.10

Abbreviations: n, number of preparations of different lungs.
* P < 0.05; ** P < 0.01, mediator is significant factor in ANOVA.
[a] First CCRC's were obtained before addition of an inflammatory mediator, second CCRC's in the presence of a certain inflammatory mediator and third CCRC's after washout.
[b] P < 0.05, epithelium is significant factor in ANOVA.

$PGF_{2\alpha}$, the contraction to identical doses of an agonist was somewhat bigger in strips without than in strips with epithelium. This effect was significant only in the case of methacholine (8.0 ± 1.6 versus 10.1 ± 1.4 %MACR, n = 17, $P < 0.05$) and for anti-IgE, where the difference was much bigger (6.7 ± 2.4 versus 18.5 ± 4.4 %MACR, n = 9, $P < 0.05$).

12.5. Discussion

This study gives insight in the effects of different factors that modulate the cholinergic responsiveness of isolated human airways. Removal of the epithelium causes a parallel leftward shift of the CCRC of peripheral human airways to methacholine, corresponding to a 1.6 to 1.8-fold increase in sensitivity. Furthermore, low levels of histamine, PGD_2, U46619 cause a non-parallel leftward shift of the CCRC to methacholine. When taken together, epithelial denudation

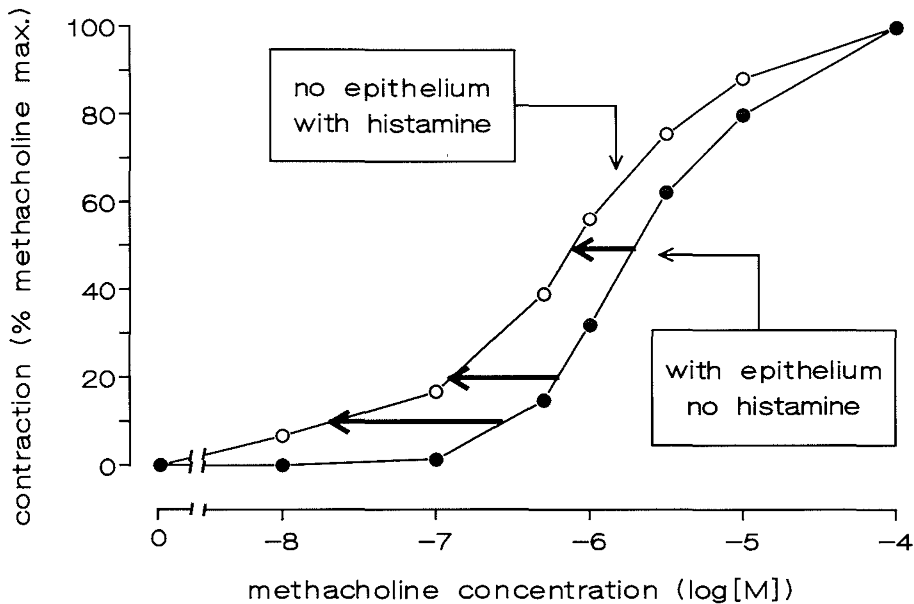

Figure 12.2. CCRC to methacholine of strips of human airways **with** epithelium in the **absence** of histamine (closed symbols) and airways **without** epithelium in the **presence** of histamine (3 x 10^{-7} M) (open symbols). The arrows indicate the leftward shift at 10, 20, and 50% of the maximal contraction to methacholine. There is a non-parallel leftward shift of the CCRC: the sensitivity to low doses of methacholine increases more than that to higher doses of methacholine. The horizontal axis depicts the logarithm of the methacholine concentration in the organ bath, the vertical axis represents the magnitude of the contractions which is expressed as a % of the difference between the maximal contraction to methacholine (10^{-4} M) and baseline contractile state before starting the CCRC to methacholine. For statistical analysis see *Tables* 12.1, 12.2 and 12.3 and *Section* 12.4. For reasons of clarity SEM bars were omitted. The SEM was $< 7\%$. ($n = 6$).

and low levels of these inflammatory mediators cause a 4.0 to 9.1-fold increase in sensitivity based on the $-\log EC_{10}$, a 2.5 to 5.2-fold increase based on the $-\log EC_{20}$ and a 1.8 to 3.0-fold increase in sensitivity based on the $-\log EC_{50}$. In addition, removal of the epithelium increases the contractile response to a single dose of anti-IgE.

Using isometric force transducers and cartilaginous bronchial rings dissected from human lung tissue obtained at thoracotomy, others[1,22] have found that, based on the $-\log EC_{50}$, rings without epithelium were 2 to 3 times more sensitive to acetylcholine than rings with epithelium. Maximal responses were not affected by removal of the epithelium. Although we used isotonic transducers and strips of non-cartilaginous airways and methacholine as the agonist, our findings were similar, indicating that the effect of epithelium on airway sensitivity is similar in central and peripheral airways and can be measured both isometrically and isotonically.

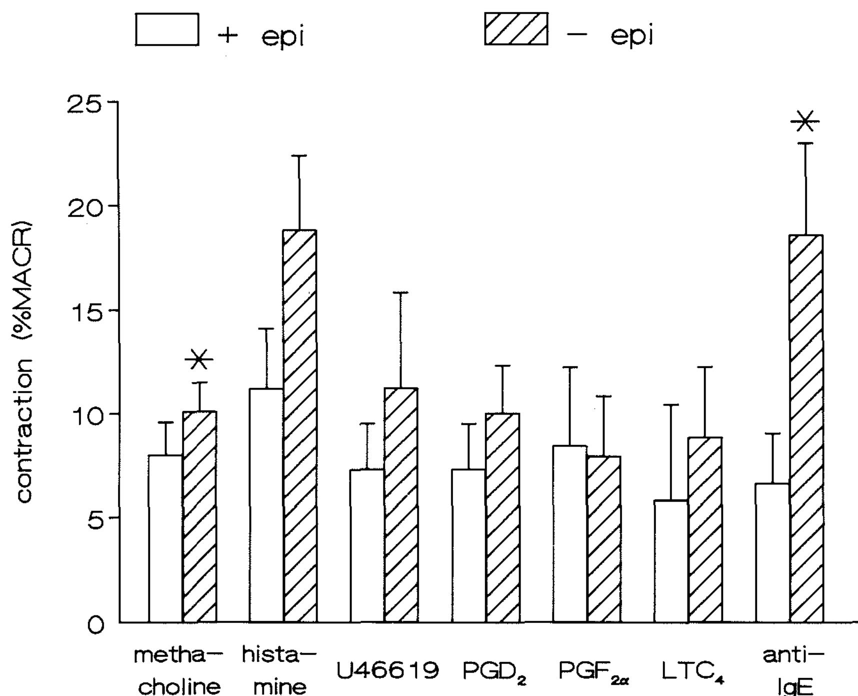

Figure 12.3. Contractile effect of low doses of different agonists on strips **with** epithelium (open bars) and **without** epithelium (hatched bars). The following agonists were used: methacholine (3 x 10^{-7} M, n = 17), histamine (3 x 10^{-7} M, n = 6), U46619 (10^{-9} M, n = 6), PGD_2 (3 x 10^{-7} M, n = 5), $PGF_{2\alpha}$ (3 x 10^{-7} M, n = 6), LTC_4 (10^{-9} M, n = 5) and anti-IgE (24.4 ± 4.0 μg/ml, n = 9). The vertical axis gives the magnitude of the contractions (departing from the baseline contractile state) expressed as a percentage of the maximal active contractile range (%MACR) which represents the difference between maximal contraction to methacholine (10^{-4} M) (100%) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer containing EDTA (1.5 x 10^{-3} M) (0%). * $P < 0.05$, compared to response of strip with epithelium.

We have previously found that, on the basis of the $-\log EC_{50}$, low concentrations of histamine, U46619, PGD_2 and $PGF_{2\alpha}$ significantly increase the sensitivity of isolated non-asthmatic human airways to methacholine[15], possibly via a mechanism of functional interaction such as threshold amplification[17,24]. The present study confirms our previous findings. We now show that the leftward shift caused by the inflammatory mediators is a non-parallel one. We also show that the combined effect of the presence of an inflammatory mediator and removal of the epithelium on the sensitivity of the airways to methacholine roughly equals the sum of the separate effects of these factors. This indicates that the effects are additive.

Considering our finding of mediator-induced muscle hypersensitivity it is somewhat unexpected that we did not find a significant effect of mast cell activation with anti-IgE on smooth muscle sensitivity, since this stimulus will

release inflammatory mediators in the airway preparation[12,18]. Our results do, however, not exclude the existence of such an effect. The 95% confidence interval for the mean difference in $-\log EC_{50}$ of curves obtained before and during stimulation of mast cells with anti-IgE was [-0.20, 0.31] for strips with epithelium and [-0.65, 0.53] for strips without epithelium. This means that mast cell activation may cause a leftward shift of the CCRC to methacholine of up to 0.3 to 0.5 of a logstep, i.e. more than the separate effects of histamine or PGD_2. The wideness of these confidence intervals in spite of the considerable number of experiments ($n = 9$) probably reflects the variable effect of an immunologic stimulus compared to that of a pharmacological one. This is also reflected by the difference between pairs of strips in the dose of anti-IgE needed to induce a small contraction.

From our data it is not clear whether removal of the epithelium increases the sensitivity of the airways via the loss of an EpDRF, via the loss of a diffusion barrier or both. Two of our findings argue against the involvement of an EpDRF. Firstly, the similarity of BCS in strips with and without epithelium suggests that, at least at baseline, the epithelium does not release a relaxing factor. Secondly, for 6 of the 7 agonists the response to identical doses of the agonist was higher in strips without epithelium than in strips with epithelium. This favours the involvement of a non-specific mechanism such as the loss of a diffusion barrier. That the difference between the contractile response in strips with and without epithelium to stimulation with anti-IgE was bigger than for stimulation with the other agonists could also be explained by the loss of a diffusion barrier. One would expect the epithelium to provide a more efficient diffusion barrier against large protein molecules such as anti-IgE (molecular weight approx. 150.000 g/mol) than against the smaller mediator molecules (molecular weight < 1000 g/mol). Thus, although the mechanism requires further study, our results indicate that *in vitro* the airway epithelium may be an important determinant of the response to anti-IgE. This suggests that, *in vivo*, the epithelium may also be an important determinant of responses to inhaled mast cell-activators such as allergens.

For several reasons it is difficult to know how the changes in airway responsiveness that we observed *in vitro* would translate into changes in airway responsiveness *in vivo*. First of all, airway responsiveness *in vivo* and *in vitro* do not correlate[3,7]. Secondly, we do not know how the mediator concentrations that we have used relate to those in asthmatic airways. Thirdly, in asthma the epithelium is damaged[16] but not almost absent as in our experiments. Fuller *et al.*[8], however, have found that prior inhalation of a small doses of PGD_2 caused a twofold increase in the responsiveness to methacholine of mild asthmatic subjects, which is similar to the effect of PGD_2 in our *in vitro* experiments. Therefore it seems possible that *in vivo* the presence of inflammatory mediators together with damage of the epithelium can explain part of the leftward shift of the dose response curve to inhaled methacholine. Based on our results we would predict that the presence of inflammatory mediators together with damage

of the epithelium will substantially lower the concentration of inhaled methacholine at which the airways just start narrowing (i.e. the threshold concentration), but will not affect the maximal response. Finally, it should be recognized that in our *in vitro* model we might underestimate the effect of epithelial denudation on airway responsiveness since the stimuli that we have given *in vitro* reached the airways both from the luminal and the serosal side, while *in vivo* many of these stimuli will reach the airway from the luminal side only. Munakata *et al.*[20] have shown that luminally perfused guinea-pig trachea is 35 to 115-fold more sensitive to stimulation with histamine or acetylcholine from the serosal side than from the luminal side. This difference disappears after epithelial denudation[20].

In *conclusion*, removal of the epithelium causes a greater increase of the response to a single dose of anti-IgE than to methacholine, U46619, PGD_2, $PGF_{2\alpha}$ or LTC_4, suggesting that the airway epithelium is an important determinant of the response to inhaled mast cell-activators such as allergens. Removal of the epithelium also produces a parallel leftward shift of the CCRC to methacholine of peripheral human airways. Both in airways with and without epithelium low levels of histamine, PGD_2, and U46619 cause a similar leftward shift of the CCRC especially in the lower concentration range showing that removal of the epithelium adds to the increase in airway sensitivity caused by an inflammatory mediator but does not potentiate it. When taken together epithelial denudation and low levels of these inflammatory mediators cause a 4.0 to 9.1-fold increase in sensitivity based on the $-logEC_{10}$ and a 1.8 to 3.0-fold increase in sensitivity based on the $-logEC_{50}$.

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Chapter 13

Effects of Zymosan-activated Human Granulocytes on Isolated Human Airways*

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13.1. Summary

In asthma a temporal association exists between the late allergic reaction (LAR), the influx of granulocytes into the airway wall, and an increase in bronchial responsiveness. Therefore we tested the hypothesis that activated human granulocytes constrict isolated human airways and increase their sensitivity to cholinergic stimuli. Bronchial rings were dissected from 23 lung tissue specimens collected at thoracotomy, and studied isotonically in organ baths. Airways were incubated with 1, 2, 5, 10 or 20 x 10^6 granulocytes from normal or atopic donors. Activation of the cells with serum treated zymosan (STZ) (0.2 mg/ml), which itself did not alter baseline airway caliber, resulted in a bronchoconstriction proportional to the number of zymosan activated granulocytes (ZAG) present (r_s = 0.79, $P<0.001$). This contraction was reduced by about 70% with the leukotriene C_4/D_4 receptor antagonist FPL 55712 (11.5 x 10^{-6} M) ($P<0.001$) or with the lipoxygenase inhibitor nordihydroguaiaretic acid (10^{-5} M) ($P<0.001$). The scavengers of activated oxygen molecules superoxide dismutase (300 U/ml) and bovine catalase (5000 U/ml), the cyclooxygenase inhibitor indomethacin (10^{-5} M) or the histamine (H_1) receptor antagonist mepyramine (2.8 x 10^{-6} M) had no effect. Granulocyte suspensions from atopic donors contained more eosinophils ($P<0.001$) and the magnitude of the contraction to 10 x 10^6 ZAG was related to the proportion of eosinophils (r_s = 0.66, $P<0.01$). The sensitivity of the airways to methacholine was unchanged in the presence of 1,2 or 5 x 10^6 ZAG and decreased with 10 or 20 x 10^6 ZAG ($P<0.05$). The cholinergic

* Jongejan RC, de Jongste JC, Raatgeep HC, Bonta IL, Kerrebijn KF. Effects of zymosan-activated human granulocytes on isolated human airways. Am Rev Respir Dis (In Press). Printed with permission of the American Review of Respiratory Disease.

twitch and the non-adrenergic relaxation to near-maximal electric field stimulation (EFS) were unchanged in the presence of 5 x 10^6 ZAG, but the slow contractile response to EFS was slightly potentiated (P<0.01). If during the LAR the number of granulocytes and their activation resembles those used in these *in vitro* experiments the results suggest that during the LAR granulocytes do not increase airway muscle sensitivity but may contribute to muscle contraction. Most of this muscle contraction seems secondary to the release of LTC_4 which is related to the proportion of eosinophils among the granulocytes.

Key words: isolated human bronchi; bronchial smooth muscle; electric field stimulation; neutrophils; eosinophils; leukotriene C_4

13.2. Introduction

In atopic asthmatics exposure to allergen produces an early bronchoconstriction which may be followed by a late broncho-obstructive phase[7,32]. This late asthmatic reaction (LAR) is characterized by an influx of inflammatory cells into the airway wall[3]. Increases in the number of eosinophils, neutrophils and other inflammatory cells have been described in bronchial lavage fluid obtained 6 to 48 hours after allergen challenge[17,19,31], in conjunction with a transient increase of bronchial responsiveness[10,35]. Thus, there is a temporal association between the influx of granulocytes into the airway wall during the LAR and an influx of granulocytes might be associated with an increase in bronchial responsiveness. Therefore we tested the hypothesis that activated human granulocytes constrict isolated human airways and increase the sensitivity of these airways to a cholinergic stimulus. Furthermore, to asses whether activated granulocytes affect the function of autonomic nerves in the airway wall, we studied the effect of activated granulocytes on the responses of central human bronchus to electric field stimulation (EFS), which selectively activates intramural postganglionic nerves[12].

13.3. Methods

13.3.1. Patients

Human lung tissue was obtained from 18 male and 5 female patients with a mean age of 64.9 years (range 41 to 81 years) who underwent a thoracotomy for lung cancer. Twenty were smokers, 14 had chronic obstructive pulmonary disease (COPD) according to the criteria of the American Thoracic Society[1], none had characteristics of asthma. Preoperative lung function showed mean values for Inspiratory Vital Capacity (IVC) of 93.9 ± 4.2 % of the predicted value and a forced expiratory volume in 1 second as a percentage of IVC of 72.7 ± 3.6% of the predicted value. Medication during anesthesia was the same

for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane and pancuronium. Ten patients received steroids and/or theophylline before and during the operation.

13.3.2. Airway preparations

Thirty to sixty minutes after surgical resection a macroscopically normal part of the resected tissue was immersed in Krebs-Henseleit buffer of room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 5.55), which had been aerated with carbogen (95% O_2, 5% CO_2) to produce a pH of 7.35, a Pco_2 of 4.7 kPa and a Po_2 of 71.8 kPa. The tissue remained in fresh aerated buffer throughout the dissection procedure and the experiments. On the cut surface airways were identified, cannulated, taken out and dissected free from parenchyma and vessels under a stereo microscope (20x magnification) using iris-scissors and forceps. The cleaned airway was cut into segments of 4 to 5 mm length. For experiments with electric field stimulation (EFS), which were done on the day of operation, central cartilaginous airways (3rd to 6th generation) with a diameter of 3 to 6 mm were used. For pharmacological experiments segments from peripheral airways (6th to 11th generation) with a diameter of 2 to 3 mm were used. These were stored overnight in a slow flow of aerated buffer of 4°C, containing penicillin (3×10^{-5} g/l) and tobramycin (5×10^{-3} g/l). We formerly demonstrated that this storage procedure does not affect the contractility of the preparations to methacholine[11]. The next day the segments were mounted between two small polished stainless steel hooks (diameter 0.3 mm), and placed between a glass hook at the bottom of a double jacketed organ bath of 10 ml and a high precision isotonic angular position transducer (Penny and Giles, 3810/60, United Kingdom). The bath was siliconized (dimethyldichlorosilane, BDH, United Kingdom) to prevent adherence of the granulocytes. The signal from the transducer was monitored with a digital voltmeter (Fluke 73 multimeter, USA) and a pen-recorder (Kipp BD 40, The Netherlands). This method has been described in detail previously[25]. The preparations contracted against an isotonic load of 250 mg which has been shown to be optimal for human bronchial segments[25].

In all experiments the preparations were contracted twice with methacholine (10^{-5} M, 10^{-4} M) during an equilibration period of 2 hours in order to assess smooth muscle function. Between stimulations the preparations were washed 4 times with intervals of 2 minutes and every 15 minutes afterwards until stabilization of resting length which took 20 to 30 minutes.

13.3.3. Effect of cell activators on isolated peripheral airways

To find a cell activator that would not change airway caliber, cumulative concentration response curves (CCRC) were obtained with the following stimulants: calcium ionophore A23187, N-formyl-methionyl-leucyl-phenylalanine

(FMLP), phorbol 12-myristate 13-acetate, concanavalin A and serum treated zymosan (STZ). As shown in *Table* 13.1 STZ (0.01 to 1.0 mg/ml) was the only compound which did not alter the contractile state of the airways. The highest concentration of STZ that would remain in suspension was 0.2 mg/ml and therefore this concentration was used to activate the granulocytes. It gives maximal H_2O_2 production by neutrophils and eosinophils[42] and induces about 40% of the maximal LTC_4 formation from purified human eosinophils[8]. STZ was prepared by homogenizing zymosan at 4 mg/ml in fresh human serum with a Potter-Elvejhem homogenizer, followed by incubation of the mixture for 30 minutes at 37°C. After centrifugation and 2 washings, the STZ was suspended in 0.9% NaCl at 20 mg/ml[36].

13.3.4. Donors and isolation of granulocytes

Granulocytes were isolated either from laboratory personnel (n = 5) or subjects under control for hemostasis (n = 5) who denied symptoms of allergy. Granulocytes from atopic donors (n = 3) were also used to see if these had similar effects. The characteristics of the atopic donors are given in *Table* 13.2. The number of observations with granulocytes from atopic donors is indicated with the results. The granulocytes were isolated according to the method by Roos *et al.*[37]. Briefly, heparinized blood (20 to 30 ml) was diluted 2 times with phosphate buffered saline (PBS) of pH 7.4 containing 13 mM trisodium citrate, and centrifuged (1000g) at room temperature over a pyrogen-free Ficoll-Metrizoate gradient of 1.077 g/ml density (Nycomed, Norway) for 20 min. The erythrocytes in the pellet were lysed by repeated incubation (15 min) with an excess of ice-cold isotonic ammoniumchloride. After centrifugation the cells were washed twice and suspended in PBS containing 0.5% (wt/wt) bovine albumin (Sigma, USA). Total cell number was counted in a Bürker cell chamber. Viability was tested with the trypan blue exclusion test. Cytocentrifuge smears of each isolate were

Table 13.1. Sensitivity and maximal responses of peripheral human airways to different cell stimulants

stimulant	n	concentration range	$-\log EC_{50}$	maximal contraction (% max)[a]
A23187	6	10^{-9} M - 10^{-6} M	6.4 ± 0.14	69.5 ± 12.1
FMLP	7	10^{-9} M - 5 x 10^{-6} M	6.9 ± 0.18	66.2 ± 8.3
PMA	6	10^{-8} M - 5 x 10^{-6} M	–[b]	19.0 ± 10.0[b]
CON A	3	10^{-8} M - 10^{-6} M	6.4 ± 0.28	28.5 ± 6.3
STZ	5	0.01 - 1.0 mg/ml	no effect	

Abbreviations: n, number of preparations from different lungs; A23187, calcium ionophore A23187; FMLP, N-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; Con A, concanavalin A; STZ, serum treated zymosan.

[a] Maximal contraction is expressed as a % of the maximal contraction to methacholine.

[b] The $-\log EC_{50}$ could not be computed. PMA slightly relaxed the airway up to 10^{-7} M and caused contraction at higher concentrations.

Table 13.2. Characteristics of the atopic patients from whom granulocytes were obtained

age	sex	RAST	asthma/ eczema/ rhinitis	$PD_{20}FEV_1$ histamine (μg)[a]	% eo	medication[b]
28	M	C, HDM	a, e	100	5.6%	none
22	F	C, HDM, P	a, r	120	7.5%	none
30	M	C, HDM	a	78	5.8%	none

Abbreviations: RAST, strongly positive (> 3+) radioallergosorbent test; $PD_{20}FEV_1$, the provocative dose that caused a 20% fall in FEV_1 from baseline; C, cat danders; HDM, house-dust mite; P, grass pollens; a, asthma defined as paroxysms of wheezing; e, eczema; r, rhinitis; %eo, eosinophils as a percentage of leukocytes in peripheral blood.

[a] dosimeter method[9].

[b] within 24 hours prior to donation.

prepared from 10 μl of cell suspension and differential counts were obtained after staining according to May-Grünwald/Giemsa.

13.3.5. Influence of granulocytes activated with serum-treated zymosan on peripheral airways

The influence of ZAG on the contractile state and sensitivity to methacholine of the airway preparations was investigated as follows. Half an hour after a first a CCRC to methacholine (10^{-8} M to 10^{-4} M) either 1, 2, 5, 10, or 20 x 10^6 granulocytes were added to the organ bath. Time-parallel controls received no granulocytes. After stabilization, usually 10 minutes later, STZ was added (0.2 mg/ml), which elicited a contractile response. *Figure* 13.1 (upper panel) illustrates the response to addition and activation of 10 x 10^6 granulocytes. Once this response had reached a plateau a second CCRC was obtained in the presence of the ZAG (not shown in *Figure* 13.1). After washing and stabilization a third CCRC to methacholine was made.

13.3.6. Mechanism of bronchoconstriction by zymosan-activated granulocytes

To elucidate if eicosanoids or histamine contributed to the bronchoconstriction evoked by ZAG, portions of 10 x 10^6 granulocytes suspended in 0.5 ml of PBS were incubated at 37°C with either the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA)(10^{-5} M), the cyclooxygenase inhibitor indomethacin (10^{-5} M), the LTC_4/D_4 receptor antagonist FPL 55712 (11.5 x 10^{-6} M) or the histamine-receptor antagonist mepyramine (2.8 x 10^{-6} M). At this concentration NDGA will totally inhibit the formation of lipoxygenase products and indomethacin will totally block the formation of cycloxygenase but not lipoxygenase products by human granulocytes stimulated with calcium-ionophore[33]. FPL 55712 at this concentration will totally prevent the response to 10^{-7} M LTC_4 which causes maximal contraction of human trachealis[24], while mepyramine at this concen-

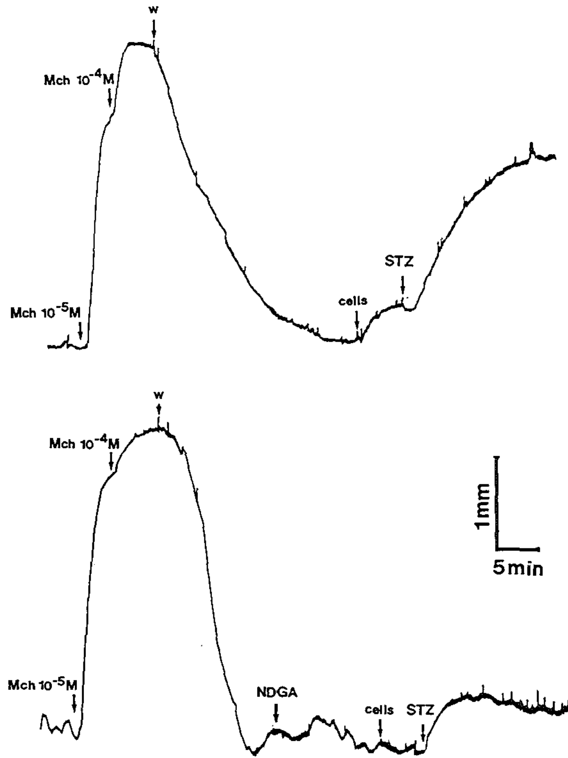

Figure 13.1. Recording of a typical response of human bronchus to 10 x 10^6 ZAG in the absence (upper tracing) and presence (lower tracing) of the lipoxygenase inhibitor NDGA (10^{-5} M), preceded by a graded contraction to methacholine. At the points indicated by the arrows methacholine (Mch), NDGA, 10 x 10^6 granulocytes (cells) or serum-treated zymosan 0.2 mg/ml (STZ) were added to the organ bath. Washing is indicated with "w".

tration blocks the contraction of isolated human bronchi to 10^{-4} M histamine[5]. Simultaneously, bronchial segments were incubated with the same concentrations of these drugs. After 15 minutes of incubation the granulocytes were transferred to the corresponding organ baths. STZ (0.2 mg/ml) was added after stabilization. Time-parallel control preparations and granulocyte suspensions were also incubated without a drug at 37°C for 15 minutes.

To see if oxygen metabolites were involved in the contraction of airway smooth muscle to ZAG a combination of superoxide dismutase (SOD) (300 U/ml) and bovine catalase (5000 U/ml) was added to the organ bath just before addition of 10 x 10^6 granulocytes. At these concentrations SOD will immediately metabolize O_2^- into H_2O_2[30] and catalase will degrade H_2O_2 into H_2O and O_2[40] so that the formation of free O_2^- or H_2O_2 is inhibited. After stabilization STZ (0.2 mg/ml) was added.

13.3.7. Effect of zymosan-activated granulocytes on responses to electric field stimulation in central airways

Two segments of central cartilaginous airways from each tissue specimen were mounted in an organ bath under a 500 mg isotonic load and stimulated twice with methacholine (10^{-5} M, 10^{-4} M). As described in detail before[12] EFS was applied with a custom-made tissue stimulator that produced voltage-constant rectangular pulses of alternating polarity, via platinum plate electrodes positioned parallel to the preparation (see *Figure* 9.1). 30-s Tetani of supra-maximal voltage (50V), a short pulse duration (0.3 ms) and a frequency of 30Hz were used to produce near-maximal responses. We have previously shown that these stimuli lead to a triphasic contraction-relaxation-contraction response of the airway muscle. The initial contractile phase results from activation of cholinergic excitatory nerves. The relaxation phase is non-adrenergic and partly inhibited by the neurotoxin tetrodotoxin[12,13]. In addition, EFS gives rise to a tetrodotoxin-insensitive slow contractile response which results from the release of metabolites of arachidonic acid in the bronchial wall[12]. A typical response is shown in *Figure* 13.2. First, a control response to EFS was obtained in each of two airway segments from 11 tissue specimens. After 30 minutes 5 x 10^6 granulocytes, the highest

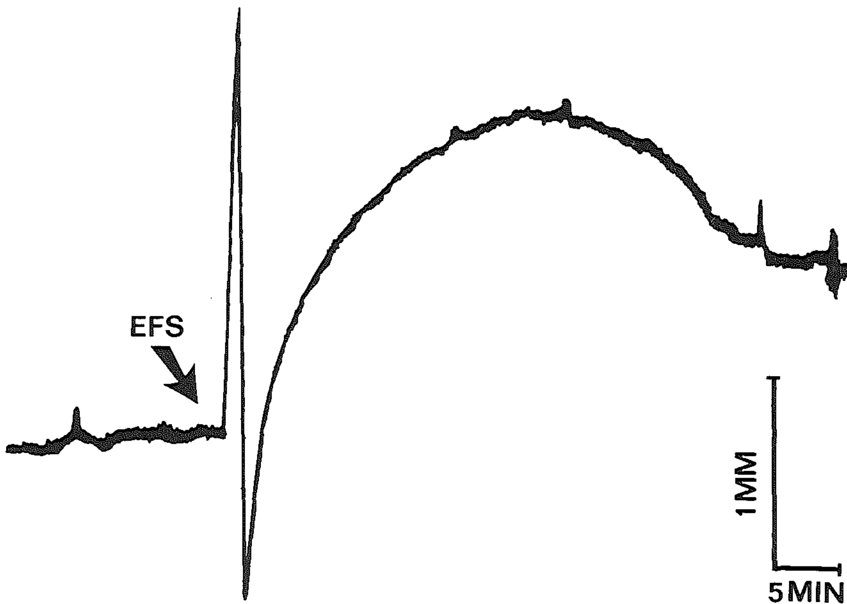

Figure 13.2. Tracing of a typical response of a fresh human bronchus to electric field stimulation (EFS) *in vitro*. At the point indicated by the arrow a 30 s tetanus of supramaximal voltage (50 V), short pulse duration (0.3 ms) and a frequency of 30 Hz was given. A cholinergic contraction is followed by a deflection below baseline (non-adrenergic relaxation) and a slow contraction[12].

number that would not significantly alter smooth muscle sensitivity to methacholine (see *Table* 13.3), were added to one of the two organ baths and activated with STZ (0.2 mg/ml). The control preparation received methacholine to match the increase in tone induced by 5 x 10^6 ZAG because earlier findings had indicated that the response to EFS depended on baseline airway tone[14]. Half an hour later a third response to EFS was obtained after contracting both the control and the ZAG treated airway with methacholine to a similar level as before the second EFS stimulation. The effect of ZAG on the non-adrenergic relaxation response to EFS was examined in separate experiments. Two airway segments from each of 7 tissue specimens were incubated with a combination of FPL 55712 (11.5 x 10^{-6} M), atropine (1.2 x 10^{-6} M) and indomethacin (6 x 10^{-6} M) for 20 minutes prior to stimulation, to inhibit the cholinergic peak and the slow contractile phase of the response to EFS[12]. Next, one preparation received 5 x 10^6 granulocytes, which were stimulated with STZ (0.2 mg/ml), while the other remained in normal buffer. Then the segments were precontracted with histamine (5 x 10^{-6} M). After stabilization a graded pulse train was applied (0.3 ms, 50V, 1 - 2 - 5 - 10 - 20 - 50 Hz) to obtain frequency response curves of EFS-induced relaxation[13].

13.3.8. Data analysis

Contractions were expressed as a percentage of the second response to methacholine 10^{-4} M, obtained at the beginning of each experiment (see 13.3.2). Relaxations were expressed as a percentage of the maximal relaxation in the presence of isoprenaline (10^{-4} M) and ethylenediamine tetra-acetic acid (EDTA) (4 x 10^{-3} M), determined at the end of the experiment. The sensitivity to methacholine was expressed as the negative logarithm of the methacholine concentration that caused 50% of the maximal narrowing ($-\log EC_{50}$). The sensitivity to EFS was defined as the effective frequency that causes half-maximal relaxation to EFS (EF_{50}). The $-\log EC_{50}$ and EF_{50} were calculated using the BMDP software module for non-linear regression[6]. The curves were fitted to a four parameter logistic function[16]. Contractions, relaxations and EF_{50} were compared with paired t-tests. Values of $-\log EC_{50}$ were analyzed with repeated measurements analysis of variance (ANOVA) using the MANOVA program of SPSS^x [38]. Correlations were examined by calculating Spearman's rank correlation coefficient (r_s). P values <0.05 (twosided) were considered significant. All data are presented as mean $\pm$ SEM.

13.3.9. Drugs

Methacholine, histamine (both Janssen Pharmaceutica, Belgium), atropine (Brocacef, The Netherlands), mepyramine (Rhône-Poulenc, France), bovine catalase (Boehringer Mannheim, W-Germany), superoxide dismutase, EDTA (both Sigma, USA) and concanavalin A (Pharmacia, Sweden) were dissolved in saline.

Indomethacin (Duchefa, The Netherlands), nordihydroguaiaretic acid (Sigma, USA) and FPL 55712 (Fisons, United Kingdom) were dissolved in methanol. Phorbol 12-myristate 13-acetate, calcium ionophore A23187 and N-formyl-methionyl-leucyl-phenylalanine (all from Sigma, USA) were dissolved in dimethylsulfoxide. L-isoproterenol sulphate (Janssen Pharmaceutica, Belgium) was dissolved in water containing ascorbic acid (88 mg/l). Preliminary experiments showed that ascorbate, methanol and dimethylsulfoxide in these concentrations have no effect on airway muscle function.

13.4. Results

13.4.1. Granulocytes

Granulocyte suspensions from healthy volunteers consisted of 93.8 ± 0.8% neutrophils, 4.0 ± 0.6% eosinophils, 2.2 ± 0.4% lymphocytes and those of the atopic donors of 81.4 ± 2.3% neutrophils, 14.0 ± 1.9% eosinophils and 3.3 ± 0.9% lymphocytes ($P<0.001$ for difference in % neutrophils and eosinophils). Viability exceeded 96%.

13.4.2. Influence of zymosan-activated granulocytes on peripheral airways

Figure 13.3 shows that activation of 1, 2, 5, 10 or 20 x 10^6 granulocytes with STZ (0.2 mg/ml) produced significantly more bronchoconstriction than unactivated granulocytes ($n = 4$ to 7, $P<0.05$). The bronchoconstriction was proportional to the number of zymosan activated granulocytes (ZAG) present ($r_s = 0.79$, $P<0.001$). *Table* 13.3 shows that in these experiments the sensitivity ($-\log EC_{50}$) of the airways to methacholine was unchanged in the presence of 1, 2 or 5 x 10^6 ZAG, but was significantly lower with 10 or 20 x 10^6 ZAG ($P<0.05$). ZAG from atopic donors were used in 2 to 3 experiments.

13.4.3. Mechanism of bronchoconstriction by zymosan-activated granulocytes

Figure 13.4 shows the contraction induced by 10 x 10^6 granulocytes in the presence of different drugs compared to the effect on paired control preparations of the same number of granulocytes from the same donors without drugs. The lipoxygenase inhibitor NDGA reduced both the contraction after addition of granulocytes (0.4 ± 3.5% versus 10.2 ± 2.7%, $n = 16$, $P<0.05$) and the contraction after activation of the granulocytes (16.2 ± 7.2% versus 49.6 ± 5.6%, $n = 16$, $P<0.001$). This is illustrated in *Figure* 13.1. FPL 55712 did not reduce the contraction to unstimulated granulocytes (8.6 ± 5.1% versus 10.2 ± 5.7%, $n = 16$, $P>0.05$) but significantly diminished the contraction to activated granulocytes (49.6 ± 5.6 versus 15.2 ± 8.3%, $n = 16$, $P<0.001$). Neither indomethacin ($n = 7$), nor mepyramine ($n = 5$), nor the combination of superoxide dismutase

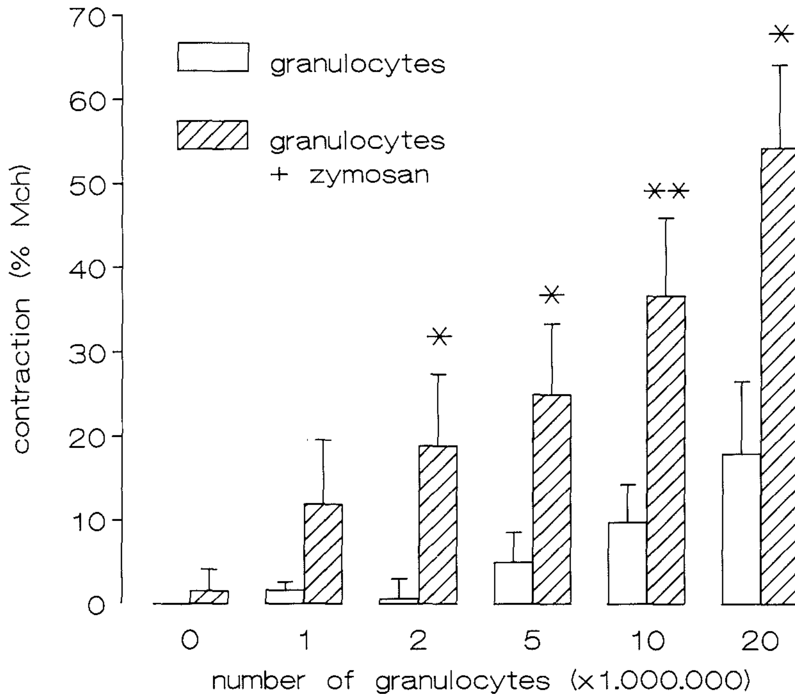

Figure 13.3. Mean contractile responses to increasing numbers of granulocytes. The open bars show the contractile response to unstimulated granulocytes. The hatched bars show the contraction caused by the same granulocytes after stimulation with STZ (0.2 mg/ml). The horizontal axis gives the number of granulocytes. The vertical axis represents the contractile state of the preparations expressed as percent of the maximal contraction to methacholine (%Mch). n = 4 to 7.
* P<0.05, ** P<0.01, compared to the response with unstimulated granulocytes.

Table 13.3. $-\text{LogEC}_{50}$ values of methacholine before, during and after addition of granulocytes activated with serum-treated zymosan

		$-\log\text{EC}_{50}$ methacholine[a]		
number of granulocytes	n	before granulocytes	with granulocytes	after granulocytes
none	7 (3)	5.94 ± 0.10	6.00 ± 0.10	5.95 ± 0.09
1×10^6	6 (3)	6.00 ± 0.13	6.07 ± 0.12	5.99 ± 0.14
2×10^6	6 (3)	6.05 ± 0.10	6.01 ± 0.18	5.95 ± 0.11
5×10^6	7 (3)	5.98 ± 0.05	5.97 ± 0.15	5.97 ± 0.04
10×10^6	6 (3)	6.03 ± 0.06	5.88 ± 0.11*	6.00 ± 0.07
20×10^6	4 (2)	6.12 ± 0.12	5.76 ± 0.14***	6.07 ± 0.08

Abbreviations: n, number of preparations from different lungs, the numbers between brackets indicate the number of observations with ZAG from atopic donors.
* P<0.05, *** P<0.001, presence of ZAG is a significant factor in ANOVA.
[a] Of each preparation the first CCRC was obtained before ZAG, the second in the presence of ZAG and the third after washout.

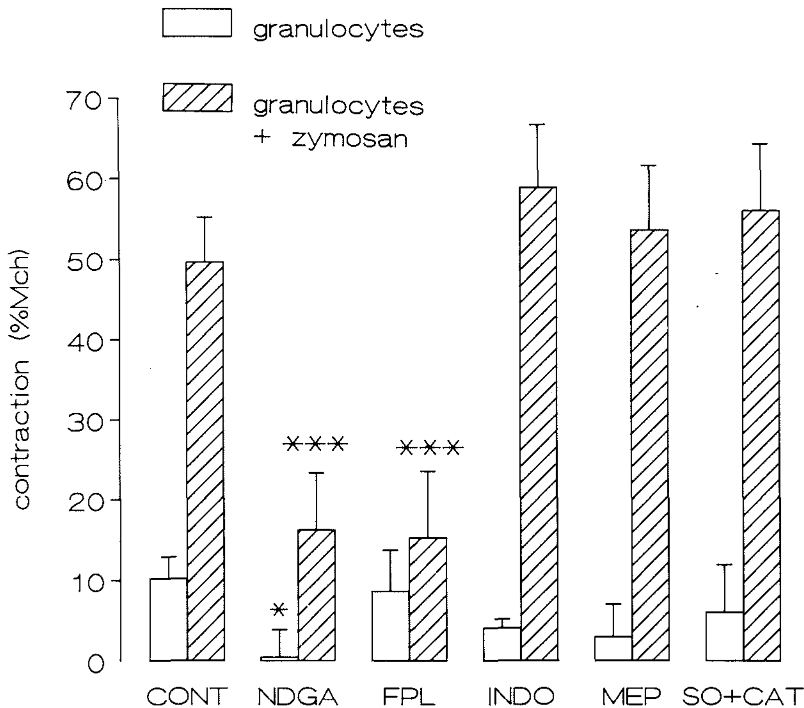

Figure 13.4. Influence of different drugs on the contractile response to 10 x 10^6 granulocytes. The open bars show the contractile response after incubation with unstimulated granulocytes in the presence of the different drugs. The hatched bars show the contraction caused by the same granulocytes after stimulation with STZ (0.2 mg/ml). The following drugs were used: NDGA (10^{-5} M, n = 16), FPL 55712 (11.5 x 10^{-6} M, n = 16), indomethacin (10^{-5} M, n = 7), mepyramine (2.8 x 10^{-6} M, n = 5) and a combination of superoxide dismutase (300 units/ml) and bovine catalase (5000 units/ml, n = 5). Controls (n = 16) received granulocytes but no drugs. Vertical axis, the contractile state of the preparations expressed as a % of the maximal contraction to methacholine (%Mch). * $P<0.05$, *** $P<0.001$, compared to controls.

and bovine catalase (n = 5) significantly changed the contractile effect of unactivated or activated granulocytes. *Figure* 13.5 shows that the magnitude of the contraction induced by 10 x 10^6 activated granulocytes in control preparations correlated positively with the logarithm of the number of eosinophils present in a particular cell-isolate ($r_s = 0.66$, $P<0.01$, n = 16). Granulocytes from atopic donors were used in 6 experiments with FPL 55712 and NDGA, in 4 experiments with indomethacin and in 2 experiments with mepyramine or superoxide dismutase + catalase. *Figure* 13.5 also illustrates that in these experiments ZAG from atopic donors contained a higher percentage of eosinophils than ZAG from non-atopic donors and caused more contraction than ZAG from non-atopic donors (68.2 ± 6.2% versus 45.5 ± 5.3%, $P<0.02$).

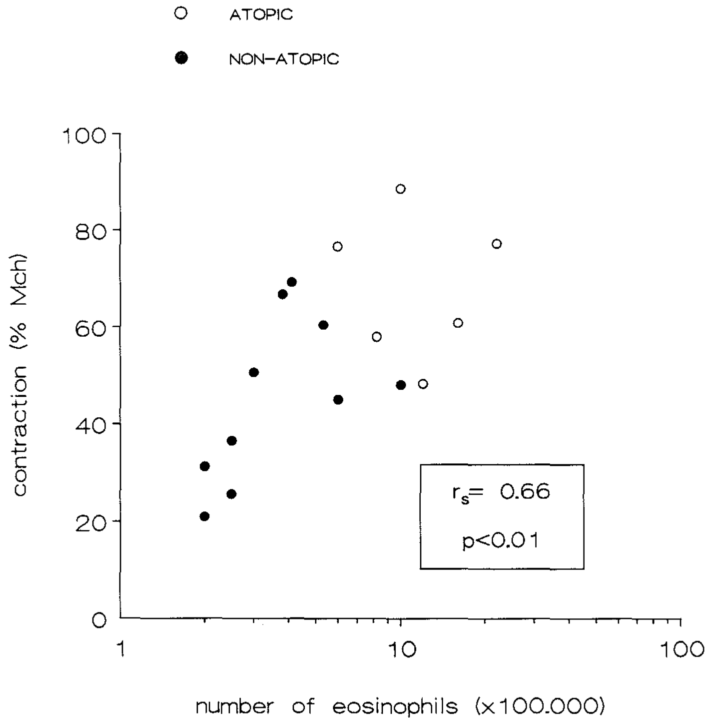

Figure 13.5. Correlation between the number of eosinophils present in 10 x 10^6 granulocytes stimulated with STZ (0.2 mg/ml) and the magnitude of the contraction induced by these granulocytes. The logarithmic horizontal axis gives the number of eosinophils present in 10 x 10^6 granulocytes. Vertical axis, the contraction induced by these granulocytes expressed as percent of the maximal contraction to methacholine (%Mch). The closed circles represent the mean results from granulocytes from normal donors (n = 10) and the open circles the mean results with granulocytes from atopic donors (n = 6) (see 13.4.3). *Abbreviations*: r_s, Spearman's rank correlation coefficient.

13.4.4. Responses of airways from smokers and patients on steroids

The responses of airway segments from non-smokers (n = 3) and smokers (n = 20) to 10 x 10^6 ZAG were similar (50.9 ± 13.3% versus 54.7 ± 5.4%; % eosinophils: 4.8 ± 1.2% versus 7.7 ± 1.7%) and the responses of airways from patients who were treated (n = 10) or were not treated (n = 13) with steroids were also similar (50.7 ± 7.2% versus 57.2 ± 6.7%; % eosinophils: 6.7 ± 1.4 versus 7.6 ± 2.3). None of the responses differed significantly nor did the percentage of eosinophils.

13.4.5. Effect of zymosan-activated granulocytes on responses of central airways to electric field stimulation

Table 13.4 shows that the magnitude of the cholinergic contraction to EFS did not differ significantly between controls and preparations treated with 5 x 10^6 ZAG. In the presence of 5 x 10^6 ZAG the slow contractile response was 16.7 ± 6.8% higher than in the absence whereas in controls this was 2.0 ± 2.9% ($P<0.05$). The increase was reversible so that after washout of the cells the slow phase was not different from its initial value. *Table* 13.4 also shows the results with ZAG from atopic donors which were used in 5 of the 11 experiments. With these cells the results were similar, but the effect of the cells on the slow contractile response failed to achieve significance ($P = 0.11$). *Figure* 13.6 shows frequency response curves of relaxation to EFS. Precontraction was similar in the presence and absence of 5 x 10^6 ZAG (59.6 ± 5.7% versus 58.4 ± 6.2% in controls, NS, n = 7). The maximal relaxation to EFS was similar in control preparations and in preparations treated with 5 x 10^6 ZAG (51.3 ± 8.0% versus 53.0 ± 7.2%, NS, n = 7). EF_{50} was also similar in both groups (2.34 ± 0.63 versus 2.53 ± 0.70 in controls, NS, n = 7). ZAG from atopic donors which were used in 4/7 experiments also had no effect on the relaxation to EFS.

Table 13.4. Responses to electric field stimulation before, during and after addition of granulocytes activated with serum-treated zymosan[a]

		before granulocytes	during granulocytes	after granulocytes
non-atopics[b]				
treated:	cholinergic	44.6 ± 5.9	43.7 ± 9.5	43.8 ± 10.1
	slow phase	21.8 ± 5.7	38.5 ± 8.0*	25.1 ± 7.1
controls:	cholinergic	43.7 ± 6.1	46.5 ± 8.7	44.2 ± 9.4
	slow phase	32.6 ± 9.3	34.7 ± 9.9	27.5 ± 7.8
atopics[c]				
treated:	cholinergic	47.5 ± 3.3	47.1 ± 6.8	49.2 ± 8.5
	slow phase	11.7 ± 3.6	29.5 ± 7.7[d]	18.3 ± 6.2
controls:	cholinergic	37.6 ± 7.4	42.2 ± 9.0	38.6 ± 9.0
	slow phase	26.7 ± 8.5	33.4 ± 12.1	23.3 ± 7.4

* $P<0.05$, for comparison of difference between second and first response in controls and in treated preparations.

[a] The cholinergic and slow phase of the response to EFS (see *Figure* 13.2) are expressed as a percentage of the maximal contraction to methacholine (10^{-4} M) starting from baseline.

[b] Non-atopics: n = 6, granulocytes from 6 donors.

[c] Atopics: n = 5, granulocytes from 3 donors.

[d] P = 0.11

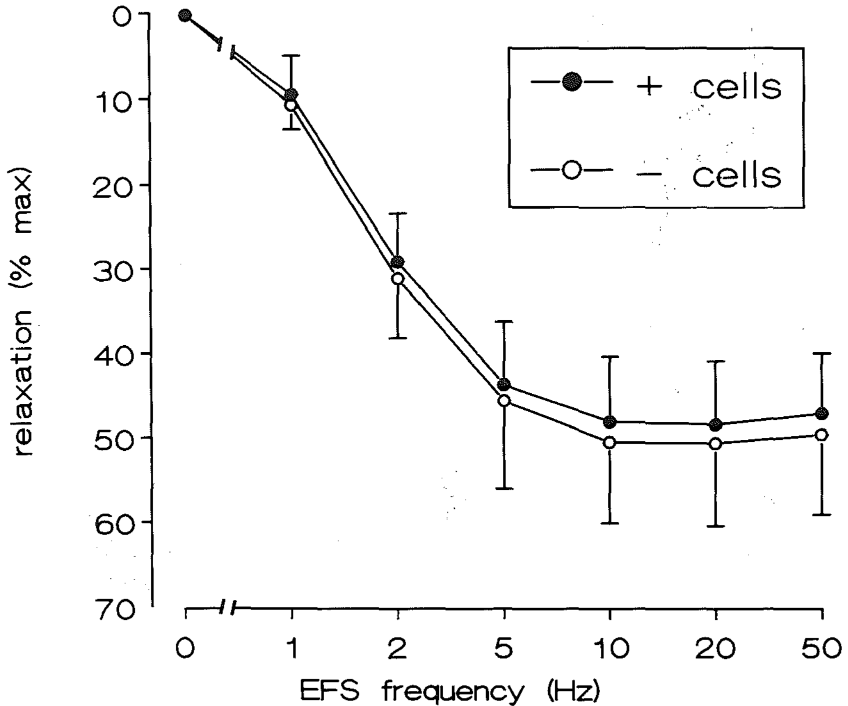

Figure 13.6. Frequency response curves of non-adrenergic relaxations of central human airways to electric field stimulation obtained in the presence (closed symbols) and absence (open symbols) of 5 x 10^6 ZAG after precontraction with histamine (5 x 10^{-6} M). To inhibit the cholinergic and sustained contractile phase (see *Figure* 13.2) the preparations were pretreated with atropine (1.2 x 10^{-6} M), indomethacin (6 x 10^{-6} M) and the leukotriene C_4/D_4 antagonist FPL 55712 (11.5 x 10^{-6} M). Vertical axis, relaxation of the preparations on a 0 to 100% scale which represents the difference between the precontraction with 5 x 10^{-6} M histamine (0%) and the maximal relaxation to isoprenaline in calcium-free buffer containing (4 x 10^{-3} M) EDTA (100%). Mean results from 7 preparations from different lung tissue specimens.

13.5. Discussion

Our results show that incubation of isolated human airways with increasing numbers of granulocytes from atopic or non-atopic donors leads to increasing bronchoconstriction. Addition of STZ, a cell-stimulator which itself does not alter airway muscle tone, leads to extra bronchoconstriction which can be prevented both with a lipoxygenase inhibitor and a LTC_4/D_4 receptor antagonist, but not with a cyclooxygenase inhibitor, a histamine (H_1) receptor antagonist or scavengers of activated oxygen molecules. The magnitude of the contraction to ZAG is related to the proportion of eosinophils in the cell-isolate. The sensitivity of the airways to methacholine is unchanged in the presence of 1, 2 or 5 x 10^6 ZAG, but decreases with 10 or 20 x 10^6 ZAG. In the presence of 5 x 10^6

ZAG the cholinergic contraction and the non-adrenergic relaxation to near-maximal EFS remain unchanged and the slow response is slightly potentiated.

Hallahan *et al.*[21] have recently found that supernatants from 10^6 neutrophils or 10^5 eosinophils which had been activated with calcium ionophore did not contract isolated human airways. Our data seem to confirm these findings since *Figure* 13.3 shows that 10^6 activated granulocytes (mostly neutrophils) will not contract the airways significantly and *Figure* 13.5 suggests that 10^5 eosinophils, even in the presence of millions of neutrophils will hardly contract the airways. Furthermore, Hallahan *et al.*[21] found that their supernatants increased the response to bolus doses of histamine by 54 to 60% while the cholinergic twitch to EFS increased by only 18% with the neutrophil supernatant and was unchanged with the eosinophil supernatant. We did not find an increase in cholinergic responsiveness with ZAG but, as suggested by the findings of Hallahan *et al.*[21] on the cholinergic twitch to EFS, this may be due to the small effect that products from granulocytes seem to have on the cholinergic responsiveness of isolated human airways. It is also possible that we missed an effect of the cells on airway responsiveness since we started stimulating the airway after the response to ZAG had reached a plateau while the increase in cholinergic responsiveness described by Hallahan *et al.* seems to be short-lived (J.L. Black, personal communication). Alternatively, this discrepancy may be due to differences in methods of cell stimulation (ionophore versus zymosan), parameters of EFS (4 to 16 Hz versus 30 Hz) or numbers of cells (10^5 to 10^6 versus 5 x 10^6).

Since STZ itself did not contract the airway segments the contraction to ZAG must be secondary to activation of the granulocytes. This contraction is most likely due to the release of LTC_4/D_4 because it was reduced by about 70% both with NDGA and FPL 55712. The significant correlation between the number of activated eosinophils in the organ bath and the magnitude of the contraction suggests that eosinophils are implicated in the production of LTC_4. There seem to be two possible ways in which eosinophils could play a role in the release of LTC_4. Firstly, activated eosinophils might activate other cells (e.g. mastcells) to produce LTC_4. Although such a mechanism would be in keeping with our results it seems unlikely since in that case we would have expected some release of histamine and we found that the response to ZAG was unaltered with a H_1-receptor antagonist. Secondly, the LTC_4 could be released by the eosinophils themselves probably through interaction of STZ with complement receptors on the cell surface[8]. Thus, the most likely explanation for our findings seems that the eosinophils among the activated granulocytes release LTC_4 which contracts the airways. However, an indirect role for the neutrophils in the granulocyte-induced contraction seems likely. It has been shown that mixing of pure fractions of eosinophils and neutrophils in ratios varying from 1/10 to 6/10 results in a synergistic increase of LTC_4 production by eosinophils of 100% to 300%[27,28].

The moderate correlation ($r_s = 0.66$) between the proportion of eosinophils among the ZAG and the magnitude of the contraction (see *Figure* 13.5) suggests that other factors also contribute to the variability in the contractile response

to 10 x 10^6 ZAG. In *Figure* 13.5 each data point represents the contraction of an airway segment of a given lung tissue specimen to the granulocytes of a particular donor isolated on a particular day. Therefore, differences in sensitivity to LTC_4 between airways from different lung tissue specimens, qualitative differences between eosinophils of different donors, and the inter-day variability in the procedure of granulocyte-isolation will also contribute to the variability in the contractile response.

We found no differences in the contraction to 10 x 10^6 ZAG (with a similar proportion of eosinophils) between airways from current smokers and non-smokers and airways from patients who received steroids and those who did not. Therefore the inflammatory state of the airways does not seem to influence the magnitude of the contraction to ZAG. If we accept that the contraction to ZAG is predominantly due to LTC_4, this finding is not unexpected. We have previously shown that although the sensitivity to LTC_4 of airways from different lung tissue specimens can differ by up to a factor 10, the variability in the responsiveness to LTC_4 is not related to the severity of airway inflammation[15].

The small contraction to unactivated granulocytes suggests that the isolation procedure might have primed or activated the granulocytes to some extent. It is possible that the cells were exposed to traces of lipopolysaccharides during the isolation procedure and this is known to modulate neutrophil function[22]. Also, the 3% lymphocytes or occasional platelets could have influenced the activation state. Alternatively, the hooks, the silk threads, and the airway preparation itself may have been acting as a mild stimulus.

The contraction to unactivated granulocytes was prevented by the lipoxygenase inhibitor NDGA, but not the LTC_4/D_4 antagonist FPL 55712. This indicates that the contraction to unactivated granulocytes may be due to a lipoxygenase product other than LTC_4/D_4, e.g. leukotriene B_4 (LTB_4) that can be released by neutrophils[39] and is a weak, indirect constrictor of isolated guinea pig trachea[29].

Incubation with up to 5 x 10^6 ZAG did not alter the sensitivity of the airways to methacholine or the cholinergic contraction to optimal EFS. This is in agreement with previous findings that low doses of LTC_4 do not increase the responsiveness of human airway muscle to methacholine[26]. It suggests that ZAG do not release any substance which affects smooth muscle sensitivity or cholinergic neurotransmission. We can not exclude that ZAG increases the sensitivity to EFS since we used only a single frequency (30 Hz) to stimulate the airways near-maximally. We could not make frequency response curves with the cells present because the slow contractile phase which follows one pulse train interferes with subsequent responses[12]. This can only be circumvented by unpractically long intervals between succesive stimulations or the addition of eicosanoid inhibitors[12].

It has been hypothesized that asthmatic bronchoconstriction could be due to inactivation of muscle relaxing neuropeptides by peptidases released from granulocytes in the airway wall[2]. Our finding that ZAG did not reduce the non-adrenergic EFS-induced relaxation suggests that if peptides mediate this

EFS-induced relaxation[34], they are not acutely metabolized by products from ZAG. Therefore our finding argues against this hypothesis.

The slow response to EFS is potentiated in the presence of ZAG. In an earlier study[12] we found that the sustained contraction which follows the cholinergic twitch (see *Figure* 13.1) was almost abolished by pretreatment with the LTC_4/D_4 receptor antagonist FPL 55712 (11.5 x 10^{-6} M). Consequently, the small increase of the sustained contraction may well be due to an extra production of LTC_4 after EFS in the presence of ZAG.

The reversible decrease in the sensitivity of the airways to methacholine with 10 or 20 x 10^6 ZAG could be due to several factors. The contraction caused by ZAG may have influenced $-\log EC_{50}$, but this seems unlikely because $-\log EC_{50}$ does not vary with BCS[26]. Therefore, it seems most likely that the reversible decrease in sensitivity was specifically due to the ZAG, but the mechanism remains to be elucidated.

The effects of ZAG from atopics were similar to ZAG from non-atopics. The only difference was that ZAG from atopics caused bigger contractions than ZAG from non-atopics (see *Figure* 13.5), and this was related to the significantly higher proportion of eosinophils in the ZAG of atopic donors. This suggests that granulocytes from atopic and non-atopic donors may be qualitatively similar in their acute effects on human airways.

Our results suggest that if granulocytes contribute to smooth muscle contraction during the late asthmatic reaction (LAR) they will mainly do so via the production of LTC_4, which is related to the proportion of eosinophils. However, we do not know how the concentration of granulocytes in the organ bath, the proportion of eosinophils among the granulocytes and the activation method that we have used compare to those in the airway wall during the LAR. Based on data from recent biopsy studies and morphometric studies[3,23] we have roughly estimated the number of granulocytes per mm^3 of bronchial tissue shortly after the LAR (see 13.6) and it seems possible that in our experiments the concentration of granulocytes in the organ bath is comparable to that in bronchial tissue shortly after the LAR. In BAL fluid obtained during the LAR about half the granulocytes are eosinophils[18]. If this reflects the proportion of eosinophils in the bronchial tissue the proportion of eosinophils in the granulocyte suspension that we used (2 to 22%, see *Figure* 13.5) may have been lower than that in the bronchial tissue during the LAR. Since we found that a higher proportion of eosinophils was related to more bronchoconstriction our results may underestimate the bronchoconstrictor potential of granulocytes during the LAR. However, we do not know if activation of granulocytes with zymosan is comparable to activation of granulocytes during the LAR.

There is some *in vivo* evidence to suggest that LTC_4 is produced during the LAR. Diaz *et al.*[18] found increased numbers of eosinophils together with a small elevation of LTC_4 in bronchoalveolar lavage fluid obtained during allergen-induced LAR. However, it is difficult to know if increased LTC_4-levels contribute to bronchoconstriction during the LAR since studies with leukotriene antagonists

in humans are scanty. One study[20] suggested that the LTD_4-antagonist LY171883 was not active during the LAR but the plasma concentrations of the drug during the LAR were not measured. Preliminary results from Bel *et al.*[4] suggest that the leukotriene synthesis inhibitor MK-886 delays the LAR as long as the plasma concentrations of the drug are high enough to inhibit lipoxygenase in blood cells. Yamai *et al.*[41] reported that the LTC_4/D_4 receptor antagonist ONO-178 inhibits the late asthmatic response by about 50%, suggesting that LTC_4 is indeed involved in the LAR. Taken together these data seem to support a role for LTC_4 in the pathogenesis of the LAR.

In *conclusion*, we have shown that incubation of isolated human airways with ZAG from atopic or non-atopic donors leads to bronchoconstriction which is mainly due to the release of LTC_4, probably by activation of eosinophils. ZAG do not increase the sensitivity of the airways to methacholine or alter the cholinergic twitch or the non-adrenergic relaxation to near-maximal EFS. The slow response after EFS however is slightly potentiated in the presence of 5 x 10^6 ZAG, probably due to an enhanced production of LTC_4. If the activation method and the concentration of granulocytes that we have used are relevant to those in the airway wall during the LAR our results suggest that during the LAR activated granulocytes may do not increase airway muscle sensitivity but may contribute to muscle contraction probably via synthesis of LTC_4 by eosinophils.

13.6. Appendix

We have estimated the concentration of granulocytes in bronchial tissue shortly after the LAR as follows.

Bronchial biopsies taken from dual responders 18 h after allergen provocation contain around 2000 cells/mm^2 of submucosa, 70/mm^2 (i.e. 3.4%) being granulocytes[3]. In non-cartilaginous airways from asthmatics with dimensions similar to those we used (internal perimeter $>$ 2mm) the cross sectional area of the submucosa is about 0.14 mm^2[2,23] and would contain 0.14 mm^2 x 70 granulocytes/mm^2 = 10 granulocytes. If we consider that the thickness of a granulocyte is some 10μm, we can picture a 4 mm-long bronchial ring as composed of 400 sections with a thickness of 10μm, each containing 10 granulocytes. This means that the submucosa of a 4 mm-long bronchial ring from an asthmatic would contain 4mm/10μm x 10 = 4000 granulocytes. We have assumed that the volume in which these 4000 granulocytes release their products does not exceed that of the bronchial ring itself. If we model the airway segment as a cylinder with a length of 4 mm, a diameter of 2 to 3 mm in which the wall takes up 25% of the cross-sectional area such as it does in asthmatic airways[23], the segment would have a volume of 3 to 7 mm^3. This would imply a granulocyte concentration of 600 to 1300 granulocytes/mm^3 of bronchial ring tissue. To obtain a similar

concentration of granulocytes in a 10 ml (= 10.000 mm^3) organ bath 6 to 13 x 10^6 granulocytes would need to be present, which is comparable to the 10 x 10^6 granulocytes that we have used.

13.7. References

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Part IV

Physical Stimuli

Chapter 14

Effect of Cooling on Responses of Isolated Human Airways to Pharmacological and Electrical Stimulation*

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14.1. Summary

We studied the effect of cooling on the responses of isolated human airways to the β-agonist isoprenaline, the α/β-agonist noradrenaline in the presence of the β-blocker timolol, methacholine, leukotriene C_4 (LTC_4) and histamine. In addition, the effect of cooling on baseline airway tone and responses to electric field stimulation (EFS) was studied. At 27°C the sensitivity ($-logEC_{50}$) and maximal response to isoprenaline were unchanged. No measurable response was found to α-adrenergic stimulation with noradrenaline + timolol either before or during cooling. At 27°C and 21°C the sensitivity and maximal contraction to methacholine and LTC_4 as well as the contraction to a single dose of histamine were reduced. Cooling diminished baseline airway tone. EFS produced a rapid cholinergic contraction followed by a deflection below baseline and a sustained non-cholinergic contractile response, which was substantially reduced by the LTC_4/D_4 receptor antagonist FPL 55712 (11.5 x 10^{-6} M) at all three temperatures. Cooling decreased the cholinergic response to EFS and increased the sensitivity to EFS-induced relaxation. In contrast, the sustained non-cholinergic contractile response to EFS was not changed suggesting that cooling facilitates the synthesis of LTC_4/D_4 which follows EFS and/or inhibits its inactivation. We conclude that in non-asthmatic isolated human airways slow cooling of the airway wall down to 21°C does not cause bronchoconstriction and does not increase the responsiveness to contractile or relaxing agonists. However, cooling inreases the sensitivity to EFS-induced relaxation and might facilitate the accumulation of leukotriene C_4/D_4 in the airway wall. Whether these effects of airway cooling

* Jongejan RC, de Jongste JC, Raatgeep HC, Bonta IL, Kerrebijn KF. Effect of cooling on responses of isolated human airways to pharmacological and electrical stimulation. Am Rev Respir Dis 1990: (In Press). Printed with permission of the American Review of Respiratory Disease.

play a role in the pathogenesis of cold air-induced bronchoconstriction in asthma remains to be elucidated.

Key words: isolated human bronchi; bronchial smooth muscle; temperature; electric field stimulation; non-adrenergic relaxation; leukotrienes

14.2. Introduction

In asthmatics, in some patients with chronic bronchitis and occasionally in normal subjects[8,30,33,28] respiratory heat loss due to hyperpnea of cold air may initiate a bronchoconstrictor response[6,7]. The mechanism of this response is subject to debate[16]. Cooling of the airway wall has been thought to influence the regulation of airway tone by stimulation of afferent nerve endings, leading to reflex bronchoconstriction[31], or by release of inflammatory mediators from immunocompetent cells in the airway wall[15,18,24]. Others have hypothesized that functional conversion of adrenoreceptors from β to α may occur during cooling[35].

Because a better understanding of the direct effect of cooling on the human airway wall may improve the insight in the pathophysiology of cold air-induced bronchoconstriction we studied the effect of cooling on isolated human airways obtained at thoracotomies. We also investigated the effect of low temperatures on the responses to the β-agonist isoprenaline, the α/β-agonist noradrenaline in the presence of the β-blocker timolol, methacholine, leukotriene C_4 (LTC_4) and histamine. In addition, we assessed the effect of cooling on baseline intrinsic airway tone and on responses to electric field stimulation (EFS).

14.3. Methods

14.3.1. Patients and airway preparations

Human lung tissue specimens were obtained from 27 patients, 19 male and 8 female, with a mean age of 64.0 years (range 36 to 79 years). All were operated because of bronchial carcinoma. Twenty-five were smokers, 15 had chronic obstructive pulmonary disease according to the criteria of the American Thoracic Society[1], none had characteristics of asthma. Preoperative lung function showed mean values for Inspiratory Vital Capacity (IVC) of $93.6 \pm 4.3\%$ of the predicted value and a forced expiratory volume in 1 second as a percentage of IVC of 62.7 ± 2.3 %. Medication during anesthesia was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane and pancuronium. Eleven patients received steroids and/or theophylline before and during the operation. Within 30 minutes after surgical resection a macroscopically normal part of the resected tissue was immersed in Krebs-Henseleit buffer of room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25,

Glucose 5.55), which had been aerated with carbogen (95% O_2, 5% CO_2) to produce a pH of 7.35, a P_{CO_2} of 4.7 kPa and a P_{O_2} of 71.8 kPa. The tissue remained in fresh aerated buffer throughout the dissection procedure and the experiments. On the cut surface bronchi were identified, cannulated, taken out and dissected free from parenchyma and vessels under a 20x magnification stereo microscope. For pharmacological experiments these cleaned bronchial tubes were cut into segments which were mounted between two small polished stainless steel hooks (diameter 0.3 mm) and studied after overnight storage in a slow flow of aerated buffer of 4°C, containing penicillin (3 x 10^{-5} g/l) and tobramycin (5 x 10^{-3} g/l). We formerly demonstrated that this storage procedure does not affect the contractile function of the preparations[10]. For EFS experiments the cleaned bronchial tubes were spiralized under a 45° pitch to obtain strips, which were studied on the day of operation. All preparations were mounted in double jacketed 10 ml organ baths, which contained aerated Krebs-Henseleit buffer at 37°C, between a glass hook at the bottom of the bath and a high precision isotonic angular position transducer (Penny and Giles, 3810/60, Great Britain) which was connected to a digital voltmeter (Fluke 73 multimeter, USA) and a pen-recorder (Kipp BD 40, The Netherlands). To assess contractile function and to assure a stable contractility for the rest of the day the preparations were contracted twice with methacholine (10^{-5} M, 10^{-4} M). Between stimulations the preparations were washed 4 times with intervals of 2 minutes and every 15 minutes afterwards until stabilization of resting length. The strips contracted against an isotonic load of 1 g[11] and the segments against a load of 0.25 g[22].

14.3.2. Cooling and responses to pharmacological stimuli

For each agonist three segments from the same tissue specimen were studied. From each segment three consecutive Cumulative Concentration Response Curves (CCRC) to a single agonist were obtained. The first CCRC was made at 37°C; the second at either 37°C, 27°C or 21°C; and the third again at 37°C. The preparations were cooled by lowering the thermostat-setting of the circulating pump after which the desired temperature was reached in 15 to 20 minutes. The CCRC to the β-agonist isoprenaline (10^{-10} M to 10^{-5} M, n = 7), the α/β-agonist noradrenaline (10^{-8} M to 10^{-4} M, n = 4) in the presence of the β-receptor antagonist timolol (10^{-6} M), methacholine (10^{-8} M to 10^{-4} M, n = 7) or LTC_4 (10^{-10} M to 10^{-7} M, n = 7) were obtained by adding the agonist in small volumes ($< 100\ \mu L$) to the bath. Relaxation curves to isoprenaline were started after precontraction with methacholine (10^{-5} M).

14.3.3. Cooling and responses to electric field stimulation

EFS was applied with a custom-made tissue stimulator that produced voltage-constant rectangular pulses of alternating polarity via platinum plate electrodes parallel to the strips (see *Figure* 9.1). 30-s Tetani of supramaximal voltage (50V),

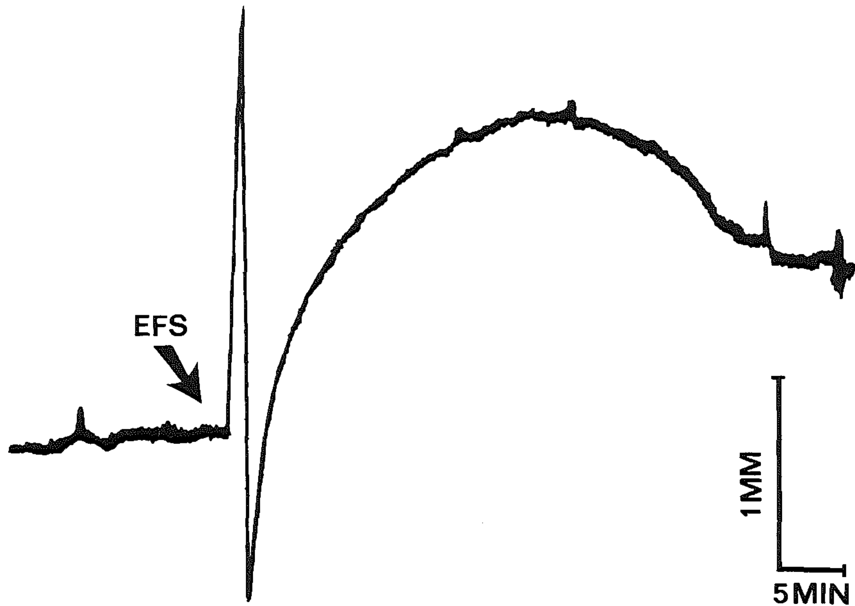

Figure 14.1. Tracing of a typical response of a fresh central human bronchus to electric field stimulation (EFS) *in vitro*. At the point indicated by the arrow a 30 s tetanus of supramaximal voltage (50 V), short pulse duration (0.3 ms) and a frequency of 30 Hz was given. A cholinergic contraction is followed by a deflection below baseline (non-adrenergic relaxation) and a slow contraction[11].

short pulse duration (0.3 ms) and a frequency of 30 Hz were used to produce near maximal responses[11]. We have previously shown that these stimuli lead to a triphasic contraction-relaxation-contraction response of the airway muscle. The contractile phase results from activation of cholinergic nerves. The relaxation phase is non-adrenergic and partially inhibited by the neurotoxin tetrodotoxin[11,12]. In addition, this stimulus gives rise to tetrodotoxin-insensitive slow contractile responses which result from the release of metabolites of arachidonic acid in the bronchial wall[11]. A typical response is shown in *Figure* 14.1. From each of 7 tissue specimens 3 bronchial strips were prepared and a first response to electric field stimulation (EFS) was obtained. Thirty minutes after the first responses, 2 of the 3 strips were cooled to 27°C and 21°C respectively. The third strip remained at 37°C and served as a time-parallel control. After stabilization, a second response to EFS was obtained. Thirty minutes later, after washout and stabilization, the preparations were rewarmed to 37°C and a third EFS response was obtained. The role of LTC_4/D_4 in the responses to EFS at these three temperatures was investigated in separate experiments where the LTC_4/D_4 receptor antagonist FPL 55712 (11.5 x 10^{-6}M M)[21] was added to the organ bath 15 minutes before EFS at 37°C, 27°C or 21°C.

To examine the effect of cooling on the non-adrenergic inhibitory response to EFS three strips from each of 7 tissue specimens were incubated for 10 minutes with atropine (1.2 x 10^{-6}M), indomethacin (6 x 10^{-6}M), and the leukotriene

C_4/D_4 antagonist FPL 55712 (11.5×10^{-6} M) before stimulation. This was done to inhibit the cholinergic peak and the slow contractile phase of the response to EFS[11] (see *Figure* 14.1). Next, two of the three preparations were cooled to 27°C and 21°C respectively, while the control preparation remained at 37°C. Then the segments were precontracted with histamine (5×10^{-6} M) and a graded pulse train was applied (0.3 ms, 50V, 1 - 2 - 5 - 10 - 20 - 50 Hz) to obtain frequency response curves of the non-adrenergic inhibitory system[12].

To investigate if cooling had any effect on the integrity of the epithelium preparations that had been treated with methacholine or EFS at 37°C and 21°C were processed for light microscopy and examined histologically. The percentage of the basement membrane covered with epithelium was estimated by an observer who was unaware of the previous treatment of the preparations.

14.3.4. Data analysis

The cholinergic and the slow contractile response to EFS (see *Figure* 14.1) were expressed as a percentage of the first cholinergic response to EFS at 37°C[11]. All other responses were expressed on a 0 - 100 % scale, which defines the maximal active contractile range (%MACR)[26]. The maximal contraction to methacholine (10^{-4} M) at the beginning of an experiment (see 14.3.1) was called 100% shortening. Maximal relaxation after 10^{-4} M isoprenaline in calcium-free buffer with 1.5×10^{-3} M ethylenediamine tetra-acetic acid (EDTA), determined at the end of each experiment, was called 0% shortening. On this MACR scale the following parameters are defined:

BCS: the baseline contractile state i.e. the spontaneous position of a preparation on the MACR scale under a given load.

E_{max}: the maximal effect of an agonist or EFS.

The sensitivity to isoprenaline, methacholine and LTC_4 was expressed as the negative logarithm of the concentration of these agonists that caused 50% of their maximal effect ($-\log EC_{50}$). The sensitivity to EFS-induced relaxations was expressed as EF_{50}, i.e. the effective frequency that caused 50% of the maximal relaxation. $-\log EC_{50}$ and EF_{50} were calculated using the BMDP software module for non-linear curvefitting[5]. The curves were fitted to a four parameter logistic function[14]. Since cooling changed BCS, E_{max} and $-\log EC_{50}$ it was important to know if the magnitude of cooling-induced changes in these parameters were related. Therefore cooling-induced changes in these parameters were plotted against each other and the correlation-coefficients computed. This was done for cooling to 27°C and 21°C both for methacholine and LTC_4.

Although for the relaxation experiments identical doses of methacholine or histamine were added at 37°C, 27°C or 21°C, the precontraction levels at these temperatures differed significantly. These differences in precontraction probably reflect differences in intracellular levels of second messengers which could have affected the values of $-\log EC_{50}$ or EF_{50}, as has been shown for isoprenaline[34]. Therefore we selected 5 pairs of preparations with similar precontraction at

the diferent temperatures (see 14.4.1), which enabled us to compare $-\log EC_{50}$ and EF_{50} values independent of the effect of temperature on precontraction. Values of $-\log EC_{50}$ and EF_{50} were compared with the Student t-test for unpaired samples (unpaired t-test). Student's t-test for paired samples was used for comparison of subgroups with similar precontraction (paired t-test). Correlations were examined with Spearman's rank correlation coefficient (r_s). Values of BCS, E_{max}, the contractile responses to EFS and the percentage of the basement mebrane covered with epithelium were evaluated with the Mann-Whitney U test (MWU-test). P values <0.05 (twosided) were considered significant. All data are presented as mean $\pm$ SEM.

14.3.5. Drugs

Methacholine hydrobromide (Janssen Pharmaceuticals, Belgium), EDTA (Sigma, USA), atropine (Brocacef, The Netherlands), timolol maleate (Merck, Sharp & Dôhme, USA) and synthetic LTC_4 (a gift from Merck Frosst, Canada) were dissolved in saline. FPL 55712 (a gift from Fisons, United Kingdom) and indomethacin (Duchefa, The Netherlands) were dissolved in methanol. L-isoproterenol sulphate (Janssen Pharmaceutica, Belgium) and L-noradrenaline hydrochloride (Fluka, Switzerland) were dissolved in water containing ascorbic acid (88 mg/l). Preliminary experiments showed that ascorbate and methanol in these concentrations have no effect on airway muscle function.

14.4. Results

14.4.1. Cooling and responses to pharmacological stimuli

Table 14.1 summarizes values of BCS, $-\log EC_{50}$ and E_{max} for isoprenaline, noradrenaline, methacholine and LTC_4. In segments exposed to isoprenaline the mean precontraction to methacholine (10^{-5} M) decreased with decreasing temperature. Mean $-\log EC_{50}$ was similar at all three temperatures and mean E_{max} was similar at 37°C and 27°C, but was lower ($0.05 < P < 0.10$) at 21°C. In 5 pairs of preparations with similar precontraction to methacholine (10^{-5} M) at 37°C and 27°C (85.7 $\pm$ 2.1% versus 81.4 $\pm$ 2.6%, NS, n = 5), -logEC50 and E_{max} remained similar at 37°C and 27°C ($-\log EC_{50}$: 6.80 $\pm$ 0.14 versus 6.73 $\pm$ 0.15; E_{max}: 25.6 $\pm$ 6.5% versus 35.0 $\pm$ 11.0%; NS, n = 5). This indicates that at 27°C the sensitivity to isoprenaline was unchanged, independent of the precontraction level. Since there were no preparations with comparable precontraction at 37°C and 21°C a similar subgroup-analysis for the preparations studied at these temperatures was not possible. We found no measurable α-adrenergic activity either before or during cooling. In preparations treated with methacholine the mean BCS was reduced at 27°C and significantly lower at 21°C. Cooling reduced both the $-\log EC_{50}$ and E_{max} of methacholine. In

Table 14.1. Effect of cooling on responses to isoprenaline, methacholine and leukotriene C_4

		temperature		
		37°C	27°C	21°C
isoprenaline	precontraction[a]	87.9 ± 2.9	$69.3 \pm 8.0^{*}$	$43.9 \pm 9.4^{**}$
(n = 7)	$-logEC_{50}$	6.64 ± 0.17	6.58 ± 0.22	6.75 ± 0.31
	E_{max}	30.3 ± 5.1	28.5 ± 8.7	$12.6 \pm 6.0^{\#}$
noradrenaline	BCS	45.7 ± 6.6	36.6 ± 12.7	33.0 ± 7.9
+ timolol	$-logEC_{50}$[b]	–	–	–
(n = 7)	E_{max}[b]	–	–	–
methacholine	BCS	37.0 ± 5.0	32.5 ± 8.7	$23.1 \pm 3.7^{*}$
(n = 7)	$-logEC_{50}$	5.94 ± 0.10	$5.59 \pm 0.04^{**}$	$5.37 \pm 0.10^{**}$
	E_{max}	101.5 ± 1.0	$83.6 \pm 7.2^{*}$	$75.7 \pm 8.2^{**}$
LTC_4	BCS	43.5 ± 8.3	33.4 ± 8.9	$20.0 \pm 2.8^{*}$
(n = 7)	$-logEC_{50}$	7.85 ± 0.10	$7.60 \pm 0.06^{*}$	$7.45 \pm 0.12^{*}$
	E_{max}	78.4 ± 2.4	75.0 ± 2.8	$63.9 \pm 5.7^{*}$

Abbreviations: $-logEC_{50}$, the negative logarithm of the agonist concentration that caused 50% of the maximal effect; E_{max}, the maximal effect of an agonist in %MACR; BCS, the baseline contractile state in %MACR; LTC_4, leukotriene C_4.

\# $0.05 < P < 0.10$.

* $P < 0.05$, $P < 0.01$.

[a] Precontraction with methacholine (10^{-5} M), expressed as a percentage of the maximum active contractile range (%MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M).

[b] Noradrenaline (10^{-8} M to 10^{-4} M) in the presence of timolol (10^{-6} M) had no effect on the airways.

preparations treated with LTC_4 mean BCS was also lower at 27°C and significantly lower at 21°C. Here, cooling also reduced $-logEC_{50}$ and E_{max}. In preparations treated with LTC_4, cooling-induced changes in BCS were not correlated to cooling-induced changes in E_{max} or $-logEC_{50}$ ($-0.25 < r_s < 0.35$ for the different combinations, NS, n = 7). A similar lack of correlation was found for preparations treated with methacholine ($-0.40 < r_s < 0.40$ for the different combinations, NS, n = 7). The effects of cooling were reversed after rewarming to 37°C.

In the control preparation the mean $-logEC_{50}$ values for the first, the second and the third CCRC at 37°C were 6.79 ± 0.11, 6.64 ± 0.17, 6.71 ± 0.08 for isoprenaline; 5.89 ± 010, 5.94 ± 0.10 and 5.85 ± 0.09 for methacholine; and 7.88 ± 0.09, 7.85 ± 0.10 and 7.93 ± 0.08 for LTC_4, indicating that no tachyphylaxis occurred with any of these three agonists.

14.4.2. Cooling and responses to electric field stimulation

Table 14.2 shows that cooling to 27°C or 21°C decreases the cholinergic phase of the response to EFS significantly but does not affect the slow contractile

Table 14.2. Effect of cooling and FPL 55712 on the cholinergic and slow contractile responses to electric field stimulation[a]

	temperature		
	37°C	27°C	21°C
without FPL 55712			
cholinergic phase	122.3 ± 5.1	106.3 ± 14.2*	57.2 ± 7.4**
slow phase	102.2 ± 35.7	105.7 ± 36.3	113.2 ± 28.5
with FPL 55712			
cholinergic phase	128.0 ± 14.8	96.9 ± 14.6*	41.7 ± 6.1**
slow phase	1.0 ± 0.9##	13.1 ± 6.5##	30.8 ± 8.8#

* $P<0.05$; ** $P<0.01$, compared to response at 37°C.

# $P<0.05$, ## $P<0.01$, compared to responses in the absence of FPL 55712.

[a] Phases of the response to EFS (see *Figure* 14.1) are expressed as a percentage of the first cholinergic response to EFS at 37°C. Mean results from 6 to 7 different lung tissue specimens.

phase. The slow contractile phase was substantially inhibited by FPL 55712 at all three temperatures, although the inhibitory effect seemed less strong at lower temperatures. The amplitude of the cholinergic phase reassumed its initial value after rewarming to 37°C.

Table 14.3 shows that the precontraction induced by histamine (5 x 10^{-6} M) was smaller at lower temperatures, indicating that cooling decreased the effect of histamine. EF_{50} to EFS was significantly reduced at 27°C and 21°C. E_{max} to EFS did not differ significantly at the three temperatures. *Figure* 14.2 illustrates that the increase in sensitivity to EFS-induced relaxation is also seen in subgroups of preparations of which the precontraction levels did not differ significantly at 21°C, 27°C and 37°C (1.96 ± 0.32 Hz at 27°C versus 4.10 ± 0.56 Hz at 37°C and 1.53 ± 0.20 Hz at 21°C versus 4.50 ± 0.74 Hz at 37°C, unpaired t-test, $P<0.01$ for both comparisons, n = 5). E_{max} to EFS-induced relaxations

Table 14.3. Effect of cooling on the relaxation component of the response to electric field stimulation[a]

	temperature		
	37°C	27°C	21°C
precontraction[b]	69.8 ± 2.4	56.2 ± 3.5*	44.1 ± 5.2*
EF_{50}	3.97 ± 0.56	2.14 ± 0.32**	1.92 ± 0.40**
E_{max}	38.7 ± 4.8	33.6 ± 5.6	29.4 ± 4.9

Abbreviations: EF_{50}, the effective frequency (Hz) that caused 50% of the maximal relaxation; E_{max}, the maximal effect of EFS is expressed as a percentage of the maximum active contractile range (%MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5 x 10^{-3} M). * $P<0.05$, ** $P<0.01$.

[a] To inhibit the cholinergic and sustained contractile phase (see *Figure* 14.1) the preparations were incubated with atropine (1.2 x 10^{-6} M), indomethacin (6 x 10^{-6} M) and the leukotriene C_4/D_4 antagonist FPL 55712 (11.5 x 10^{-6} M) before stimulation. Mean results from 7 different lung tissue specimens.

[b] Contraction to histamine (5 x 10^{-6} M), expressed as %MACR.

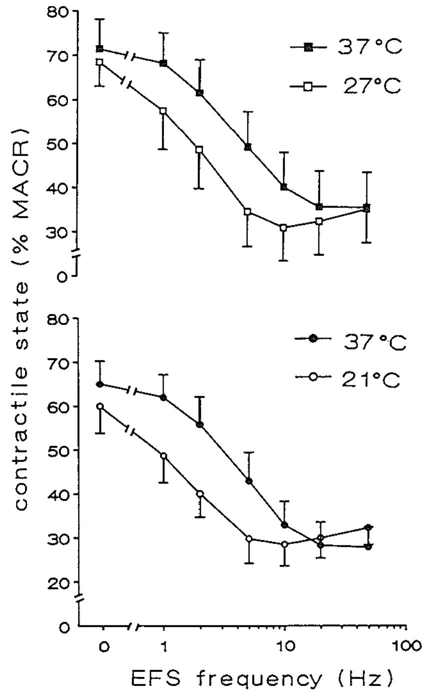

Figure 14.2. Frequency response curves of non-adrenergic relaxations of central human airways to EFS obtained at 37°C, 27°C or 21°C. The preparations were matched for similar levels of precontraction. Cooling to 27°C or 21°C gave a leftward shift of the frequency response curve. To inhibit the cholinergic and sustained contractile phase (see *Figure* 14.1) the preparations were incubated with atropine (1.2 x 10^{-6} M), indomethacin (6 x 10^{-6} M) and the leukotriene C_4/D_4 antagonist FPL 55712 (11.5 x 10^{-6} M). Airways were precontracted with histamine (5 x 10^{-6} M). The vertical axis gives the contractile state of the preparations on a 0 to 100% scale which represents the difference between maximal contraction to methacholine (10^{-4} M) (100%) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5 x 10^{-3} M) (0%). The horizontal axis gives EFS-frequencies. Mean results from 5 preparations from different lung tissue specimens.

at 37°C, 27°C and 21°C was also similar in these subgroups of preparations. Thus, the increase in sensitivity to EFS-induced relaxation is probably not related to differences in precontraction at different temperatures, but represents a real effect of cooling on EFS-induced relaxation.

14.4.3. Epithelial integrity after cooling

There were no differences between the proportion of the basement membrane that was covered with epithelium in preparations that had been cooled to 21°C and those that had remained at 37°C (66.7 ± 6.1 % versus 62.2 ± 7.0 %, n = 14, NS). Results were similar for central and peripheral airways.

14.5. Discussion

The present study gives insight into the effects of cooling on the baseline contractile state and on the sensitivity of human airways to contracting and relaxing stimuli. Our results indicate that in human airways the sensitivity ($-logEC_{50}$) to the β-agonist isoprenaline is unchanged at 27°C and we found no measurable α-adrenergic activity either before or during cooling. Cooling reduced BCS as well as the sensitivity and maximal response to the contractile agonists methacholine and LTC_4. The magnitude of the cooling-induced decrease in BCS was unrelated to the magnitude of the cooling-induced decrease in E_{max} or $-logEC_{50}$. Cooling reduced the cholinergic contraction to EFS, increased the sensitivity to EFS-induced relaxation, but left the sustained contraction after EFS unchanged, probably due to accumulation of LTC_4/D_4.

Studies into the effect of cooling on responses of isolated human airways to β- or α-agonists, to LTC_4 or to EFS have, to our knowledge, not yet been published. Black *et al.*, who studied strips of human airways isometrically, showed that cooling causes both a drop of the baseline tension and a decrease in sensitivity to carbachol[4]. In addition they found an increase in the maximal tension to carbachol at 20°C. Our data confirm a loss of sensitivity during cooling and we also observed a decrease in BCS. Our finding that cooling reduced the maximal shortening to methacholine seems in disagreement with the increased maximal tension to carbachol reported by Black *et al.* This difference might however be explained by the different agonists (methacholine versus carbachol), the different preparations (segments versus strips) or the different methods of measurement (isotonic versus isometric).

We found no significant difference between the sensitivity to isoprenaline at 37°C and that at 27°C when comparing preparations with similar precontraction. Although this does not exclude the possibility that cooling to 27°C has some effect on the $-logEC_{50}$ to isoprenaline, the small range (–0.25 to 0.12) of the 95% confidence interval of the mean difference in $-logEC_{50}$ between segments with similar precontraction at 37°C and 27°C indicates that if such a difference exists it must be small. Because it was not possible to make groups with similar precontraction at 37°C and 21°C, it seems unwarranted to draw conclusions on the effect of cooling to 21°C on β-adrenoreceptor function.

Which are the possible mechanisms for the effects of cooling that we have found? Cooling decreased BCS and reduced the sensitivity to the contractile agonists such as methacholine, LTC_4 and histamine as well as the cholinergic response to EFS, but not the relaxation to isoprenaline. A possible explanation is that cooling only decreases the affinity for the receptor of the three contractile agonists that were studied, and leaves the affinity of the β_2-receptor for isoprenaline unchanged. Such a mechanism would be in contrast with findings in rat tracheal smooth muscle where cooling does not change the affinity of acetylcholine or carbachol for their receptor[19]. A more likely explanation for the decrease of $-logEC_{50}$, E_{max} and cholinergic response to EFS seems that cooling

has a non-specific effect on the coupling between excitation and contraction. Indeed, a study by Stephens *et al.*[32] on canine tracheal smooth muscle suggested that cooling leaves the number of force generating sites in the muscle unaffected but reduces the rate at which each of them liberates energy for contraction. BCS probably reflects a continuous contractile activity of the muscle which, since BCS can be reduced with isoprenaline, requires the liberation of energy. Therefore the mechanism suggested by Stephens *et al.* could also explain the decrease in BCS at 27°C and 21°C.

In an earlier study we found that in about 90% of fresh human central airway preparations the cholinergic twitch to EFS was followed by a mono-phasic sustained contraction (see *Figure* 14.1) which was almost abolished by pre-treatment with the LTC_4/D_4 receptor antagonist FPL 55712 (11.5 x 10^{-6} M)[11]. The effect of FPL 55712 was confirmed in the present study and suggests that at all three temperatures the sustained non-cholinergic response is due to the synthesis of LTC_4/D_4 in the airway wall. Therefore the stability of the sustained phase at 21°C and 27°C, in spite of the decreased responsiveness to exogenous LTC_4 during cooling, suggests that in human airways low temperatures may enhance the formation and/or inhibit the inactivation of bronchoconstrictive leukotrienes. This seems in accordance with findings by Huang *et al.*[18] who showed that stimulation of guinea pig airways with a phorbol ester, which activates the second messenger protein kinase C, induces relaxation at 37°C but contraction at 22 °C, which could be blocked by FPL 55712 (20 x 10^{-6} M).

That FPL 55712 seemed to be less effective in blocking the sustained response to EFS at 27°C and 21°C (*Table* 14.2) may also be due to an accumulation of LTC_4/D_4 at these teperatures. However, this effect may also be caused by a less effective blockade of leukotriene receptors by FPL55712 at 27°C and 21°C.

Can our findings contribute to the understanding of the mechanisms underlying bronchoconstriction which follows hyperventilation of cold air? Firstly, the temperatures to which we have cooled our airways may well be relevant. Recordings in the tracheobronchial tree and the esophagus of humans during isocapnic hyperpnea with air of –10 to –20°C have revealed that temperatures in the proximal airways drop to 20.5°C and in the distal airways to 31.6°C[27] and that oesophageal temperature drops to around 33°C[9]. This suggests that, especially in central airways, the full thickness of the airway wall may be cooled to temperatures similar to those that we have applied in our experiments. Nevertheless, the mechanistic interpretation of our data is difficult for two reasons. Firstly, because we could not study tissue resected from asthmatics since this is rarely available for *in vitro* studies[13]. Secondly, because inhalation of cold, dry air causes transient cooling of the airway wall and the bronchoconstriction usually becomes apparent during rewarming[9], whereas we have applied cooling in an equilibrium. Therefore, our indirect evidence for an increased formation and/or a decreased inactivation of leukotrienes during cell stimulation at 21°C and 27°C should be interpreted cautiously. It is conceivable that an accumulation

of LTC_4/D_4 in the airway wall during cooling is more pronounced in asthmatics than in normals since in asthmatics the number of inflammatory cells which can release LTC_4/D_4, such as eosinophils[17] or mastcells[23,25], is increased[3]. This could be especially important in asthmatic airways in which we found the maximal response to LTC_4 to be increased[12]. Our data suggest that an accumulation of leukotriene C_4/D_4 during airway cooling need not lead to pronounced bronchoconstriction since the responsiveness of the airway muscle to leukotrienes diminishes at lower temperatures. However, it could be that during and after rewarming of the airways, following hyperventilation with cold air, leukotrienes cause bronchoconstriction since our data show that the sensitivity of the airway muscle returns to normal as it rewarms. A role for leukotrienes in bronchoconstriction induced by hyperpnea of cold air is suggested by a study from Israel *et al.* who showed that treatment of asthmatics with a LTD_4 receptor antagonist gives a limited but significant protection against bronchoconstriction to hyperpnea of cold air[20]. However, given the limited effect of this drug, definite conclusions about the role of leukotrienes in cold air-induced hyperpnea in asthma await further studies with leukotriene antagonists or lipoxygenase inhibitors.

Our finding of an increased responsiveness to EFS-induced relaxation during cooling suggests that during cooling of normal airways the efficiency of the non-adrenergic inhibitory system to oppose contractile stimuli is increased. In normals this may protect against cooling-induced bronchoconstriction. Whether this is also the case in asthmatic airways is unclear since the integrity of the non-adrenergic bronchodilatory system in asthma is controversial. Recent histological evidence suggests that VIP, a putative mediator of non-adrenergic inhibitory system, may be lacking in asthmatic airways[29]. On the other hand *in vitro* studies with isolated human airways suggest that in asthmatics at least the efferent pathway of the non-adrenergic bronchodilatory system is intact[2,12].

In *conclusion*, cooling to 27°C does not alter the sensitivity of isolated human airways to isoprenaline but increases their responsiveness to EFS-induced relaxation. There is no measurable α-adrenergic activity either before or during cooling. Lowering of the temperature reduces BCS and also decreases the sensitivity to methacholine, LTC_4 and histamine, possibly by lowering metabolic rates. Cooling reduces the cholinergic, but not the sustained contraction of central human airways to EFS. Considering that this sustained contraction is reduced by a LTC_4/D_4 receptor antagonist and that the sensitivity to LTC_4 decreases during cooling, these results suggest that in human airways cooling may facilitate the accumulation of bronchoconstrictive leukotrienes in the airway wall which follows EFS.

14.6. References

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Chapter 15

Effects of Changes in Osmolarity on Isolated Human Airways*

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15.1. Summary

The effects of hypo- and hyperosmolarity on the functioning of isolated human airways were studied. Changes in osmolarity induced an increasing bronchoconstriction which was proportional to the magnitude of the change in osmolarity. Hypertonicity-induced airway narrowing resulted when buffer was made hypertonic with sodium chloride or mannitol, but not with urea. The airways showed no tachyphylaxis to repetitive exposure to hypo- and hypertonic buffer of 200 and 600 mosM respectively. The bronchoconstriction was not secondary to stimulation of H_1- or leukotriene C_4/D_4 receptors, or to the release of prostaglandins in the preparation. The bronchoconstriction in hypotonic buffer was totally dependent on extracellular calcium, whereas in hypertonic buffer the bronchoconstriction seemed partially dependent on intra-cellular calcium release. Isoprenaline prevented the bronchoconstriction in hyper- or hypotonic buffer of 450 mosM and 250 mosM, but not in buffer of 600 mosM and 150 mosM. It is concluded that both hypo- and hypertonic buffer lead to bronchoconstriction via different mechanisms, which relate to influx of extracellular calcium in hypoosmolar buffer, and, probably, to release of calcium from intracellular stores in hypertonic buffer. In strongly hypertonic buffer, part of the bronchoconstriction may be due to osmotic shrinkage. The relevance of our data for the mechanism of bronchoconstriction after inhalation of hypo- or hypertonic saline depends on whether changes in osmolarity around the airway smooth muscle occur in asthmatics but not in normals and this has not yet been established.

Key words: isolated human bronchi; bronchial smooth muscle; hypertonicity; hypotonicity; calcium

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15.2. Introduction

Inhalation of nebulized hypo- or hypertonic saline leads to bronchoconstriction in asthmatics[27]. Studies on the effect of such stimuli on isolated airways could lead to more insight in the mechanism behind this phenomenon, which is poorly understood. It has been reported that hypertonic solutions contract bovine tracheal smooth muscle via activation of the contractile apparatus[19]. In contrast, in the guinea pig trachea, perfusion with hypertonic medium produces an epithelium-dependent relaxation[25]. In human airways, hypertonic solutions induced a transient reduction of the resting tone whereas hypotonic solutions caused contraction. Changes in osmolarity were found to diminish the contractile responses of isolated human airways to carbachol and histamine[13]. The mechanisms of these findings were, however, not investigated[13]. Because these data suggest that several, maybe opposing, effects on airway muscle might play a role in osmolarity-induced phenomena we investigated the effect of increasingly hypo- or hypertonic media on isolated segments of human airways.

In the present study we found that hypo- and hypertonic solutions lead to an osmolarity-dependent constriction of isolated human bronchus and that in a mildly hyperosmolar environment the bronchi were more sensitive to methacholine. To characterise this response we examined the effects of specific blocking drugs, depletion of intra- en extracellular calcium and a β-receptor agonist. We found that the contraction of the bronchi does not depend on stimulation of H_1- or leukotriene C_4/D_4 receptors, or on the release of prostaglandins in the airway. The bronchoconstriction to hypotonic solutions was fully dependent on extracellular calcium whereas the bronchoconstriction to hypertonic solutions was probably dependent on the release of intracellular calcium. A β-agonist prevented the contraction in mildly hypo- or hyperosmotic environments.

15.3. Methods

15.3.1. Patients and airway preparations

Human lung tissue was obtained from patients who underwent a thoracotomy because of bronchial carcinoma. Within 30 minutes after surgical resection a macroscopically normal part of the resected tissue was immersed in Krebs-Henseleit buffer of room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 5.55), which had been aerated with carbogen (95% O_2, 5% CO_2) to produce a pH of 7.35, a Pco_2 of 4.7 kPa and a Po_2 of 71.8 kPa. The tissue remained in fresh aerated buffer throughout the dissection procedure and during the experiments.

On the cut surface, airways with a diameter of 1 to 2 mm (8th to 11th generation)[29] were identified, cannulated, taken out and dissected free from parenchyma and

vessels under a 20x magnification stereo microscope using iris-scissors and forceps. The cleaned airway was cut into segments of 3 to 4 mm length. Segments were mounted between two small polished stainless steel hooks[15] and studied in double jacketed 10 ml organ baths, which contained aerated Krebs-Henseleit buffer at 37°C. Contractions were measured isotonically with high precision isotonic angular position transducers (Penny and Giles, 3810/60, Great Britain)[23], connected to a digital voltmeter (Fluke 73 multimeter, USA) and pen-recorders (Kipp BD 40, The Netherlands). The preparations were stored overnight in a slow flow of aerated buffer of 4°C, containing penicillin (3 x 10^{-5} g/l) and tobramycin (5 x 10^{-3} g/l). This method has been described in detail previously and it was demonstrated that this storage procedure does not affect the contractile function of the preparations[7]. To asses contractile function and to assure a stable contractility for the rest of the day the preparations were rewarmed to 37°C the next day and contracted twice with methacholine (10^{-5} M) followed by wash-out and stabilization of resting length. The preparations contracted against an isotonic load of 250 mg which has been shown to be optimal for segments of human bronchi[18]. The use of human lung tissue for *in vitro* experiments has been approved by the ethical committee of the University Hospital.

15.3.2. Effect of changes in osmolarity on muscle contractile state

2 segments from each of 6 lung tissue specimens were repeatedly exposed to a single concentration of methacholine (10^{-5} M) while in one of each pair of segments the osmolarity of the buffer was changed as follows: 317 mosM - 450 mosM - 600 mosM - 317 mosM; and in the other: 317 mosM - 250 mosM - 150 mosM - 317 mosM. The osmolarity of the Krebs buffer was changed by varying its sodium chloride concentration. Between stimulations the segments were washed 4 times with 2-minute intervals and every 15 minutes afterwards until stabilization of resting length.

15.3.3. Potency of methacholine in hypo- or hypertonic solutions

Three consecutive cumulative concentration response curves (CCRC) to methacholine were obtained from each of 6 triads of segments from 6 lung specimens. The first methacholine CCRC on each segment was obtained at 317 mosM, the second at either 317 mosM, 450 mosM or 250 mosM and the third again at 317 mosM. Intervals between CCRC were 30 minutes, necessary for stabilization of resting length. Differences in potency of methacholine between the first curves at 317 mosM and the second at either 317 mosM, 450 mosM or 250 mosM were compared.

15.3.4. Effect of different solutes on hyperosmolarity-induced shortening

In order to examine whether the effect of hyperosmolarity depended on the

solute with which the osmolarity was raised, osmolarity response curves to different solutes were made. The osmolarity of the buffer was raised by adding appropriate amounts of either NaCl, urea or mannitol. In each type of buffer 5 segments were challenged repeatedly with 10^{-5} M methacholine while the osmolarity of the buffer changed as follows: 317 mosM – 450 mosM – 600 mosM – 850 mosM – 317 mosM.

15.3.5. Repetitive exposure to non-isotonic buffer

A possible development of tachyphylaxis to hyper- or hypotonic stimulation was investigated by challenging 5 airway segments 4 consecutive times with buffer of 600 mosM or 200 mosM, while control segments remained in buffer of 317 mosM. Between two challenges the preparations were washed with normal buffer and at the end of the experiment the preparations were contracted to 10^{-5} M methacholine in normal buffer of 317 mosM. To examine whether hypo- and hyperosmolar buffer caused damage to the airways, several tissues were processed for light microscopy and examined histologically.

15.3.6. Effect of drugs interfering with mediator release

To find out if osmolarity acts indirectly via the release of mediators, airway segments from each of 6 lungs, were incubated with buffer containing either indomethacin (6×10^{-6} M), mepyramine (2.8×10^{-6} M), FPL 55712 (11.5×10^{-6} M), or disodiumcromoglycate (10^{-4} M). One segment of each specimen was kept in normal buffer as a control. Only one drug was studied on a given preparation. After an incubation period of 20 minutes the segments were exposed to buffer of different osmolarities as follows: 317 mosM – 450 mosM – 600 mosM – 317 mosM. In a separate series of experiments ($n = 6$) the segments were submitted to the same protocol but now the osmolarity of the buffer was changed from: 317 mosM – 250 mosM – 150 mosM – 317 mosM.

15.3.7. Effect of calcium depletion and β-receptor stimulation

To determine whether osmolarity-induced muscle shortening represented active muscle contraction, we examined the effects of calcium depletion, and of the muscle relaxing β-agonist isoprenaline.

From each of 6 lungs, five airway segments were prepared. One pair of segments was incubated in normal buffer, another pair in calcium-free buffer containing 0.5 mM EGTA to chelate residual calcium ions. In an attempt to exhaust intracellular calcium stores the fifth segment was incubated with calcium-free buffer with 2.5 mM EGTA and repeatedly exposed to methacholine (10^{-5} M) until this no longer contracted the preparation[19]. After equilibration, one of each pair of segments, and the fifth segment, were exposed to buffer of 317 mosM – 450 mosM – 600 mosM – 317 mosM and the two remaining segments

to 317 mosM - 250 mosM - 150 mosM - 317 mosM. Each osmolarity step was made after stabilization of baseline, which took approximately 15 minutes.

Pairs of segments of 6 other tissues were exposed to the same sequences of hypo- and hyperosmolar buffer in the presence and absence of the β-agonist isoprenaline (10^{-5} M). The treated preparations were incubated with this agonist for 20 minutes prior to the changes in osmolarity.

15.3.8. Data analysis

The contractile state of the preparations was expressed on an arbitrary 0 - 100% scale, which defines the maximal active contractile range[21]. The maximal response to methacholine was called 100% shortening. Maximal relaxation after 10^{-4} M isoprenaline and 4 x 10^{-3} M EDTA, determined at the end of each experiment, was called 0% shortening. For all studies, except for 15.3.3, the following parameters were defined on this % scale:

S: muscle shortening to a contractile agonist expressed as a % of the maximal active contractile range.

BCS: the baseline contractile state, i.e. the spontaneous position of a preparation on the 0 - 100% scale under a 250 mg load, which reflects intrinsic muscle activity.

In 15.3.3 the potency of methacholine was expressed as the $-\log EC_{50}$ i.e. the negative logarithm of the agonist concentration that caused 50% of the maximal shortening.

To calculate this parameter BCS was taken as 0% and the maximum of a given curve as 100%. On this scale CCRC were constructed and the $-\log EC_{50}$ was calculated using the BMDP software module for non-linear curvefitting[4]. The curves were fitted to a four parameter logistic function[8].

The design of each protocol was such that the first response was obtained at standard conditions, whereas in further responses a change in osmolarity was introduced. Differences in parameters of functioning between first and subsequent curves for treated preparations and for untreated time-parallel controls were compared and evaluated with the Mann-Whitney U test (MWU). Differences in the parameters for first and subsequent curves for treated preparations were compared with Wilcoxon's matched-pairs signed rank test (Wilcoxon). $P<0.05$ was considered to be significant. All data are presented as means $\pm$ SEM.

15.3.9. Drugs

Methacholine hydrobromide (Janssen Pharmaceuticals, Belgium), ethylenediamine tetra-acetic acid disodium salt (EDTA) (Sigma, USA), sodium cromoglycate (Fisons, UK), mepyramine (Rhône-Poulenc, France) and ethyleneglycol-2-(2-aminoethyl)-tetracetic acid (EGTA) (Fluka, Switzerland) were prepared in 0.9% NaCl. Mannitol (ICN Pharmaceuticals, USA) and urea (Merck, W-

Germany) were dissolved in Krebs-buffer. FPL 55712 (a gift from Fisons, UK) and indomethacin (Duchefa, The Netherlands) were dissolved in methanol. L-isoproterenol sulphate (Janssen Pharmaceuticals, Belgium) was dissolved in water containing ascorbic acid (88 mg/l). Preliminary experiments showed that methanol and ascorbic acid in these concentrations have no effect on airway smooth muscle. Fresh drug solutions were prepared daily and kept on ice during the experiments.

15.4. Results

15.4.1. Patients

Lung tissue specimens were obtained from 40 patients, 33 male and 7 female, with a mean age of 63.5 years (range 33 to 80 years). All were operated because of bronchial malignancies. None had a history of asthma; 33 were smokers and 16 had chronic obstructive pulmonary disease (COPD) according to the criteria of the American Thoracic Society[1]. Preoperative lung function showed mean values for Inspiratory Vital Capacity (IVC) of 3.6 ± 0.15 l and forced expiratory volume in 1 second (FEV_1) as a percentage of VC of 65.8 ± 2.3 %. Medication during anesthesia was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane and pancuronium. 14 Patients received steroids and/or theophylline before and during the operation.

15.4.2. Effect of changes in osmolarity on muscle contractile state

Figure 15.1 shows that addition of hypertonic buffer of 450 mosM and 600 mosM significantly increased BCS from 53.7 ± 7.1% to 68.6 ± 6.5% and 88.2% ± 3.3% respectively ($P<0.05$). Addition of hypotonic buffer of 250 mosM and 150 mosM also significantly increased BCS from 56.2 ± 5.4% to 68.1 ± 4.7% and 79.9% ± 4.6% ($P<0.05$). These increases were reversible in iso-osmolar buffer of 317 mosM. The bronchoconstriction after a change in osmolarity started within three minutes and reached a plateau after 15-25 minutes. The increase in BCS at 250 mosM was transient whereas the increase in buffer of 150 mosM was sustained and more slowly reversible than that in hypertonic buffer. In hypertonic buffer S to 10^{-5} M methacholine decreased slightly but significantly after re-establishing iso-osmolarity (99.3 ± 1.4% initially versus 92.1 ± 2.0% at the final contraction in 317 mosM, $P<0.05$). S to 10^{-5} M methacholine was significantly attenuated in buffer of 250 and 150 mosM and returned only partially to its initial value within the duration of the experiment (100.3 ± 1.2% initially at 317 mosM versus 88.5 ± 2.2% at 250 mosM, 83.9 ± 2.3% at 150 mosM and 94.1 ± 3.0% at 317 mosM at the end of the experiment, $P<0.05$).

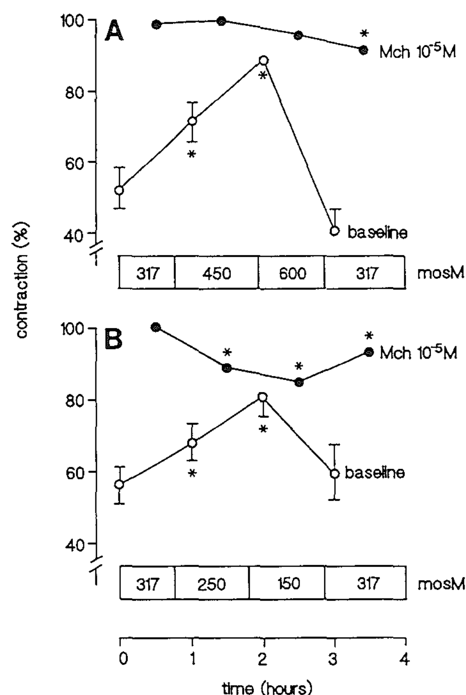


Figure 15.1. Baseline contractile state (open symbols) and mean contractile responses to 10^{-5} M methacholine (Mch) (closed symbols) of segments of airways exposed to increasingly hypertonic Krebs-Henseleit buffer (A) or increasingly hypotonic buffer (B). Osmolarity of the buffer was changed by varying its sodium chloride content. Vertical axes, shortening as a percentage of the maximal active contractile range (see 15.3.8). Values are mean ± SEM; n = 6. SEM < 5% not indicated. * $P<0.05$, significantly different from initial value at 317 mosM (Wilcoxon).

15.4.3. Potency of methacholine in hypo- or hypertonic solutions

Table 15.1 shows the mean $-\log EC_{50}$ values of methacholine in buffer of 317, 450 and 250 mosM. In buffer of 450 mosM the CCRC shifted leftwards and $-\log EC_{50}$ increased 0.29 ± 0.07. In buffer of 250 mosM the opposite was seen and $-\log EC_{50}$ decreased 0.28 ± 0.09. This was significant compared to a difference of 0.01 ± 0.03 between the first and the second curves in controlpreparations kept in 317 mosM ($P<0.05$). This effect was reversible and after re-establishing iso-osmolarity $-\log EC_{50}$ was no longer significantly different from the initial values. Like S to 10^{-5} M methacholine in the previous experiment, S to 10^{-4} M methacholine decreased significantly in buffer of 250 mosM from 103.2 ± 2.9% to 93.2 ± 1.5% ($P<0.05$), whereas it remained unchanged in buffer of 450 mosM.

Table 15.1. Responses of human airway segments to methacholine at different osmolarities[a]

	before	during	after
450 mosM			
$-\log EC_{50}$	6.01 ± 0.14	6.30 ± 0.18*	6.17 ± 0.08
S, %	101.0 ± 1.9	99.5 ± 1.8	97.6 ± 2.4
250 mosM			
$-\log EC_{50}$	6.04 ± 0.14	5.76 ± 0.06*	5.95 ± 0.20
S, %	103.2 ± 2.9	93.2 ± 1.5*	104.7 ± 5.2
317 mosM (control)			
$-\log EC_{50}$	6.01 ± 0.15	6.03 ± 0.16	5.95 ± 0.14
S, %	97.9 ± 1.1	97.8 ± 2.0	94.9 ± 3.3

Abbreviations: S, maximal shortening to 10^{-4} M methacholine expressed as a percentage of the initial contraction to 10^{-4} M methacholine (see 15.3.8); $-\log EC_{50}$, the negative logarithm of the methacholine concen tration that caused 50% of the maximal shortening.

* $P<0.05$ (MWU).

[a] For each preparation a CCRC was obtained before, during and after a change in the osmolarity of the bathing fluid. In controls the osmolarity remained unchanged.

15.4.4. Effect of different solutes on hyperosmolarity induced shortening

Figure 15.2 shows the effect of different hypertonic buffers on BCS. Sustained bronchoconstriction was seen with increasing concentrations of mannitol or sodium chloride, but not with urea, which caused an initial transient contraction (not shown in *Figure* 15.2) followed by a small decrease in mean BCS at 600 and 850 mosM. At 850 mosM the contraction to 10^{-5} M methacholine was significantly reduced compared to baseline values ($P<0.05$), returning only partly to its initial value within the duration of the experiment.

15.4.5. Repetitive exposure to non-isotonic buffer

Figure 15.3 shows that repetitive exposure to solutions of 600 mosM or 200 mosM with intervals of 1 hour caused some decrease in BCS but no significant decrease in the net response. This indicates that there is no tachyphylaxis of the contractile response to hypo- or hypertonic buffer. The response to 10^{-5} M methacholine in buffer of 317 mosM determined at the end of the experiment was significantly higher in preparations that had been challenged repetitively with hypotonic buffer of 200 mosM, than in controls in which the osmolarity had remained unchanged (107.8 ± 2.5% after repetitive exposure to 200 mosM versus 99.5 ± 3.2% in controls, $P<0.05$). Histologic examination showed that most preparations had intact epithelium covering 50 to 80% of the lumen. There were no significant differences between preparations exposed to hypo- or hypertonic buffer and paired control preparations which had been kept in isotonic buffer.

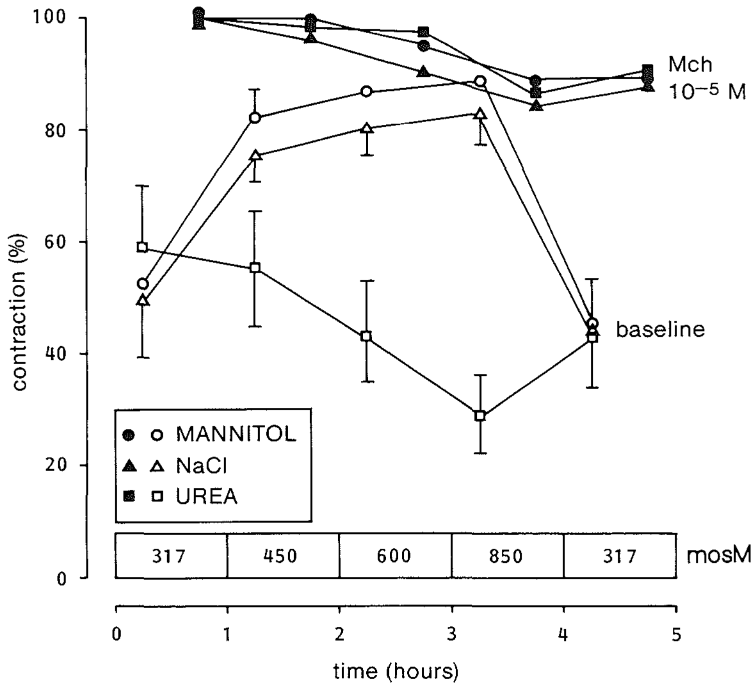


Figure 15.2. Baseline contractile state (open symbols) and mean contractile responses to 10^{-5} M methacholine (Mch) (closed symbols) of segments of airways exposed to Krebs-Henseleit buffer of different osmolarities. The osmolarity of the buffer was raised with either mannitol (circles), sodium chloride (triangles) or urea (squares). Vertical axes, shortening as percent of the maximal active contractile range (see 15.3.8). Values are mean ± SEM; n = 5. SEM < 5% not indicated.

15.4.6. Effect of drugs interfering with mediator release

Figure 15.4 shows that neither the cyclooxygenase inhibitor indomethacin (6 x 10^{-6} M) nor mepyramine, which blocks H_1 receptors selectively at the concentration used (2.8 x 10^{-6} M)[10], the LTC_4/LTD_4 antagonist FPL 55712 (11.5 x 10^{-6} M)[17] or disodiumcromoglycate (10^{-4} M) prevented the contraction induced by hypertonic buffer of 450 and 600 mosM or the bronchoconstriction at 250 mosM and 150 mosM. The initial baseline contractile state at 317 mosM did not differ significantly between controls and the treated groups of preparations ($P>0.3$). During incubation with mepyramine the spontaneous decrease in BCS with time was significantly greater than in parallel controls which were not incubated with this drug (a decrease of 2.4 ± 1.4% in 1 hour in controls and of 12.1 ± 3.2% in preparations treated with mepyramine, $P<0.01$). This suggests that histamine was being produced in the preparations.

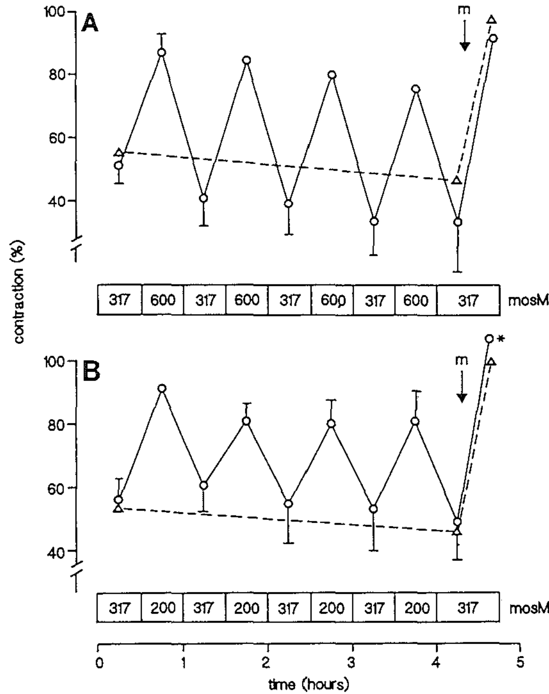
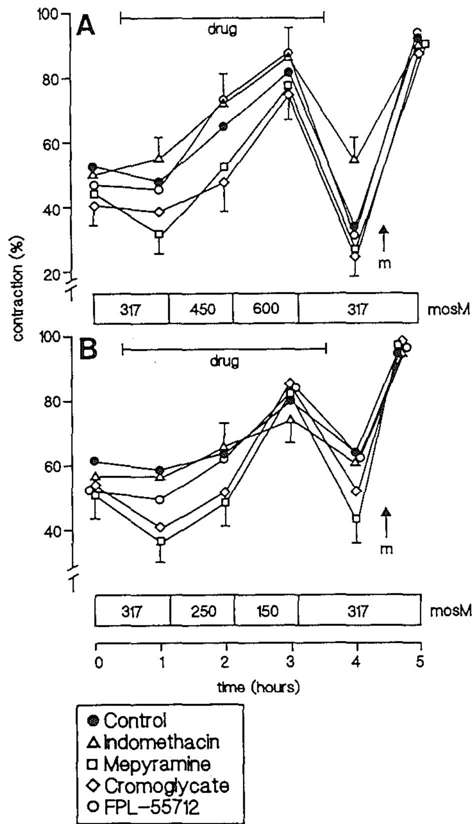


Figure 15.3. Effects of repetitive challenge with Krebs-Henseleit buffer of 600 mosM (A) or 200 mosM (B) alternated with buffer of 317 mosM. In the control preparations (open triangles) the osmolarity remained 317 mosM throughout the experiment. The addition of 10^{-5} M methacholine at the end of the experiment is indicated with the arrows marked "m". Vertical axes, shortening as a percentage of the maximal active contractile range of a given preparation (see 15.3.8). Values are mean ± SEM; n = 5. SEM < 5% not indicated.
* $P<0.05$, significantly different from controls (MWU).

15.4.7. Effect of calcium depletion and β-receptor stimulation

The results of depletion of calcium are shown in *Figure* 15.5. In the preparations to be exposed to hypertonic buffer incubation with calcium-free buffer led to a drop in BCS of 59.0 ± 6.0% in calcium-free buffer with 0.5 mM EGTA and of 62.7 ± 6.4% in calcium-free buffer with 2.5 mM EGTA. In the preparations that had been repeatedly exposed to methacholine (10^{-5} M) in calcium-free buffer with 2.5 mM EGTA, the magnitude of the contraction to buffer of 450 mosM was 7.3 ± 4.1% compared to a magnitude of 31.0 ± 9.1% in calcium-free buffer with 0.5 mM EGTA, where no depletion of intracellular calcium stores had been attempted ($P<0.05$). There was no significant difference in the magnitude of the contraction to buffer of 600 mosM between these two groups of preparations. Calcium-free buffer with either 0.5 mM EGTA or 2.5 mM EGTA



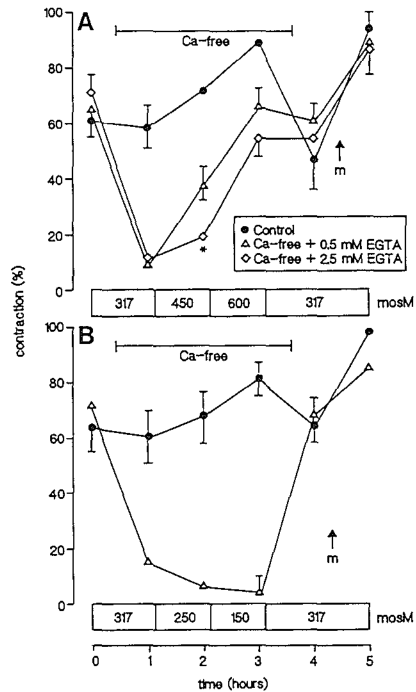


Figure 15.4. Effects of drugs interfering with mediator release on the response of airway segments to changes in osmolarity. Mean contractile responses of controls (closed circles) and preparations treated with indomethacin (6.0 x 10^{-6} M, triangles), mepyramine (2.8 x 10^{-6} M, squares), sodium cromoglycate (10^{-4} M, diamonds) and FPL 55712 (11.5 x 10^{-6} M, open circles) to increasing (A) or decreasing (B) osmolarity are shown. The addition of 10^{-5} M methacholine at the end of the experiment is indicated by the arrows marked "m" and the presence of a blocker by the bracket marked "drug". Vertical axes, shortening as a percentage of the maximal active contractile range of a given preparation (see 15.3.8). Values are mean $\pm$ SEM; n = 6. For clarity SEM bars are not always indicated. SEM never exceeded 9%.

Figure 15.5. Effect of calcium depletion on the response of airway segments to changes in osmolarity. Mean contractile responses of control preparations in the presence of 2.5 mM calcium (closed circles) and preparations treated with calcium-free buffer with 0.5 mM EGTA (triangles) or calcium-free buffer with 2.5 mM EGTA (diamonds) to increasing (A) or decreasing (B) osmolarity are shown. The addition of 10^{-5} M methacholine at the end of the experiment is indicated by arrows marked "m" and the presence of calcium-free buffer by the bracket marked "Ca-free". The vertical axes depict shortening as a percentage of the maximal active contractile range of a given preparation (see 15.3.8). Values are mean $\pm$ SEM; n = 6. SEM $<$ 5% not indicated.
* $P<0.05$, significantly different from calcium-free buffer containing 0.5 mM EGTA (MWU).

did not significantly reduce the magnitude of the contraction to buffer of 450 mosM or 600 mosM in comparison to controls.

In the preparations to be exposed to hypotonic buffer addition of calcium-free buffer with 0.5 mM EGTA led to a drop in BCS of 59.9 ± 7.6%. In the absence of extracellular calcium the contraction to hypotonic buffer was completely prevented.

There were no significant differences in the BCS and the responses to 10^{-5} M methacholine and BCS at the end of the experiments between controls and preparations that had been exposed to calcium-free buffer.

The influence of the β-agonist isoprenaline (10^{-5} M) is shown in *Figure* 15.6. Isoprenaline decreased the BCS by 52.2 ± 5.8% in preparations to be incubated with hypertonic buffer and by 50.0 ± 7.3% in those to be treated with hypotonic buffer. Isoprenaline significantly reduced the magnitude of the contraction to

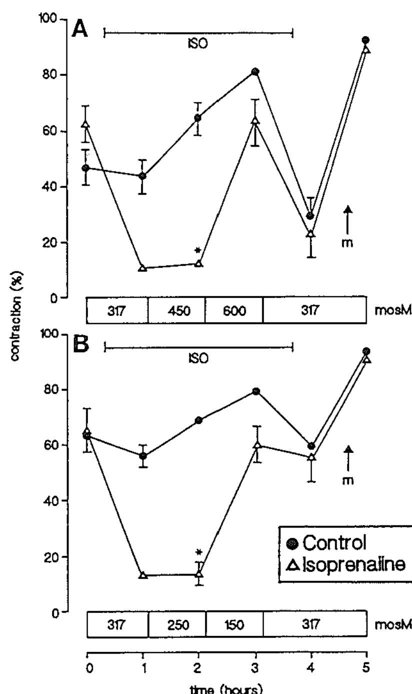


Figure 15.6. Effect of isoprenaline (10^{-5} M) on the response of airway segments to changes in osmolarity. Mean contractile responses of control preparations (closed circles) and preparations treated with 10^{-5} M isoprenaline (triangles) to increasing (A) or decreasing (B) osmolarity are shown. The responses to 10^{-5} M methacholine at the end of the experiment are indicated by the arrows marked "m" and the addition of isoprenaline by the brackets marked "ISO". The vertical axes depict shortening as a percentage of the maximal active contractile range of a given preparation (see 15.3.8). Values are mean ± SEM; n = 6. SEM < 5% not indicated.

* $P<0.05$, net increase in baseline contractile state at 450 and 250 mosM significantly less in the presence of isoprenaline than in control experiments (MWU).

buffer of 450 mosM and 250 mosM ($P<0.001$). In 600 mosM and 150 mosM however, there was a marked bronchoconstriction despite isoprenaline. After removal of isoprenaline there were no significant differences in S to 10^{-5} M methacholine and BCS between controls and preparations that had been incubated with isoprenaline.

15.5. Discussion

Our results show that Krebs-Henseleit buffer made increasingly hypo- or hypertonic by varying its sodium chloride content induces an osmolarity-dependent constriction of isolated human airways. In a mildly hyperosmolar environment the bronchi are more sensitive to methacholine. Hypertonicity-induced smooth muscle shortening not only results with extra NaCl, but also with mannitol. Urea, however, which readily crosses the cell membrane, only caused a small and transient contraction. There is no tachyphylaxis to repetitive exposure to hypo- and hypertonic buffer of 200 and 600 mosM and experiments with pharmacological blockers suggest that osmolarity-induced bronchoconstriction is not secondary to stimulation of histamine H_1, leukotriene C_4/D_4 receptors or to the release of prostaglandins in the preparation. In hypotonic, but not in hypertonic, medium the bronchoconstriction is totally dependent on extra-cellular calcium. In a mildly hypertonic environment (450 mosM) the contraction is significantly attenuated in preparations in which depletion of intracellular calcium stores had been attempted. Isoprenaline prevents the bronchoconstriction in mildly hyper- or hypotonic buffer (450 and 250 mosM), but not in strongly hyper- or hypotonic buffer (600 and 150 mosM).

A sustained, osmolarity-dependent increase in BCS of airway smooth muscle in hypertonic buffer containing extra sodium chloride has been reported previously in bovine trachealis[19]. In this study buffer made hypertonic with urea only caused a transient contraction, which is similar to our findings. The small but significant potentiation of the effect of methacholine that we found at 450 mosM has not been reported before. Others have observed relaxations to hypertonic solutions in human airways and guinea-pig trachea[13,25]. Although we used similar solutes and changes in osmolarity, we could not confirm these findings. We do, however, confirm the findings of Finney and co-workers[13] that hypotonic medium leads to bronchoconstriction and reduces the potency of a cholinergic agonist.

Hypertonicity caused by sodium chloride or mannitol induces a sustained bronchoconstriction, which was not seen with urea. This suggests that the ability of the solute to maintain an osmotic gradient over the cell membrane determines whether the preparation shortens. Moreover, changes in extracellular NaCl may give rise to ion fluxes over the cell membrane. This would lead to changes in membrane potential, which could modify muscle contraction. Various alternative mechanisms are possible. First, the airway narrowing could result from muscle

stimulation by mediators released by mast cells. Eggleston *et al.*[10] have shown that mast cells release histamine, but not eicosanoids, in a hypertonic environment. This explanation now seems unlikely because we showed that repetitive challenge did not cause tachyphylaxis as could be expected in the case of depletion of a preformed mediator such as histamine. Additionally neither mepyramine nor sodiumcromoglycate reduced the response to hypertonic buffer. At 2.8×10^{-6} M mepyramine can be regarded as a specific H_1 receptor antagonist[11]. Therefore, the possibility that H_2 receptors are involved remains open, but seems unlikely because H_2 agonists do not contract airway muscle[9]. Moreover, the degree of hypertonicity that we have used in our pharmacological studies (extra sodium chloride up to 600 mosM) would cause the lung mast cell to release only a small amount of its histamine content in buffer containing 2.5 mM calcium[10]. In addition, the experiments with indomethacine and FPL 55712 suggest that neither prostaglandins nor leukotrienes C_4/D_4 play an important role in the bronchoconstriction induced by hyper- or hypotonicity *in vitro.*

A second explanation may be that osmotic stimulation directly activates the contractile apparatus of airway smooth muscle cells. Kirkpatrick *et al.* have reported that incubation of bovine trachealis with hypertonic buffer of 600 mosM caused muscle contraction which was largely due to an increase in intracellular calcium[19]. Our experiments with calcium depletion also suggest that contractions both in hypotonic and in mildly hypertonic buffer are calcium dependent, and are therefore due to active contraction of muscle cells. We found that calcium-free medium abolished the effects of 250 and 150 mosM but not of 450 and 600 mosM. In experiments where we attempted to deplete intracellular calcium, the response to 450 mosM was inhibited. Although we did not directly measure calcium fluxes and concentrations, these data suggest that hypo- and hypertonic media contract the muscle via different mechanisms: hypotonicity induced contraction seems to depend on influx of extracellular calcium, whereas hypertonicity may selectively release calcium from intracellular stores. That osmolarity-induced bronchoconstriction is due to muscle activation is further supported by our finding that isoprenaline inhibits the response to 250 and 450 mosM. This inhibition may relate to removal of intracellular calcium by isoprenaline, which stimulates cyclic AMP, and thereby activates protein-kinase dependent ionic efflux pumps in the muscle cell membranes[26,28]. Isoprenaline did not prevent bronchoconstriction at 150 mosM and 600 mosM. This may be explained by the possibility that, at 150 mosM, the permeability of the cell membrane has increased to such a degree that, despite isoprenaline, net influx of calcium ions occurs, leading to contraction. The lack of effect of isoprenaline at 600 mosM may be due to a third alternative mechanism of osmolarity-induced shortening of muscle, passive osmotic shrinkage. Studies on other types of smooth muscle have shown that smooth muscle behaves like an osmometer[6]. As we were unable to block the bronchoconstriction in 600 mosM, it seems likely that this response was mainly due to passive shrinkage of cells.

In the presence of hypotonic buffer of 250 mosM, there was a significant

10% reduction of the maximal effect of methacholine. This could be due to osmotic swelling of muscle cells, which impairs further shortening. In contrast, we have found a significant increase in the effect of methacholine after repetitive exposure to hypotonic buffer of 200 mosM, but not after a single exposure to 250 mosM. It has previously been shown that the contraction to methacholine depends on the release of calcium from intracellular stores[22]. It can therefore be speculated that 'loading' of calcium in the muscle cells may occur after repeated exposure to hypotonic buffer, which leads to an exaggerated response of the muscle to subsequent cholinergic stimulation. This hypothesis could even be applied to explain why *in vivo* bronchial responsiveness increases after inhalation of hypotonic aerosol[3]. Clearly, this theory needs further study.

In the present study, osmolarities between 150 and 600 mosM were applied. Although it is likely that the osmolarity of the airway lining fluid changes after inhalation of non-isotonic aerosols, the exact magnitude of these changes *in vivo* is not known. Direct measurements have not been done, and accurate calculations are not possible due to uncertainty about the rate at which the bronchial circulation compensates for changes in water and solutes on the airway surface. However, in most earlier studies similar osmolarities have been applied[10,13,14,19,25].

Can our findings contribute to the understanding of the mechanisms which underlie the bronchoconstriction to inhaled hypo- or hypertonic saline in asthmatic subjects? In interpreting the data, one should realise that normal subjects do not bronchoconstrict after inhalation of non-isotonic aerosols. We used only non-asthmatic airways, and the relevance of our findings to asthmatic osmolarity-induced bronchoconstriction can therefore be questioned. However, if an altered osmolarity of the airway lining fluid extends to the airway muscle in asthmatics but not in normals, the direct bronchoconstricting effect of non-isotonic solutions could contribute to bronchoconstriction. We speculate that this might be the case for several reasons. First, asthmatic airway epithelium probably has an abnormal structure[20] and recent *in vivo* evidence suggests that airway permeability is increased in asthmatics[16]. Second, epithelial shedding in asthmatics[20] might expose the airway smooth muscle more directly to the bronchial lining fluid. These possible mechanisms might facilitate changes in osmolarity around the airway smooth muscle in asthma. However it can be argued that the submucosal vascular plexus might normalize an increase in osmolarity before it extends to the airway smooth muscle, and therefore a possible contribution of osmolarity induced muscle shortening must remain speculative.

Results from others suggest that, in asthmatics, the bronchoconstriction to inhalation of hypertonic saline can be attenuated with several drugs. Sodium-cromoglycate (4 mg) gives a protection 46.3%, ipratropium bromide (80 mg) gives a protection of 68.9% and salbutamol (200 μg) of 85.5%[2,5]. The H_1-receptor antagonist terfenadine (180 mg) gives a mean reduction of the area under curve of 68.5%[12]. The bronchoconstriction after inhalation of ultrasonically nebulised distilled water can be prevented by inhalation of salbutamol or cromoglycate[24].

These findings suggest that *in vivo*, mediator release contributes to the response of asthmatic airways to inhalation of non-isotonic solutions. However, in a study where human asthmatic and nonasthmatic airway segments were lavaged *in vivo* with hypertonic saline of 900 mosM the mediator content of the lavage fluid was not related to the osmotic stimulus[14]. Our results also indicate that mediator release to an extent that causes muscle contraction does not occur in isolated human airway segments after osmotic challenges. We only studied non-asthmatic human lungs, however, and therefore we cannot exclude that osmolarity-induced mediator release may occur in asthmatic airways.

In *conclusion*, our results show that hypo- and hyperosmotic stimuli lead to an osmolarity dependent constriction of isolated human airways. This contraction is not caused by the release of histamine, prostaglandins or leukotrienes C_4/ D_4 in the airway wall. In hypertonic medium the response seems due to both the mobilization of intracellular calcium and, at higher osmolarities, shrinkage. In hypotonic solutions the contraction is fully dependent on extracellular calcium. Furthermore, we could prevent the bronchoconstriction at moderate hypo- and hyperosmolarity with isoprenaline. Therefore, an important part of the response to non-isotonic solutions is due to direct activation of the contractile apparatus of airway smooth muscle cells. The relevance of our data for the mechanism of bronchoconstriction after inhalation of hypo- or hypertonic saline depends on whether changes in osmolarity around the airway smooth muscle occur in asthmatics but not in normals and this has not yet been established.

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Chapter 16

Effect of Hyperosmolarity on Isolated Central Human Airways*

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16.1. Summary

1. We studied the effect of hyperosmolarity on isolated human airways because a better understanding of the effect of hyperosmolarity on the human airway wall may improve the insight in the pathophysiology of hyperosmolarity-induced bronchoconstriction in asthma.
2. In cartilaginous bronchial rings dissected from fresh human lung tissue hyperosmolar Krebs-Henseleit buffer (450 mosM, extra sodium chloride added) evoked a biphasic response: a rapid relaxation phase (peak after 5.0 $\pm$ 0.3 min) followed by a slow contraction phase (peak after 25.4 $\pm$ 0.8 min).
3. With the histamine (H_1) receptor antagonist mepyramine, the contraction phase was reduced to 41.2% of the control value ($P<0.001$), with atropine to 50.0% ($P<0.01$), with the local anesthetic lidocaine to 48.7% ($P<0.05$) and with mepyramine + atropine to 19.2% ($P<0.001$)
4. With the inhibitor of neutral metalloendopeptidase phosphoramidon the contraction phase increased to 128.0% of the control value ($P<0.05$) and after removal of epithelium to 131.8% ($P<0.05$).
5. Indomethacin, the LTC_4/D_4 antagonist FPL 55712 or the nervous conductance blocker tetrodotoxin had no effect on the contractile phase.
6. The relaxation phase was not altered by any of these drugs nor by epithelial denudation. The relaxation phase was also unchanged in the presence of α-

* Jongejan RC, de Jongste JC, Raatgeep HC, Stijnen T, Bonta IL, Kerrebijn KF. Effect of hyperosmolarity on isolated central human airways. Br J Pharmacol 1991: (In Press). Printed with permission of the British Pharmacological Society.

chymotrypsin, which degrades muscle relaxing peptides such as vasoactive intestinal peptide.

7. Hyperosmolar buffer slightly increased the sensitivity and maximal response to methacholine as well as the cholinergic twitch to electric field stimulation.
8. We conclude that hyperosmolarity releases acetylcholine, histamine and neuropeptides in the human airway wall in sufficient quantities to contract airway smooth muscle. This release itself or its effect on airway muscle is modulated by the airway epithelium. The mechanism of the relaxation phase may be due to an unknown smooth muscle relaxing substance or to a direct effect on the airway muscle related to ion fluxes.

16.2. Introduction

In asthmatics inhalation of nebulized hypertonic saline leads to bronchoconstriction[29]. The mechanism behind this phenomenon is unknown. *In vivo*, the maximal fall in FEV_1 after inhalation of hyperosmolar saline is reduced after pretreatment with the anticholinergic ipratropium bromide[4] or the H_1-receptor antagonist terfenadine[11,13] whereas flurbiprofen, a potent inhibitor of cyclooxygenase, gives minor protection[11]. These data suggest that in asthmatics the release of histamine and a vagal reflex component contribute to hyperosmolarity-induced bronchoconstriction.

Because a better understanding of the effect of hyperosmolarity on the human airway wall may improve the insight in the pathophysiology of hyperosmolarity-induced bronchoconstriction in asthma we evaluated the role of histamine, acetylcholine, metabolites of arachidonic acid, neuropeptides and airway epithelium in the response of fresh human airways to a hyperosmolar stimulus. In addition, we investigated the influence of hyperosmolarity on the responses to methacholine, an analogue of acetylcholine, and on the effect of electric field stimulation (EFS), which selectively activates postganglionic nerves in the bronchial wall[8].

16.3. Methods

16.3.1. Patients and airway preparations

Lung tissue specimens were obtained from 35 patients, 32 male and 3 female, with a mean age of 65.6 years (range 50 to 80 years). All were operated because of bronchial carcinoma. None had a history of asthma, 29 were smokers and 19 had chronic obstructive pulmonary disease according to the criteria of the American Thoracic Society[2]. Preoperative lung function showed mean values for Inspiratory Vital Capacity (IVC) of 95.6 ± 3.5 % of the predicted value and a forced expiratory volume in 1 second as a percentage of IVC of 60.2

± 1.2 % of the predicted value. No provocation tests were done on these patients so that information about their airway reactivity was not available. Medication during anesthesia was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane and pancuronium. 14 patients received steroids and/or theophylline before and during the operation. Thirty to 60 minutes after surgical resection a macroscopically normal part of the resected tissue was immersed in Krebs-Henseleit buffer of room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 5.55), which had been gassed with carbogen (95% O_2, 5% CO_2) to produce a pH of 7.35, a Pco_2 of 4.7 kPa and a Po_2 of 71.8 kPa. The tissue remained in fresh aerated buffer throughout the dissection procedure and the experiments. On the cut surface cartilaginous bronchi with a diameter of 3 to 5 mm (3rd to 7th generation) were identified, cannulated, taken out and dissected free from parenchyma and vessels under a 20x magnification stereo microscope using iris-scissors and forceps. The cleaned airways were cut into segments of 3 to 4 mm length which were mounted between two small hooks made of polished stainless steel (diameter 0.3 mm) in a double jacketed 10 ml organ bath and fixed to a glass hook at the bottom of the bath and a high precision isotonic angular position transducer (Penny and Giles 3810/60, Great Britain) which was connected to a digital voltmeter (Fluke 73 multimeter, USA) and a pen-recorder (Kipp BD 40, The Netherlands). This method has been described in detail previously[6,17]. To prevent adhesion of peptides the organ baths were siliconized (dimethyldichlorosilane, BDH, United Kingdom). The bronchi contracted against an isotonic load of 500 mg which was found to be optimal in preliminary studies. During an equilibration period of 2 hours at 37°C, the preparations were contracted twice with methacholine (10^{-5} M, 10^{-4} M), to assess their contractile function. Between stimulations the preparations were washed 4 times with intervals of 2 minutes and every 15 minutes afterwards until stabilization of resting length.

16.3.2. Effect of hyperosmolarity and its mechanism

A first osmotic challenge was carried out by raising the osmolarity of the organ bath fluid to 450 mosM with 180 μl of a 3.84 M NaCl solution. This stimulus was chosen because we had previously found that 450 mosM was the highest osmolarity that would not cause osmotic shrinkage[16]. Furthermore, the osmolarity of the airway lining fluid has been estimated to surpass 640 mosM in the first 10 airway generations within 5 minutes after the initiation of a standard challenge with nebulized saline of 1330 mosM[3]. Hence, we expected buffer of 450 mosM to be a stimulus that is relevant to the *in vivo* situation, without causing osmotic shrinkage *in vitro*. The response to hyperosmolar buffer consisted of a rapid deflection below baseline followed by a slow contractile phase (see *Figure* 16.1). After a maximum response was reached the hypertonic buffer was replaced by isotonic buffer and 30 minutes later, the segments were incubated with one of the following drugs[8] : the cyclooxygenase inhibitor indomethacin (6 x 10^{-6} M);

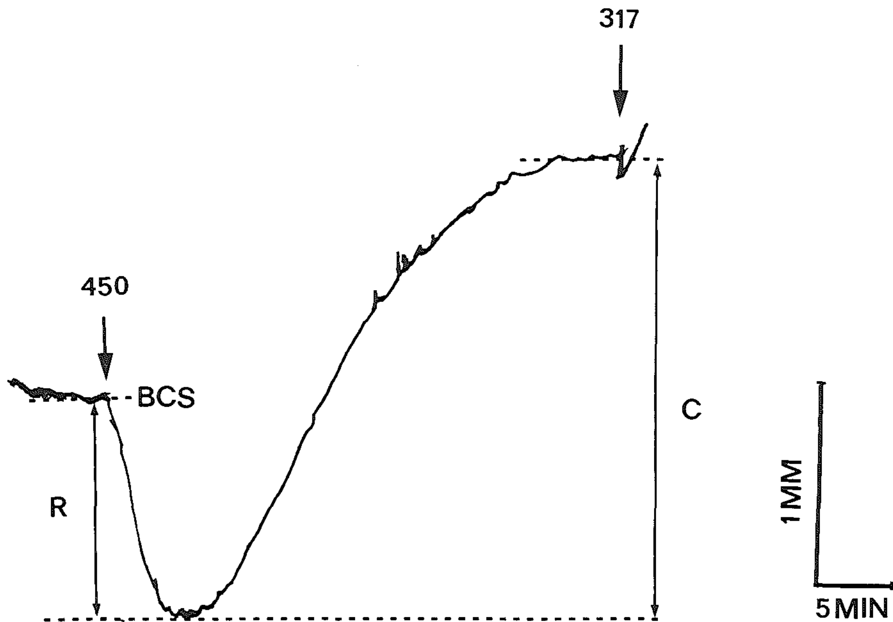

Figure 16.1. Recording of a typical response of a fresh central human bronchus to hyperosmolarity. At the point indicated by the arrow (450) the osmolarity of the Krebs-Henseleit buffer in the organ bath was raised from 317 mosM to 450 mosM by adding extra NaCl. BCS indicates the baseline contractile state. R indicates the relaxation phase and C the contraction phase.

the leukotriene C_4/D_4 (LTC_4/D_4) receptor antagonist FPL 55712 (11.5×10^{-6} M); the histamine H_1 receptor antagonist mepyramine (5×10^{-6} M); the muscarine receptor antagonist atropine (1.2×10^{-6} M); a combination of mepyramine (5×10^{-6} M) and atropine (1.2×10^{-6} M); the local anesthetic lidocaine (10^{-5} M); or the nervous conductance blocker tetrodotoxin (TTX, 9.4×10^{-6} M). The role of neuropeptides in the bronchial response to hyperosmolarity was studied by incubating the airway with drugs which inhibit the degradation of these peptides, such as the inhibitor of neutral metalloendopeptidase (NEP) phosphoramidon (5×10^{-6} M) or a combination of phosphoramidon (5×10^{-6} M), captopril (10^{-5} M) the inhibitor of angiotensin converting enzyme which also has enkephalinase activity, and the aminopeptidase inhibitor bacitracin (10^{-5} M)[15]. To examine the possible role of airway smooth muscle relaxing peptides such as vasoactive intestinal polypeptide (VIP) and peptide histidine isoleucine (PHI)[26] segments were incubated with α-chymotrypsin (2U/ml). This enzyme cleaves aromatic amino acyl bonds and abolishes relaxations to VIP and PHI in guinea-pig airways[10]. After 20 minutes of incubation with these drugs the osmolarity was raised to 450 mosM. Time-parallel control preparations from the same lung tissue specimens were challenged with hyperosmolar buffer twice, but without adding drugs.

To study the role of airway epithelium in the response to hyperosmolarity bronchial rings from which the epithelium had been removed by gentle rubbing with a forceps were challenged with hyperosmolar buffer of 450 mosM. The responses were compared to those of paired bronchial rings with intact epithelium from the same lung tissue specimen. The absence of epithelium was confirmed histologically.

Since lidocaine has been reported to bind to muscarinic receptors[25] we evaluated its anticholinergic properties in separate experiments by obtaining cumulative concentration response curves (CCRC) to methacholine (10^{-8} M to 10^{-4} M) in the presence or absence of lidocaine (10^{-5} M).

16.3.3. Hyperosmolarity and the cholinergic responsiveness of the airways

To investigate the influence of hyperosmolarity on the cholinergic responsiveness of the airway preparations, first a CCRC to methacholine (10^{-8} M to 10^{-4} M) was obtained and, after washout and return to baseline, hyperosmolar buffer of 450 mosM was added to the organ bath. Once the contractile response to hyperosmolar buffer had reached a plateau (after about 30 minutes) a second CCRC to methacholine was obtained in the presence of hyperosmolar buffer and, after washout, a third CCRC again at 317 mosM. In time-parallel control preparations three consecutive CCRC to methacholine were obtained. Here methacholine was added to the organ bath before the second CCRC to match the increase in BCS caused by hyperosmolar buffer in the treated segment.

16.3.4. Hyperosmolarity and airway responses to electric field stimulation

Electric field stimulation (EFS) was applied with a custom-made tissue stimulator that produced voltage-constant rectangular pulses of alternating polarity, via platinum plate electrodes positioned parallel to the preparation (see *Figure* 9.1). 30 s Tetani of supramaximal voltage (50V), a short pulse duration (0.3 ms) and a frequency of 30 Hz were used[8]. We have previously shown that these stimuli elicit a triphasic contraction-relaxation-contraction response of the airway muscle. The first contractile phase results from activation cholinergic excitatory nerves. The relaxation phase is non-adrenergic and partially inhibited by the neurotoxin tetrodotoxin (TTX)[8,9]. In addition, EFS gives rise to tetrodotoxin-insensitive slow contractile responses which result from the release of metabolites of arachidonic acid in the bronchial wall[8]. A typical response is shown in *Figure* 16.2.

First, a control response to EFS was obtained in each of two airway segments. After washout, half an hour later, the osmolarity was raised to 450 mosM in one organ bath. Since earlier findings had indicated that the response to EFS depended on baseline airway tone[7] the control preparation received methacholine to match the increase in tone caused by hyperosmolarity. The second response to EFS was obtained at the peak of the contraction to methacholine or

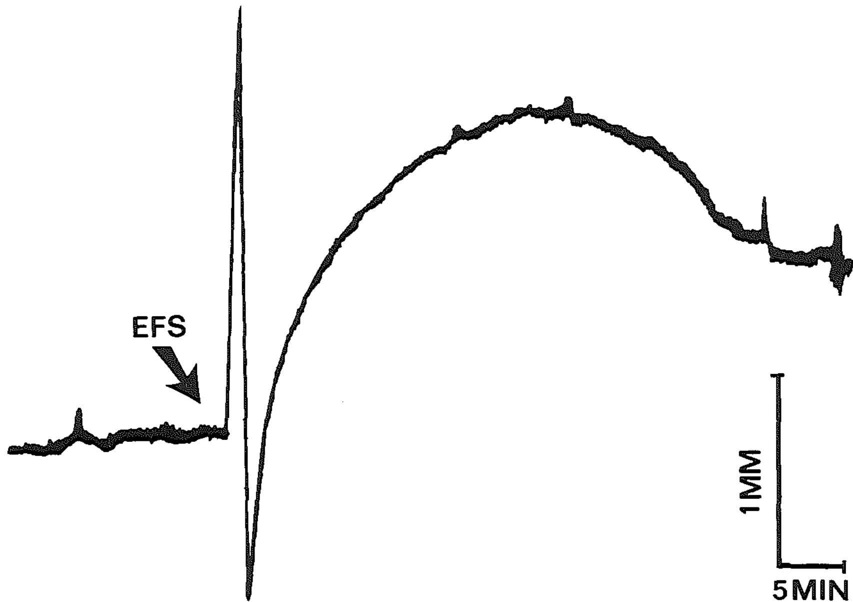

Figure 16.2. Tracing of a typical response of a fresh central human bronchus to electric field stimulation (EFS) *in vitro*. At the point indicated by the arrow a 30 s tetanus of supramaximal voltage (50 V), short pulse duration (0.3 ms) and a frequency of 30 Hz was given. A cholinergic contraction is followed by a deflection below baseline (non-adrenergic relaxation) and a slow contraction[8].

hyperosmolar buffer. After washout half an hour later a third response to EFS was obtained after contracting both airways with methacholine to the same level as before the second EFS-stimulation.

16.3.5. Data analysis

The spontaneous position of a preparation under a 500 mg load is defined as the baseline contractile state (BCS). BCS is expressed on a 0 to 100% functional scale which defines the maximal active contractile range (%MACR)[16,17,23]. The second maximal response to 10^{-4} M methacholine (see 16.3.1) was taken as 100 %MACR and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M), which was determined at the end of each experiment, was taken as 0% MACR. Since BCS before the first (BCS_1) and the second (BCS_2) response were different it was important to know if the relaxation and contraction phase were dependent on BCS. Therefore the relation between BCS_1, the relaxation and the contraction phase was analyzed for the first responses of all the bronchial rings using a standard analysis of covariance with lungs as groups and BCS_1 as the covariable[30]. The analysis revealed that the relaxation phase depended on BCS_1 (range of BCS_1 8.9 to 79.8 %MACR)

but only for values of BCS_1 below 30 %MACR (regression coefficient for covariate B = –0.137, P<0.002). The contraction phase was independent of BCS_1 (B = –0.056, P>0.45). The analysis also showed significant differences in the mean relaxation and contraction phase between rings from different lung tissue specimens (P<0.001). Therefore the contraction and relaxation phase of the second response are expressed as a percentage of the contraction and relaxation phase of the first response (%first). Since pairs of rings with and without epithelium were stimulated with hyperosmolar buffer only once the relaxation and contraction phase were expressed as %MACR. The sensitivity to methacholine is expressed as $-\log EC_{50}$. The maximal effect (E_{max}) of methacholine and the different phases of the responses to EFS are expressed as %MACR. Values of the contraction and relaxation phase as well as differences between sequential responses to EFS were compared for treated preparations and controls from the same tissue specimens using paired Student t-tests (after a log-transformation if appropriate). P values <0.05 (two-tailed) were considered significant. Data are expressed as means ± SEM.

16.3.6. Drugs

Indomethacin (Duchefa, The Netherlands) and FPL 55712 (a gift from Fisons, United Kingdom) were dissolved in methanol. Mepyramine (Rhône-Poulenc, France), atropine (Brocacef, The Netherlands), lidocaine (Astra, Sweden), tetrodotoxin (Sigma, USA), N-(α-rhamnopyranosyloxy-hydrophosphinyl)-Leu-Trp (phosphoramidon, Sigma, USA), bacitracin (Sigma, USA), captopril (Squibb, USA), α-chymotrypsin type I-S from bovine pancreas (Sigma, USA), methacholine hydrobromide (Janssen Pharmaceuticals, Belgium) and ethylenediamine tetra-acetic acid disodium salt (EDTA) (Sigma, USA) were dissolved in saline. L-isoproterenol sulphate (Janssen Pharmaceutica, Belgium) was dissolved in water containing ascorbic acid (88 mg/l). Preliminary experiments showed that ascorbate and methanol in these concentrations have no effect on airway muscle function.

16.4. Results

16.4.1. Effect of hyperosmolarity and its mechanism

Addition of hyperosmolar buffer caused a rapid relaxation, which was maximal after 5.0 ± 0.3 minutes, followed by a slow contraction, which was maximal after 25.4 ± 0.8 minutes. BCS before the first response was higher than BCS before the second response (41.3 ± 2.3 %MACR versus 31.2 ± 2.3 %MACR, P<0.001). The relaxation and contraction phase of the first and second response did not differ significantly (13.9 ± 1.2 %MACR versus 12.2 ± 1.3 %MACR and 26.0 ± 1.8 %MACR versus 29.5 ± 2.0 %MACR, both NS).

Table 16.1 shows the effect of several drugs on BCS, the relaxation and the contraction phase. With indomethacin, mean BCS_2 was significantly above the control value (+12.3 %MACR, $P<0.05$) and with atropine + mepyramine mean BCS_2 was significantly lower than the control value (-15.4 %MACR, $P<0.05$). In the groups where mean BCS_2 was lowest, i.e. the preparations treated with mepyramine, mepyramine + atropine and lidocaine, the relaxation phase was also significantly lower, suggesting that this reduction of the relaxation phase was non-specific and related to the low BCS. With mepyramine the contraction phase was 41.2% of the control-value ($P<0.001$), with atropine 50.0% ($P<0.01$) and with lidocaine 48.7% ($P<0.05$). The combination of mepyramine and atropine reduced contraction phase to 19.2% of the control value ($P<0.001$), and this reduction was significantly larger than the reductions caused by mepyramine or atropine alone (unpaired t-test, $P<0.001$ for both comparisons). The effect of mepyramine + atropine on BCS, the relaxation and the contraction phase

Table 16.1. Values of BCS_2, the relaxation and the contraction phase in the absence and presence of several drugs

		BCS_2 (%MACR)	relaxation phase (%first)[a]	contraction phase (%first)[a]	n
indomethacin	–	32.8 ± 5.2	88.0 ± 10.6	127.3 ± 9.6	
	+	45.1 ± 3.5*	70.9 ± 22.1	108.9 ± 17.6	7
FPL 55712	–	24.0 ± 5.5	62.9 ± 11.6	112.9 ± 4.1	
	+	23.3 ± 5.1	77.3 ± 16.6	149.3 ± 20.2	6
mepyramine	–	28.5 ± 4.1	101.8 ± 14.4	128.4 ± 11.3	
	+	21.0 ± 7.2	57.7 ± 14.1**	52.9 ± 14.2**	9
atropine	–	35.1 ± 8.3	91.9 ± 16.8	115.9 ± 11.8	
	+	29.5 ± 5.1	123.9 ± 16.5	58.0 ± 11.7**	7
mepyramine	–	31.1 ± 3.7	72.1 ± 9.3	138.9 ± 11.5	
+ atropine	+	15.7 ± 3.6*	38.0 ± 10.5*	26.6 ± 10.5***	8
lidocaine	–	24.3 ± 5.0	74.2 ± 13.2	116.6 ± 5.3	
	+	16.8 ± 2.5	36.9 ± 14.5*	56.8 ± 14.9*	9
tetrodotoxin	–	35.2 ± 5.8	103.7 ± 17.8	110.8 ± 12.5	
	+	35.7 ± 7.1	105.5 ± 24.2	126.3 ± 14.6	8

Abbreviations: BCS_2, the baseline contractile state before the second response to hyperosmolar buffer expressed as a percentage of the maximum active contractile range (%MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M).

* $P<0.05$; ** $P<0.01$; *** $P<0.001$ for comparison of responses in the absence (-) and presence (+) of a drug.

[a] The relaxation and contraction phase are expressed as a percentage of the first response on the same preparation.

is illustrated by the tracings shown in *Figure* 16.3. Indomethacin, FPL 55712 or TTX had no effect on the response to hyperosmolar buffer.

Table 16.2 shows that the presence of phosphoramidon, phosphoramidon + captopril + bacitracin, α-chymotrypsin or the absence of epithelium did not alter BCS_2 or the relaxation phase. With phosphoramidon the contraction phase increased to 128.0% of the control value ($P<0.05$) and with phosphoramidon + captopril + bacitracin to 131.0% ($P<0.01$). In the absence of epithelium the contraction phase increased to 131.8% of the control value ($P<0.05$). The effect of phosphoramidon on the contraction phase is exemplified by the recording shown in *Figure* 16.3.

Lidocaine did not affect the dose response curves to methacholine indicating that lidocaine does not block muscarinic receptors. The mean $-\log EC_{50}$ in the absence and presence of lidocaine was 5.73 ± 0.18 and 5.77 ± 0.14 respectively (NS, $n = 5$).

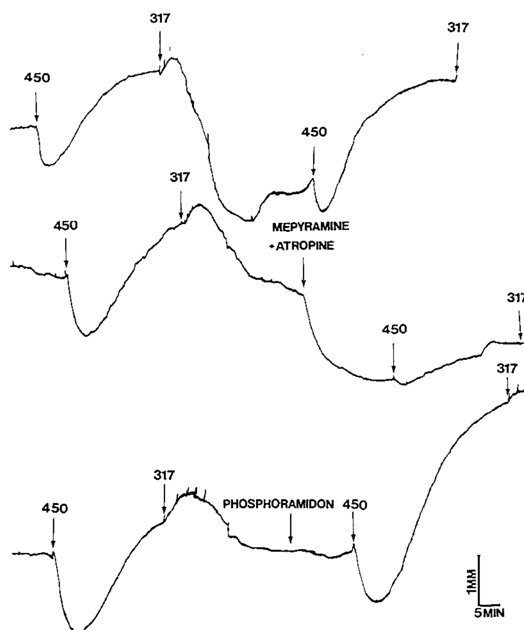

Figure 16.3. Tracings of responses of fresh human central bronchi to Krebs-Henseleit buffer of 450 mosM in the absence and presence of different drugs. The arrows indicate where the osmolarity was changed to 450 mosM (450) and back to 317 mosM (317) and where drugs were added. Upper tracing: the responses of a control preparation challenged with hyperosmolar buffer followed by washing with normal buffer and a second hyperosmolar challenge. Middle tracing: responses to hyperosmolar buffer before and after addition of mepyramine (5×10^{-6} M) and atropine (1.2×10^{-6} M). The combination of these drugs reduces the baseline contractile state as well as the relaxation and the contractile phase of the response to hyperosmolarity. Lower tracing: responses to hyperosmolarity in the absence and presence of phosphoramidon (5×10^{-6} M), which increases the contractile phase of the response to hyperosmolarity.

Table 16.2. Values of BCS_2, the relaxation and the contraction phase in the absence and presence of drugs which interfere with the degradation of neuropeptides, and of epithelium

		BCS_2 (%MACR)	relaxation phase (%first)[a]	contraction phase (%first)[a]	n
phosphoramidon	–	29.5 ± 3.6	74.9 ± 12.8	136.0 ± 9.5	
	+	32.6 ± 4.1	101.0 ± 16.8	174.0 ± 12.6*	10
phosphoramidon	–	29.2 ± 4.1	81.4 ± 15.3	126.9 ± 13.6	
+ captopril	+	32.9 ± 5.3	105.9 ± 12.3	166.0 ± 19.3**	8
+ bacitracin					
α-chymotrypsin	–	28.0 ± 5.5	91.6 ± 13.8	113.2 ± 12.3	
	+	24.3 ± 5.1	76.2 ± 14.2	104.6 ± 12.0	5
epithelium	+	30.0 ± 4.2	-8.8 ± 2.8[b]	37.4 ± 5.3[b]	
	–	32.8 ± 5.4	-12.8 ± 4.0[b]	49.3 ± 4.6[b]*	6

Abbreviations: BCS_2, the baseline contractile state before the second response to hyperosmolar buffer expressed as a percentage of the maximum active contractile range (%MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M).

* $P<0.05$; ** $P<0.01$ for comparison of responses in the presence (+) and absence (— of a drug.

[a] The relaxation and contraction phase are expressed as a percentage of the first response on the same preparation.

[b] Expressed as %MACR, see 16.3.5.

16.4.2. Hyperosmolarity and the cholinergic responsiveness of the airways

Table 16.3 shows the mean $-\log EC_{50}$ and E_{max} values of methacholine in buffer of 317 and 450 mosM. In buffer of 450 mosM the CCRC shifted leftwards and $-\log EC_{50}$ increased 0.22 ± 0.05. This was significant compared to a difference of 0.01 ± 0.03 between the first and the second curves in control preparations kept in 317 mosM ($P<0.001$). This effect was reversible so that after re-establishing iso-osmolarity $-\log EC_{50}$ was no longer significantly different from the initial values. E_{max} increased by 8.2 ± 1.7%MACR in buffer of 450 mosM, and this was significant compared to a decrease of 0.9 ± 2.4%MACR in controls ($P<0.001$).

16.4.3. Hyperosmolarity and airway responses to electric field stimulation

Table 16.4 shows the responses to EFS before, during and after exposure to hyperosmolar buffer together with the responses of time-parallel control preparations. The mean cholinergic contraction obtained during hyperosmolarity was 128.9% of the mean cholinergic contraction before hyperosmolarity whereas in controls this was only 89.4% ($P<0.001$). A similar difference between controls and treated preparations was seen if the responses during and after hyperosmolarity were compared (121.8% versus 91.1%), indicating that the increase

Table 16.3. Responses of segments of central human airways to methacholine at different osmolarities[a]

treated	317 mosM	450 mosM	317 mosM
$-logEC_{50}$	5.89 ± 0.05	6.11 ± 0.08***	5.86 ± 0.07
E_{max}	100.5 ± 2.4	108.7 ± 2.1***	93.7 ± 2.3
control	317 mosM	317 mosM	317 mosM
$-logEC_{50}$	5.90 ± 0.07	5.91 ± 0.04	5.93 ± 0.07
E_{max}	95.0 ± 1.9	94.1 ± 1.4	90.0 ± 1.5

Abbreviations: $-logEC_{50}$, the negative logarithm of the methacholine concentration that caused 50% of the maximal effect; E_{max}, maximal effect of methacholine expressed as a percentage of the maximum active contractile range (%MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) at the beginning of the experiment and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5 x 10^{-3} M).

*** $P<0.001$, compared to control.

[a] For each preparation a CCRC was obtained before, during and after a change in the osmolarity of the bathing fluid. In controls the osmolarity remained unchanged. Mean results from 7 different lung tissue specimens.

Table 16.4. Responses of human airways to electric field stimulation at different osmolarities[a]

treated	317 mosM	450 mosM	317 mosM
cholinergic twitch	20.4 ± 7.7	26.3 ± 7.2***	21.6 ± 7.5
non-adrenergic relaxation	-9.5 ± 3.3	-5.4 ± 3.4	-3.2 ± 1.6
slow contraction	7.7 ± 3.0	11.7 ± 2.8	12.0 ± 4.6
control	317 mosM	317 mosM	317 mosM
cholinergic twitch	22.8 ± 6.5	20.4 ± 5.7	22.6 ± 5.6
non-adrenergic relaxation	-10.1 ± 5.4	-11.7 ± 5.4	-10.7 ± 4.7
slow contraction	11.0 ± 4.3	12.6 ± 6.2	15.8 ± 5.1

*** $P<0.001$ compared to control.

[a] The results are expressed as a percentage of the maximum active contractile range (%MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5 x 10^{-3} M). For each preparation a response to electric field stimulation (EFS) was obtained before, during and after a change in the osmolarity of the bathing fluid. In controls the osmolarity remained unchanged. Mean results from 7 different lung tissue specimens.

of the cholinergic phase in hyperosmolar buffer was reversible. The relaxation phase tended to decrease in hyperosmolar buffer but this was not significant. The slow contractile phase did not change in hyperosmolar buffer. The concentration of methacholine prior to the third response to EFS was similar in controls and in preparations that had been treated with hyperosmolar buffer (3.5 ± 1.6 x 10^{-7} M versus 6.5 ± 3.0 x 10^{-7} M, NS).

16.5. Discussion

Our study shows that hyperosmolar Krebs-Henseleit buffer of 450 mosM elicits a biphasic relaxation-contraction response in isolated fresh central human airways. None of the drugs that we used specifically inhibited the initial rapid relaxation phase, and this phase was unchanged in the presence of α-chymotrypsin or after removal of the epithelium. The contraction phase was inhibited by a histamine (H_1) receptor antagonist, a muscarinic receptor antagonist and a local anesthetic and it was potentiated in the presence of a NEP inhibitor, a combination of a NEP inhibitor with other peptidase inhibitors and after removal of the epithelium. In hyperosmolar buffer the sensitivity and maximal response to methacholine as well as the cholinergic twitch to EFS were increased.

Our results only partly concur with those by Finney *et al.*[12] who reported that in isolated human airways hypertonic buffer gives an initial relaxation followed by a slow (30 to 40 minutes) return to baseline. We also found a relaxation phase followed by a slow contraction phase, but the contraction phase was about twice as big as the relaxation phase, resulting in a net contraction (see *Figure* 16.1). This difference might be explained by different handling of the tissue (fresh airways versus airways that had been stored overnight) and different methods of measurement (isotonic versus isometric).

In earlier experiments on peripheral human airways we found that hyperosmolar buffer of 450 mosM evoked a monophasic contractile response, while we found no evidence for the release of histamine; anticholinergics were not tested in this previous study[16]. These two differences between the present and the previous study may well be related to methodological factors. Firstly, in the present study we raised the osmolarity by adding NaCl directly to the organ bath while in the previous study[16] we washed the iso-osmolar buffer in the organ bath away with hyperosmolar buffer. During this washing procedure we probably missed the initial phase of the response. Secondly, in the present study the contribution of differences between preparations to the total variability in the responses was minimized by stimulating each preparation twice so that it could serve as its own control. In the earlier study[16] the responses of drug-treated preparations were compared to those of a time-parallel control. This increases the risk of a type II error (false negative result) since differences between preparations and differences due to drug effects will both contribute to the observed variability. Thus, it seems possible that in the previous study[16] we did not detect the relaxation phase due to different ways of increasing the osmolarity of the buffer, and that we missed a histamine-effect because of a type II error. Indeed, in later experiments on fresh peripheral airways where we added extra NaCl directly to the organ bath we also observed a rapid relaxation followed by a contraction which was virtually abolished in the presence of mepyramine + atropine (R.C. Jongejan, unpublished observations).

The effect of hyperosmolarity seems to differ between species. A study on the trachea of anesthetized dogs showed that hyperosmolar buffer had no effect

on the external tracheal diameter[5]. In precontracted guinea pig trachea luminal application of hyperosmolar buffer resulted in an epithelium-dependent relaxation[24], while we observed a biphasic relaxation-contraction response. However, we did not selectively apply the osmotic stimulus to the mucosal surface of the airway *in vitro* and this could also account for the difference in response pattern.

Which are the mechanisms of the relaxation and contraction phase? The reduction of relaxation phase in the presence of mepyramine, mepyramine + atropine and lidocaine was probably nonspecific since these drugs reduced BCS to below 25 %MACR, a level associated with low values of R. The relaxation phase is probably not caused by relaxing peptides which contain aromatic amino acyl bonds such as VIP or PHI nor by an epithelium derived relaxing factor, since it remained unchanged in the presence of α-chymotrypsin or after removal of the epithelium. We would therefore suggest, by exclusion, that the relaxation phase is caused by the release of an unknown relaxing substance or a direct effect of hyperosmolarity on the airway muscle. As suggested by Finney *et al.*[12], it seems possible that an increased extracellular Na^+ concentration leads to the influx of Na^+ into the smooth muscle cell via the Na^+/Ca^{2+}-exchanger[28]. This exchanger couples the influx of Na^+ to the efflux of Ca^{2+}, which will cause relaxation. This possibility, however, requires further investigation.

The reduction of the contraction phase with atropine or mepyramine indicates that histamine and acetylcholine contribute to this phase. That the reduction of the contraction phase with mepyramine + atropine was significantly greater than with atropine and mepyramine alone points to a separate and additive contribution of acetylcholine and histamine to smooth muscle contraction in hypertonic buffer. Several explanations are possible for the inhibitory effect of lidocaine on the contraction phase. Firstly, it has been suggested that lidocaine may block muscarinic receptors[25] but this seems unlikely since in the present study lidocaine did not change the response to methacholine. Secondly, lidocaine may inhibit nervous conductance. However, because TTX which inhibits nervous conductance too, had no effect on the contractile phase this seems an unlikely explanation for the effect of lidocaine. Thirdly, lidocaine may have inhibited the release of histamine via a direct effect on mastcells[19] but this seems unlikely since this effect is only seen at concentrations above 10^{-3} M while we used 10^{-5} M. Finally, lidocaine may have blocked the release of neuropeptides from afferent nerves[27], a process that is insensitive to TTX[22]. The potentiation of the contraction phase with phosphoramidon further supports the possibility that peptides that are sensitive to degradation by NEP are implicated in the contraction phase. A recent study by Umeno *et al.*[31] on rat trachea also indicates that hyperosmolarity releases neuropeptides from afferent nerves. The potentiating effect of epithelial denudation on the contraction phase is also in keeping with a contribution of NEP-sensitive peptides to this phase, since in the airways NEP is predominantly located to the epithelium[15]. Alternatively, removal of the epithelium increases the sensitivity of the airway muscle to acetylcholine and histamine[1] and this

could also have contributed to an increase of the contraction phase. So, the relaxation phase of the response to hyperosmolarity may involve the release of an unknown muscle relaxing substance, which is not of epithelial origin, or a direct relaxing effect of hyperosmolar buffer on the airway muscle caused by the influx of sodium into the smooth muscle cell coupled to the efflux of calcium via the Na^+/Ca^{2+} exchanger. The contractile phase may result from stimulation of afferent nerves and the release of neuropeptides, acetylcholine and histamine. These mechanisms may well be interrelated, as has been demonstrated in rat airways[18] where neuropeptides may release histamine and acetylcholine, but this remains to be elucidated for human airways.

Our finding that the sensitivity to methacholine slightly increases in hyperosmolar buffer confirms our previous results on peripheral airways[16]. This increased sensitivity of the airway muscle could also explain the increased cholinergic response to EFS that we have found. Alteratively, hyperosmolarity may facilitate the release of acetylcholine during EFS. The mechanism of this hyperosmolarity-induced airway hypersensitivity remains to be investigated.

Can our findings contribute to the understanding of bronchoconstriction to inhalation of hypertonic saline in asthmatics? It has already been pointed out (see 16.3.2) that the challenge with buffer of 450 mosM that we have used may well be relevant to the *in vivo* situation. However, several differences between the *in vivo* and *in vitro* situation make it difficult to know how the *in vitro* response relates to the *in vivo* one. First, we could not use asthmatic airways. Second, unlike *in vivo*, the osmotic stimulus *in vitro* was not selectively applied to the mucosa. It is possible that selective application of the hyperosmolar stimulus to the mucosa would result in an initial contraction, since the contraction phase seems of mucosal origin. In this case the response pattern *in vivo* and *in vitro* would be more alike. The relaxation phase, which does not seem to originate from the mucosa, may not be seen or even follow the initial contraction, in case hyperosmolarity penetrates to the level of the smooth muscle. Third, the time course of the responses *in vivo* and *in vitro* can not be simply compared since contractions *in vitro* are often slower than bronchoconstriction *in vivo*. For instance, *in vivo* the response to inhalation of a dose of methacholine will reach a maximum after about 3 min whereas in our experiments the maximum of the response to a dose of methacholine will be reached after 12 to 15 min. Based on this comparison we would estimate that the contraction phase of the response to hypertonicity *in vivo* would take 5 to 6 min, which is comparable to the time-course of hyperosmolarity induced bronchoconstriction *in vivo*. Fourth, we do not know how the BCS *in vitro* relates to baseline airway tone *in vivo*. Finally, the mucosal swelling which probably occurs *in vivo* after the release of neuropeptides or histamine and which might contribute to airway narrowing does not occur *in vitro*. However, the fact that in asthmatics H_1-receptor antagonists[11,13] and blockers of muscarinic cholinoreceptors[4] provide partial protection against the bronchoconstriction to inhaled hyperosmolar saline suggests that the response *in vivo* and *in vitro* have similar mechanisms. In this

respect two of our findings are potentially important. First, that hyperosmolarity may not only release histamine and acetylcholine in the airway wall but also neuropeptides. Secondly, that the absence of the epithelium potentiates the contractile effect of hyperosmolarity. Since in asthmatic airways the epithelium seems to be damaged[14,20,21], this might be a factor which contributes to the hyperresponsiveness to inhalation of hypertonic saline in asthma.

In *conclusion*, hyperosmolarity causes a biphasic relaxation-contraction response in central human airways. The relaxation phase is probably not due to relaxing peptides such as VIP or PHI or the release of an epithelium derived relaxing factor, but may result from a direct effect of hyperosmolar buffer on the muscle. The release of acetylcholine, histamine and neuropeptides contributes to the contractile phase. This contractile phase is increased after removal of the epithelium. Prostaglandins or LTC_4/D_4 do not seem to contribute to the contractile phase.

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Chapter 17

Summary, General Discussion and Directions for Further Research

17.1. Summary

Asthma is a syndrome characterized by paroxysms of dyspnea, wheezing and cough. Many asthmatics are atopic, which means that they have an inclination to produce IgE antibodies to common environmental antigens. Most asthmatics have an increased tendency to bronchoconstrict to allergens and to pharmacological (e.g. methacholine and histamine) and/or physical stimuli (e.g. cold air, nebulized hypo- and hypertonic saline). This phenomenon is called bronchial hyperresponsiveness. In asthmatic bronchial hyperresponsiveness the stimulus intensity at which the airways start narrowing is abnormally low and the maximum attainable reduction of airway caliber is abnormally high. Furthermore, the level of bronchial hyperresponsiveness is more or less independent of baseline airway caliber.

Histological studies almost invariably show that asthma is accompanied by inflammatory changes in the walls of the airways. These changes comprise damage of the airway epithelium, hypertrophy of the airway muscle and the mucous glands as well as an inflammatory reaction in the lamina propria with dilated capillaries, interstitial edema and a cellular infiltrate containing many eosinophils, neutrophils, mononuclear cells and lymphocytes. That bronchial hyperresponsiveness and inflammatory changes in the airway wall occur simultaneously suggests a causal relationship between the defective regulation of airway patency in asthma and these inflammatory changes.

Airway patency depends on smooth muscle tone, mucus deposition, and the thickness of the airway wall. It is difficult to say which of these is the most important in asthma, but the observation that airway smooth muscle relaxants such as β_2-agonists cause immediate dilatation of the airways during asthma attacks and that inhaled airway smooth muscle stimulants such as methacholine narrow the airways, strongly suggests that the airway muscle is an important determinant of airway caliber. To improve the understanding of mechanisms involved in asthma, it is therefore important to investigate how the different stimuli that cause airway narrowing in asthmatics interact with airway smooth muscle or its regulation and to investigate how inflammatory changes such as seen in asthmatic airways interfere with airway smooth muscle function. Doing this requires a valid model of human airway smooth muscle function and its regulation.

De Jongste *et al.*[2] have previously validated methods to measure responses

of isolated human airways both to pharmacological stimuli and to electric field stimulation. This last stimulus selectively activates postganglionic nerve endings in the airway wall which leads to a triphasic contraction-relaxation-contraction response of the airway muscle. The contractile phase results from activation of cholinergic nerves. The relaxation phase is non-adrenergic and partially inhibited by the neurotoxin tetrodotoxin. In addition, this stimulus gives rise to a tetrodotoxin-insensitive slow contractile response which results from the release of leukotrienes. The airways were dissected from lung tissue of patients operated for bronchial carcinoma. Some of these patients had chronic obstructive pulmonary disease (COPD). De Jongste *et al.* found that, except for an increased maximal response to histamine in airways from these patients, functional responses of airways from patients with and without COPD were comparable. Therefore it seems acceptable to use human airways dissected from tissue obtained at thoracotomies as a model for the function and regulation of normal human airway smooth muscle.

In this thesis the model by de Jongste *et al.* has been further improved. The improved model was used to investigate the effect of physical stimuli and activated inflammatory cells and their mediators on airway smooth muscle function and its regulation. The results are discussed in relation to mechanisms of bronchial hyperresponsiveness in asthma.

To allow for critical evaluation of the results, **Part I** contains an overview of the different aspects of bronchial hyperresponsiveness in asthma and, since for the studies in this thesis airways from smokers with lung cancer were used, in COPD.

Chapter 1 discusses clinical aspects of airway hyperresponsiveness in asthma and COPD. **Chapter 2** describes the morphology of the airways in normals, in asthmatics, and in patients with COPD. **Chapter 3** deals with the concept of airway responsiveness in vitro. It also gives an overview of the factors that determine to which extent inhaled stimuli will change airway caliber. The following chapters cover each of these factors in more detail. **Chapter 4** summarizes functional aspects of different inflammatory cells involved in asthma. **Chapter 5** describes the role of the mucosa, **Chapter 6** that of the autonomic innervation and **Chapter 7** that of airway smooth muscle and airway mechanics in the regulation of airway caliber. Possible abnormalities of the regulation of airway caliber in asthma and COPD are discussed. Finally, in **Chapter 8** the aims of the studies are derived.

Part II describes the methods used.

Chapter 9 gives a detailed description of the dissection of human airways. It is shown that isotonic responses of bronchiolar segments and spiral strips to repeated stimulation with methacholine are reproducible and comparable. The total variation in responses of both strips and segments is similar. Limited length-active shortening experiments indicate that 250 mg is a suitable load for

both strips and segments. Under this load both types of preparations show a stable intrinsic contractile activity, which is significantly higher in segments.

Part III deals with inflammation.

Chapter 10 describes the effect of subthreshold and threshold concentrations of the inflammatory mediators histamine, the stable thromboxane (Tx) A_2 analogue U46619, prostaglandin (PG) D_2, $PGF_{2\alpha}$ or leukotriene (LT) C_4 on the responsiveness of the airways to methacholine. With the exception of LTC_4, the presence of any of these mediators at either concentration increases the sensitivity to methacholine by a factor 1.1 to 2. This increase does not depend on the dose of the mediator. The maximal response to methacholine is unchanged in the presence of the mediators.

In **Chapter 11** the results of **Chapter 10** are further analyzed to see how mediator-induced muscle hypersensitivity interacts with the increased thickness of the airway wall that has been observed in asthmatic airways. The effect of these two factors on the increase in airway resistance of a single isolated human airway to increasing concentrations of methacholine is calculated. The result suggests that mediator-induced hypersensitivity to cholinergic stimuli may explain part of the leftward shift of the asthmatic dose response curve to methacholine especially at lower levels of bronchoconstriction. Increased thickness of the airway wall is likely to be more important for the increased slope and maximum of the asthmatic dose response curve.

Chapter 12 deals with the combined effect of epithelial denudation and threshold concentrations of inflammatory mediators or anti-IgE on the responsiveness of isolated airways to methacholine. Firstly, both in strips with or without epithelium histamine, U446619 and PGD_2, cause a non-parallel leftward shift of the dose response curve to methacholine. The effect of anti-IgE on the responsiveness of the airways is highly variable and, overall, not significant. Type II error analysis (false negative results) reveals that the results do not exclude an effect of anti-IgE on smooth muscle responsiveness similar to that of a single mediator. Airway strips without epithelium are 1.6 times more sensitive to methacholine than strips with epithelium. Furthermore, removal of the epithelium causes a greater increase of the response to a single dose of anti-IgE than to the other agonists, suggesting that the airway epithelium is an important determinant of the response to inhaled mast cell-activators such as allergens. When taken together epithelial denudation and threshold concentrations of histamine, U46619 or PGD_2 cause a 4.0 to 9.1-fold increase in sensitivity based on the $-\log EC_{10}$ and a 1.8 to 3.0-fold increase in sensitivity based on the $-\log EC_{50}$.

The effects of activated human granulocytes on isolated airways are described in **Chapter 13**. Incubation of the airways with increasing numbers of granulocytes activated with serum-treated zymosan results in a considerable contraction of the airways which is proportional to the number of granulocytes present. Experiments with suitable antagonists show that the contractile response is predominantly due to lipoxygenase products, mostly LTC_4/D_4, and not to

histamine, prostaglandins or activated oxygen molecules. Granulocyte suspensions from atopic donors contain more eosinophils and the magnitude of the contraction to zymosan-activated granulocytes is related to the proportion of eosinophils among the granulocytes. With zymosan-activated granulocytes the sensitivity of the airways to methacholine tends to decrease with increasing numbers of granulocytes. The response to electric field stimulation is unchanged in the presence of zymosan-activated granulocytes, except for the slow contractile response which is slightly potentiated.

Part IV deals with the effect of different physical stimuli on isolated human airways.

Chapter 14 describes the effect of cooling on the responses of isolated human airways to electric and pharmacologic stimulation. Cooling does not seem to affect β-adrenoreceptor function. The airways do not respond to α-adrenoreceptor stimulation at 37°C, 27°C or 21°C. Cooling diminishes baseline airway tone and the sensitivity and maximal contraction to methacholine and LTC4. Cooling also reduces the cholinergic twitch to electric field stimulation. In addition, cooling lowers the frequency of electric field stimulation that causes half-maximal relaxation. In contrast, the sustained non-cholinergic contractile response to electric field stimulation, which is due to the endogenous release of LTC_4/D_4, is unchanged during cooling of the airways. This indicates that cooling facilitates the accumulation of LTC_4/D_4 released during stimulation of the airways.

In **Chapter 15** the effects of hypo- and hyperosmolarity on the functioning of isolated peripheral human airways are studied. Changes in osmolarity induce an increasing bronchoconstriction which is proportional to the magnitude of the change in osmolarity. Hypertonicity-induced airway narrowing results when buffer is made hypertonic with solutes that do not permeate the cellular membrane. The airways show no tachyphylaxis to the effects of hypo- or hypertonic buffer. The bronchoconstriction is not secondary to the release of histamine, LTC_4/D_4 or prostaglandins in the preparation. A β-agonist prevents the bronchoconstriction in mildly hyper- or hypotonic buffer, but not in strongly hyper- or hypotonic buffer. The bronchoconstriction in hypotonic buffer is totally dependent on extracellular calcium, whereas in mildly hypertonic buffer the bronchoconstriction seems partially dependent on intra-cellular calcium release. In strongly hypertonic buffer the contraction may be partly due to osmotic shrinkage. The responsiveness of the airways increases in hypertonic buffer and decreases in hypotonic buffer.

Chapter 16 describes the effect of hyperosmolarity on isolated fresh central human airways. Mildly hyperosmotic Krebs-Henseleit buffer containing extra sodium chloride evokes a biphasic response: a rapid relaxation phase followed by a slow contraction phase. The magnitude of the relaxation phase but not that of the contraction phase depends on baseline airway tone. The relaxation phase is not due to prostaglandins, histamine, acetylcholine, metalloendopeptidase-, angiotensin converting enzyme- or aminopeptidase-sensitive peptides,

muscle-relaxing peptides such as vasoactive intestinal peptide or an epithelium derived relaxing factor. The contractile phase is due to release of acetylcholine, histamine and metalloendopeptidase sensitive peptides probably from afferent nerves. It is potentiated by removal of the epithelium.

17.2. Conclusions

Methods

1. The similarity between responses of strips and segments justifies the use of bronchiolar segments for in vitro experiments. This is preferable on practical and theoretical grounds.

Inflammation

1. Threshold concentrations of inflammatory mediators such as histamine, PGD_2 and TxA_2 increase the sensitivity of human airway muscle especially to low concentrations of cholinergic agonists. Epithelial damage will add to this mediator-induced muscle hypersensitivity, while thickening of the airway wall will increase the maximal response of an airway to cholinergic stimulation.

2. Damage of the airway epithelium potentiates smooth muscle responses to mast cells activated with anti-IgE.

3. The contraction of isolated human airways to activated granulocytes depends both on the number of granulocytes and the proportion of eosinophils among the granulocytes. The principal bronchoconstrictor substance released by activated human granulocytes is LTC_4/D_4.

4. Granulocytes activated with zymosan do not increase airway smooth muscle responsiveness to cholinergic stimulation.

Physical stimuli

1. Cooling reduces the sensitivity of the airway muscle to contractile agonists and enhances the accumulation of LTC_4/D_4 in the airway wall after electric field stimulation.

2. Cooling increases the sensitivity of the airways to the relaxation induced by electric field stimulation.

3. In peripheral human airways increasingly hypotonic Krebs buffer causes increasing bronchoconstriction, related to the influx of extracellular calcium. Increasingly hypertonic Krebs buffer also causes increasing bronchoconstriction, but this is related to the mobilization of intracellular calcium at mild hyperosmolarity and to osmotic shrinkage at strong hyperosmolarity.

4. In fresh central human airways mildly hyperosmotic Krebs buffer will release acetylcholine, histamine and neuropeptides in sufficient quantities to contract airway muscle. This release itself or its effect on airway muscle is potentiated by removal of the epithelium.

17.3. General discussion

Having summarized the main results of the studies it is now time to give these results a more general perspective by discussing their possible contribution to the understanding of mechanisms of airway hyperresponsiveness in asthma.

17.3.1. Inflammation

Bronchial hyperresponsiveness in asthma is characterized by a leftward shift and an increased maximum of the dose response curve to a variety of inhaled stimuli (*Chapter 1*). *Figure* 8.1 illustrates that inflammation of the airways can contribute to bronchial hyperresponsiveness in many ways. The importance of inflammation of the airways in the pathogenesis of bronchial hyperresponsiveness in asthma is stressed by the recent findings of van Essen-Zandvliet *et al.*[19] who showed that the PD_{20} to histamine in children with mild to moderate asthma increases linearly towards normal during two years of treatment with inhaled steroids.

Our studies have focused on the possibility that in asthma inflammation-induced muscle hyperresponsiveness contributes to the leftward shift and the increased maximum of the dose response curve to inhaled methacholine. Activated granulocytes will probably not increase the cholinergic sensitivity of the airway muscle, but mediator-induced muscle hypersensitivity together with epithelial damage might indeed contribute to the leftward shift of the dose response to inhaled methacholine in asthma. Presumably, these factors will mostly affect the initial part of the dose response curve and especially the threshold dose, i.e. the dose of methacholine at which the airways start narrowing. Based on the relatively small effect of these two factors on the in vitro dose response curve to methacholine, we would predict their contribution to the total leftward shift of the in vivo dose response curve in asthma to be modest. Mediator-induced muscle hypersensitivity can, however, contribute to sudden and short-lived changes in the sensitivity of the airways in asthmatics such as seen after inhalation of allergen. Such changes are difficult to explain on the basis of smooth muscle hypertrophy and epithelial damage. Thus, combining our data with those from the literature we propose that mediator-induced muscle hypersensitivity, damage of the epithelium, smooth muscle hypertrophy and thickening of the airway wall are all factors involved in bronchial hyperresponsiveness in asthma. Mediator-induced muscle hypersensitivity and damage of the epithelium will contribute to a leftward shift, while muscle hypertrophy and thickening of the

airway wall, to which muscle hypertrophy contributes, will increase the maximal response. A factor that can not be evaluated with our model, but which will add to the leftward shift of the dose response curve, is the increased accessability of the underlying structures caused by damage of the epithelium.

Damage of the airway epithelium potentiates smooth muscle responses that are seen after activation of mast cells with anti-IgE. This suggests that once the inflammatory reaction in the airways has led to damage of the airway epithelium, the airways become even more sensitive to allergen which in turn might aggravate the inflammatory reaction, thus initiating a vicious circle.

The interpretation of our results with granulocytes depends on whether the numbers of granulocytes and the activation methods we have used are relevant to those during the late asthmatic reaction. If so, it seems that granulocytes can only contribute to smooth muscle contraction during the late asthmatic reaction via the release of LTC_4. One should realize of course, that there is probably more to the late asthmatic reaction than smooth muscle contraction alone. LTC_4 may also contribute to airway narrowing during the late asthmatic reaction by stimulating mucus secretion or by thickening the airway wall. Granulocytes may also release other products with these capacities. Further confirmation of the putative role of LTC_4 in the late asthmatic reaction awaits the development of selective and potent leukotriene antagonists.

In *summary,* we would suggest that the increased accessability of the underlying structures caused by damage of the epithelium, together with smooth muscle hyperresponsiveness caused by the presence of mediators and damage of the epithelium, all contribute to the leftward shift of the dose response curve. Smooth muscle hypertrophy and thickening of the airway wall, to which muscle hypertrophy also contributes, will increase the maximal response. Activated granulocytes do not increase airway responsiveness by sensitizing the airway muscle, but they have the potential to contract the airways via leukotrienes released by eosinophils.

17.3.2. Physical stimuli

The results presented here suggest that hyperosmolarity of the airway lining fluid may release constrictor substances in sufficient quantities to cause smooth muscle contraction. Hypo-osmolarity of the airway lining fluid may directly contract the airway muscle, although the protective effect of anticholinergics and antihistaminics (see *Table* 1.2) against hypotonic aerosol-induced bronchoconstriction suggests that this direct effect of hypotoncicty is not the only mechanism that may be involved in bronchoconstriction to hypotonic aerosols. Since asthmatics are hyperresponsive to almost any stimulus that directly or indirectly contracts airway smooth muscle, it might not be so surprising that asthmatics are hyperresponsive to inhalation of nebulised hypo- or hypertonic saline. It seems possible that hyperosmolarity releases more histamine in asthmatic than in non-asthmatic airways since in asthmatic airways more mast cells are

present. Also, damage of the epithelium might expose C-fiber endings so that more neuropeptides are released during inhalation of hypertonic saline. In addition, degradation of these neuropeptides by the damaged epithelium will be impaired, because of loss of neuropeptide degrading peptidases located to the epithelium. Furthermore, damage of the epithelium might also allow inhaled hypotonic saline to reduce the osmolarity in the milieu of the airway muscle and thus to cause bronchoconstriction.

In experiments in vivo it is not possible to cool the airways without drying them and therefore it is difficult to be sure that a thermal stimulus to the airways is not also an osmotic stimulus[4,5,17]. Fot this reason it has been difficult to prove or disprove on the basis of in vivo experiments that either cooling or drying of the airways by itself initiates bronchoconstriction after exercise or isocapnic hyperventilation of cold air. Consequently the importance of our in vitro results lies in that they allow us to separately evaluate the effect of cooling and changes in osmolarity on the airways. Our results would suggest that, as far as the airway muscle is concerned, not cooling but hyperosmolarity is a bronchoconstrictor stimulus. However cooling of the airways may have a strong modulatory effect on the responsiveness of the airways to contractile stimuli. First of all it will relax the airways and decrease the responsiveness of the airways to contractile stimuli. This may explain why in asthma the airways dilate during exercise[18] and only start narrowing within 2 to 5 minutes after completion of the exercise[6], when the airways start rewarming. That this might indeed occur in vivo is suggested by a recent study by Inman *et al.*[6], which shows that during exercise the airways become less responsive to contractile stimuli. Secondly, cooling seems to increase the efficacy of the non-adrenergic inhibitory system. Since this system may be defective in asthma[16], it may only provide extra protection against bronchoconstrictor stimuli in normals but not in asthmatics. The validity of this speculation, however, depends on whether the relaxation response to electrical field stimulation in vitro is indeed due to stimulation of the non-adrenergic inhibitory system, and this remains to be proven. Thirdly, cooling seems to enhance the accumulation of leukotrienes released during stimulation of the airways, and this may also contribute to the bronchoconstrictor effect of hyperventilation of cold air or exercise, especially in inflamed airways. Finally, one should realize that cooling of the airways or changes in osmolarity may also have effects on the vascular plexus in the lamina propria for instance by direct effects or by the release of neuropeptides. In addition these stimuli might induce reflexes. All these putative additional effects, which may affect airway patency as well, can not be evaluated with our model.

In *summary,* our results would suggest that asthmatics are hyperresponsive to direct smooth muscle stimulation by hypotonic saline and to the release of histamine, acetylcholine and neuropeptides by hypertonic saline. Cooling itself may not be a bronchoconstrictor stimulus, but it may modulate the responses of the airways to other constrictor stimuli that arise during hyperventilation of (cold) air, either during exercise or in a laboratory setting.

17.4. Directions for further research

It should be noted that the above discussion must remain speculative since the in vitro conditions may differ from the in vivo ones. The reasons for this uncertainty are:

1. Asthmatic airways are almost never available for functional in vitro-studies.
2. The elastic forces that oppose narrowing of the airways in vivo are not present in vitro.
3. The model does not allow direct measurement of the effects of airway wall thickening on the development of resistance to airflow.
4. We ignore to what extent a given amount of airway smooth muscle shortening or a given increase in airway muscle responsiveness as measured in a single bronchial ring in vitro will increase the resistance or responsiveness of the whole bronchial tree in vivo.
5. The stimuli that we have given in vitro reached the airways both from the luminal and the serosal side, while in vivo many of these stimuli will reach the airway from the luminal side only. Hence, the role of the airway mucosa in regulating the responsiveness of the airway to different stimuli can not be evaluated.

Given these uncertainties the biggest possible effort needs to be put into improving the model towards the in vivo situation. One way to do this is to measure changes in the resistance to flow of isolated human bronchi which are luminally perfused with Krebs buffer under a constant pressure that approaches the in vivo transmural pressure. In such a model the role of the epithelium in regulating the responsiveness of the airways by altering the permeability of the airway mucosa can elegantly be determined. The role of the vascular plexus in the lamina propria can, however, not be determined with this model. This disadvantage could be overcome by restoring the microcirculation in the vascular plexus of the lamina propria using the method described by Kröll *et al.*[12,13] where perfusion of branches of the a. pulmonalis leads to perfusion of the vascular plexus in the lamina propria via collateral vessels. In this model the effect of swelling of this plexus on airway resistance and mucosal permeability can be assessed. Furthermore, an in vitro model should be developed where the airways contract against an elastic load that mimicks the elastic recoil forces that oppose smooth muscle contraction in vivo. Such techniques have been described for vascular and airway smooth muscle[3,8].

Apart from these methodological improvements the above discussion raises several points related to mechanisms of bronchial hyperresponsiveness that require further study:

1. Direct evidence for the existence of an epithelium derived factor that relaxes human airway muscle is still lacking.
2. Despite the evidence that in asthma the epithelium is damaged or dysfunctional, there are few in vivo studies to show that the accesability of the structures

underlying the airway epithelium is increased in asthma. Little is known about the regulation of mucosal permeability in human airways.

3. There is evidence that in asthma the epithelium, the mucosa (i.e. the lamina propria) and the airway muscle all contribute to thickening of the airway wall[8]. For the epithelium and the mucosa the mechanisms of this thickening are obscure. Can the epithelial layer in asthma be damaged and thickened at the same time? Could one expect hyperemia of the mucosa to contribute to thickening of the airway wall if experiments in dogs show that hyperemia of the tracheal mucosa hardly causes thickening of the mucosa[14]? Is thickening of the airway wall a reversible process?
4. Exactly how does airway smooth muscle hypertrophy affect the dose response curve to inhaled agonists? Does muscle hypertrophy alter the length tension characterisitcs of asthmatic airways? If so, how will this influence the dose response curve? What are the mechanisms of smooth muscle hypertrophy in asthma? Are growth factors (e.g. neuropeptides) released during the inflammatory reaction? What is the effect of anti-inflammatory drugs on smooth muscle hypertrophy?
5. It seems that steroids are less effective in reversing bronchial hyperresponsiveness in adults[10,11] than in children[19], suggesting that in the long run asthma causes irreversible changes in the airways, as suggested by the findings of Brackel *et al.*[1]. What are these irreversible changes and what is their mechanism?
6. What exactly is the mechanism of the non-adrenergic inhibtory response in human airways? Are peptides (e.g vasoactive intestinal peptide) indeed involved? Is this response deficient in asthmatic airways?
7. In rodents the existence of neurogenic inflammation through the release of neuropeptides from afferent nerves is well-established. Does this penomenon also occur in human airways? If so, which peptides are involved and what are their effects?
8. How are granulocytes that migrate into the airway wall during the late asthmatic reaction activated? How do the granule products that they release affect human airways?
9. What are the effects of activated T-cells and macrophages (e.g. interleukins) on human airways?
10. What is the effect of cooling, hypo- and hyperosmolarity if, unlike in our experiments, these stimuli are administered via the airway lumen only? What is the effect of hypotonicity on central human airways? Does it only have a direct contractile effect on the airway muscle or does it, like hyperosmolar buffer, also release contractile substances?

Answering the above questions will require a keenly orchestrated effort by specialists in the fields of pathology, pharmacology, physiology, biochemistry, immunology, epidemiology, molecular biology, genetics, and clinical medicine.

Together, these disciplines will hopefully provide a clearer view on the complex etiology of the functional abnormality called bronchial hyperresponsiveness.

17.5. References

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Samenvatting

Astma is een syndroom dat wordt gekenmerkt door aanvallen van kortademigheid, piepende ademhaling en hoest. Veel mensen met astma zijn atopisch, wat betekent dat ze een aangeboren aanleg hebben om IgE-antilichamen te maken tegen antigenen (allergenen) uit hun omgeving. Patienten met astma zijn meestal overgevoelig voor inademing van allergenen, farmacologische stimuli (methacholine en histamine) en/of fysische stimuli (koude lucht en hypo- en hypertone aërosolen). Dit verschijnsel noemt men bronchiale hyperreactiviteit. Kenmerkend voor bronchiale hyperreactiviteit is dat de prikkelintensiteit waarbij een waarneembare luchtwegvernauwing optreedt veel lager is dan bij gezonden. Met name bij astma is de maximaal induceerbare luchtwegvernauwing groter dan normaal en de mate van bronchiale hyperreactiviteit min of meer onafhankelijk van de uitgangstoestand van de luchtwegen.

Uit bijna alle histologische studies blijkt dat astma gepaard gaat met een in ernst wisselende ontstekingsreactie in de wand van de luchtweg. Deze ontstekingsreactie omvat beschadiging van het epitheel, hypertrofie van de bronchiale gladde spier en van de slijmklieren, interstitieel oedeem, verwijding van capillairen in de lamina propria, en een cellulair infiltraat bestaande uit eosinofielen, neutrofielen, mononucleaire cellen en lymfocyten. Dat bij astma een specifiek type ontstekingsreactie in de wand van de luchtweg samengaat met bronchiale hyperreactiviteit kan op een causaal verband wijzen tussen deze ontstekingsreactie en de kennelijk gebrekkige regulatie van de luchtwegdoorgankelijkheid.

De doorgankelijkheid van de luchtwegen hangt af van de contractietoestand van de gladde spier die de luchtweg omringt, de hoeveelheid slijm in het lumen van de luchtweg en de dikte van de luchtwegwand. Het is moeilijk te zeggen welke van deze factoren de belangrijkste is. De waarneming dat verslappers van de gladde spier, zoals β_2-agonisten, onmiddellijk bronchusverwijding geven tijdens astma-aanvallen en dat stimulatoren van de gladde spier, zoals methacholine, de luchtwegen vernauwen, is een sterke aanwijzing dat de gladde spier een zeer belangrijke regulator is van de luchtwegdoorgankelijkheid. Om het inzicht in de mechanismen die aan astma ten grondslag liggen te vergroten is het derhalve belangrijk te onderzoeken hoe de verschillende stimuli die bij patienten met astma bronchoconstrictie veroorzaken de regulatie van de luchtwegdiameter beïnvloeden. Ook is het van belang te onderzoeken hoe de ontstekingsreactie in de wand van astmatische luchtwegen de functie van de gladde spier beïnvloedt. Om dit te onderzoeken is een valide model voor de regulatie en functie van humaan bronchiaal glad spierweefsel nodig.

De Jongste *et al.* hebben eerder methoden gevalideerd om responsen van geïsoleerde menselijke luchtwegen op farmacologische prikkels en op stimulatie met een electrisch veld te meten. Deze laatste stimulus activeert selectief postganglionaire zenuwvezels in de wand van de luchtweg en leidt tot een respons die uit drie opeenvolgende fasen bestaat. De eerste fase bestaat uit een snelle

cholinerge contractie veroorzaakt door stimulatie van cholinerge zenuwen in het preparaat. De tweede fase is een snelle niet-adrenerge relaxatie welke wordt gevolgd door een trage contractie veroorzaakt door de vrijmaking van leukotriëne C_4/D_4. De luchtwegen worden vrijgeprepareerd uit longweefsel verkregen bij longoperaties vanwege longkanker. Sommige van de patienten hebben chronisch obstructief longlijden (COPD). De Jongste *et al.* vonden, behalve een hogere maximale respons op histamine bij luchtwegen van patienten met COPD, geen functioneel verschil tussen luchtwegen van patienten met en zonder COPD. Het lijkt daarom gerechtvaardigd om luchtwegen die langs bovenstaande weg zijn verkregen te gebruiken als een model voor de functie en regulatie van normaal bronchiaal glad spierweefsel.

In dit proefschrift is het model van de Jongste *et al.* verder verbeterd. Het verbeterde model is gebruikt om het effect van fysische stimuli en geactiveerde ontstekingscellen en ontstekingsmediatoren op de functie en regulatie van glad spierweefsel te onderzoeken. De resultaten worden besproken in relatie tot mechanismen van bronchiale hyperreactiviteit bij astma.

Om een kritische analyse van de resultaten te vergemakkelijken, bevat **Deel I** een overzicht van de verschillende aspecten van bronchiale hyperreactiviteit bij astma en, omdat luchtwegen van rokers met COPD werden gebruikt, bij COPD.

In **Hoofdstuk 1** worden klinische aspecten van bronchiale hyperreactiviteit bij astma en COPD besproken. **Hoofdstuk 2** is gewijd aan de morfologie van normale luchtwegen waarbij de morfologische veranderingen bij astma en COPD ook aan bod komen. **Hoofdstuk 3** behandelt het concept van bronchiale reactiviteit *in vitro*. Er wordt ook een overzicht gegeven van de factoren die bepalen in welke mate een geïnhaleerde prikkel de diameter van de luchtwegen zal veranderen. **Hoofdstuk 4** bevat een overzicht van de functies van ontstekingscellen die een rol spelen bij astma. **Hoofdstuk 5** bespreekt de rol van de mucosa, **Hoofdstuk 6** die van de autonome innervatie en **Hoofdstuk 7** die van de gladde spier en van mechanische factoren, bij de regulatie van de luchtwegdoorgankelijkheid. Uit deze inleiding worden in **Hoofdstuk 8** de vraagstellingen van het onderzoek afgeleid.

In **Deel II** worden de gebruikte methoden beschreven

Hoofdstuk 9 bevat een gedetailleerde beschrijving van de dissectie van menselijke luchtwegen. Er wordt aangetoond dat isotone responsen van segmenten en strips van bronchioli bij herhaalde stimulatie met methacholine reproduceerbaar en vergelijkbaar zijn. De totale variatie in de reponsen van strips en segmenten is ook vergelijkbaar. Experimenten waarin de maximale verkorting van de spier bij verschillende spierlengte (i.e. belasting) wordt vergeleken, tonen aan dat een gewicht van 250 mg een geschikte belasting is voor zowel strips als segmenten. Bij deze belasting hebben beide typen preparaten een spontane tonus, die bij segmenten significant hoger is dan bij strips.

Deel III gaat over ontsteking.

Hoofdstuk 10 beschrijft het effect van twee verschillende concentraties van de ontstekingsmediatoren histamine, de stabiele thromboxaan (Tx) A_2 analoog U46619, prostaglandine (PG) D_2, $PGF_{2\alpha}$ en leukotriëne (LT) C_4, op de gevoeligheid van de luchtwegen voor methacholine. Er worden drempel- en subdrempelconcentraties gebruikt. Drempelconcentraties worden gedefinieerd als die mediatorconcentratie waarbij de luchtweg juist begint samen te trekken en subdrempelconcentraties als een honderdvoudige verdunning daarvan. Met uitzondering van LTC_4, verhoogt elk van deze mediatoren bij beide concentraties de gevoeligheid voor methacholine met een factor 1.1 – 2. De grootte van deze toename hangt niet af van de dosis van de mediator. De maximale contractie door methacholine verandert niet in aanwezigheid van deze mediatoren.

In **Hoofdstuk 11** worden de resultaten van **Hoofdstuk 10** nader geanalyseerd. De interactie tussen mediator-geïnduceerde hyperreactiviteit van de spier en de bij astma gemeten verdikking van de luchtwegwand wordt onderzocht. Vervolgens wordt het effect van deze twee factoren op de toename in weerstand van één menselijke luchtweg berekend bij een toenemende concentratie methacholine. Het lijkt dat mediator-geïnduceerde hyperreactiviteit van de spier voor cholinerge prikkels een gedeelte van de voor astma zo typerende linksverschuiving van de dosis werkingscurve voor methacholine kan verklaren, in het bijzonder bij geringe bronchoconstrictie. Verdikking van de luchtwegwand is waarschijnlijk belangrijker voor de toename van de helling en het maximum van de dosis werkingscurve bij astma.

Hoofdstuk 12 is gewijd aan het effect van epitheelverwijdering in combinatie met drempelconcentraties van inflammatoire mediatoren of anti-IgE op de gevoeligheid van geïsoleerde luchtwegen voor methacholine. Histamine, U46619 en PGD_2 veroorzaken een niet-parallelle linksverschuiving van de dosis werkingscurve voor methacholine zowel in strips *met* als strips *zonder* epitheel. Het effect van anti-IgE op de gevoeligheid van de luchtwegen is variabel en, gemiddeld genomen, niet significant. Analyse op type II fouten (vals negatieve resultaten) laat zien dat de resultaten een effect van IgE ter grootte van dat van één enkele mediator niet uitsluiten. Strips *zonder* epitheel zijn 1.6 maal gevoeliger voor methacholine dan strips *met* epitheel. Verwijdering van het epitheel verhoogt de gevoeligheid voor anti-IgE meer dan de gevoeligheid voor de andere agonisten. In combinatie veroorzaken drempelconcentraties histamine, U46619 of PGD_2 *én* de afwezigheid van het epitheel een 4.0 tot 9.1-voudige toename van de gevoeligheid voor methacholine gebaseerd op de $-\log EC_{10}$ en een 1.8 tot 3.0-voudige toename in gevoeligheid gebaseerd op de $-\log EC_{50}$. De $-\log EC_{10}$ en de $-\log EC_{50}$ zijn gedefinieerd als de negatieve logaritme van de methacholine concentratie die 10 respectievelijk 50 procent van de maximale respons veroorzaakt.

De effecten van geactiveerde menselijke granulocyten op geïsoleerde menselijke luchtwegen worden beschreven in **Hoofdstuk 13.** Incubatie van de luchtwegen met toenemende aantallen granulocyten die zijn geactiveerd met zymosan dat

met serum is voorbehandeld geeft een aanzienlijke samentrekking van de luchtwegen, evenredig met het aantal granulocyten in het orgaanbad. Experimenten met geëigende antagonisten tonen aan dat deze contractie voor het grootste gedeelte veroorzaakt wordt door lipoxygenase producten, voornamelijk LTC_4/D_4, en niet door histamine, prostaglandines of geactiveerde zuurstofmoleculen. Granulocytensuspensies van atopische donoren bevatten gemiddeld meer eosinofielen en er is een positieve correlatie tussen de mate van bronchoconstrictie en het percentage eosinofielen onder de granulocyten. De gevoeligheid van de luchtwegen neemt af bij toenemende aantallen geactiveerde granulocyten. De repsons (zie hierboven) op stimulatie door een electrisch veld is onveranderd in aanwezigheid van zymosan-geactiveerde granulocyten, behalve de trage respons, die enigszins wordt gepotentieerd.

Deel IV is gewijd aan het effect van fysische stimuli op geïsoleerde menselijke luchtwegen.

Hoofdstuk 14 beschrijft hoe afkoeling de respons van luchtwegsegmenten op farmacologische en electrische stimulatie verandert. Koeling heeft geen effect op de functie van de β-adrenoreceptoren. De luchtwegen reageren bij geen van de gebruikte temperaturen (37°C, 27°C en 21°C) op stimulatie van α-adrenoreceptoren. Koeling verlaagt zowel de spontane tonus als de gevoeligheid en maximale respons van de segmenten voor methacholine en LTC_4/D_4. Afkoeling vermindert ook de cholinerge piek van de respons op stimulatie in een electrisch veld en verlaagt de frequentie van electrische stimulatie waarbij halfmaximale relaxatie optreedt. De trage respons na electrische stimulatie, die het gevolg is van de vrijmaking van LTC4/D4, blijft bij lagere temperaturen echter onveranderd. Dit wijst erop dat afkoeling de opeenhoping van LTC_4/D_4 in gestimuleerde luchtwegen vergemakkelijkt.

In **Hoofdstuk 15** worden de effecten van hypo- en hyperosmolariteit op geïsoleerde perifere menselijke luchtwegen bestudeerd. Veranderingen in osmolariteit veroorzaken toenemende bronchoconstrictie evenredig met de grootte van de verandering in osmolariteit. De door hypertoniciteit veroorzaakte bronchoconstrictie treedt alleen op als de osmolariteit van de buffer wordt verhoogd met stoffen die de membraan niet, of slecht, passeren. Bij herhaalde stimulatie met hypo- of hypertone buffer treedt geen tachyfylaxie op. De bronchoconstrictie is niet secundair aan de vrijmaking van histamine, LTC4/D4, prostaglandines. Een β-agonist voorkomt de bronchoconstrictie in licht hyper- of hypotone buffer, maar niet die in sterk hyper- of hypotone buffer. De bronchoconstrictie in hypotone buffer is geheel afhankelijk van extracellulair calcium, terwijl in licht hypertone buffer de bronchoconstrictie gedeeltelijk afhankelijk is van vrijmaking van calcium in de cel. In sterk hypertone buffer lijkt de contractie gedeeltelijk het gevolg van osmotische krimp. De gevoeligheid van de luchtwegen voor methacholine neemt toe in licht hypertone en neemt af in licht hypotone buffer.

Hoofdstuk 16 beschrijft het effect van hyperosmolariteit op segmenten van verse centrale luchtwegen. Licht hyperosmolaire Krebs-Henseleit buffer waaraan

extra natrium chloride is toegevoegd geeft een bifasische respons: een snelle relaxatiefase gevolgd door een langzame contractiefase. De grootte van de relaxatiefase, maar niet de contractiefase, is omgekeerd evenredig met de hoogte van de basistonus. De relaxatiefase wordt niet veroorzaakt door prostaglandines, leukotriënen, acetylcholine, metalloendopeptidase-, angiotensine converting enzyme- of aminopeptidase-gevoelige peptiden. Deze fase wordt ook niet veroorzaakt door peptiden die de spier relaxeren (vasoactief intestinaal peptide) of door het epitheel geproduceerde relaxerende factor(en). De contractiefase is het gevolg van de vrijmaking van acetylcholine, histamine en metalloendopeptidase-gevoelige peptiden. Deze peptiden zijn waarschijnlijk afkomstig uit afferente zenuwuiteinden. De contractiefase wordt gepotentieerd door verwijdering van het epitheel.

Deze resultaten leiden tot de volgende conclusies:

Methoden

1. De overeenkomst tussen de responsen van strips en segmenten rechtvaardigt het gebruik van segmenten voor *in vitro* studies. Het gebruik van deze segmenten geniet de voorkeur op practische en theoretische gronden.

Ontsteking

1. Drempelconcentraties van onstekingsmediatoren als histamine, PGD_2 en TxA_2 doen de gevoeligheid van humaan bronchiaal glad spierweefsel toenemen met name voor lage concentraties cholinerge agonisten. Beschadiging van het epitheel zal deze mediator-geïnduceerde hyperreactiviteit van de spier versterken, terwijl verdikking van de luchtwegwand de maximale respons door cholinerge stimulatie zal verhogen.

2. Beschadiging van het bronchiaal epitheel potentieert de contractie van de gladde spier na activatie van mestcellen met anti-IgE

3. De contractie van geïsoleerde menselijke luchtwegen door geactiveerde granulocyten is evenredig zowel met het aantal granulocyten als met het percentage eosinofielen onder de granulocyten. Geactiveerde granulocyten veroorzaken voornamelijk bronchoconstrictie via LTC_4/D_4.

4. Granulocyten geactiveerd met zymosan verhogen de gevoeligheid van de gladde spier voor cholinerge stimulatie niet.

Fysische stimuli

1. Afkoeling vermindert de gevoeligheid van de gladde spier voor constrictoire stoffen en vergemakkelijkt de opeenhoping van LTC_4/D_4 veroorzaakt door stimulatie van bronchi in een electrisch veld.

2. Afkoeling verhoogt de gevoeligheid van de luchtwegen voor de relaxatie geïnduceerd door stimulatie in een electrisch veld.

3. In perifere menselijke luchtwegen veroorzaakt toenemend hypotone Krebs-Henseleit buffer toenemende bronchoconstrictie, die gepaard gaat met influx van extracellulair calcium. Toenemend hypertone buffer veroorzaakt ook toenemende bronchoconstrictie, maar deze is gekoppeld aan de mobilisatie van intracellulair calcium bij lichte hyperosmolariteit en aan osmotische krimp bij sterke hyperosmolariteit.

4. In verse centrale menselijke luchtwegen maakt licht hyperosmolaire buffer voldoende acetylcholine, histamine en neuropeptiden vrij om de spier te contraheren. Dit proces zèlf of het effect ervan op de gladde spier wordt versterkt door verwijdering van het epitheel

In de algemene discussie wordt besproken wat deze *in vitro* bevindingen zouden kunnen toevoegen aan bestaande opvattingen over de mechanismen van bronchiale hyperreactiviteit.

Er wordt geopperd dat de toegenomen bereikbaarheid van de onderliggende structuren als gevolg van epitheelbeschadiging, samen met overgevoeligheid van de bronchiale spier door ontstekingsmediatoren en beschadiging van het epitheel, allemaal bijdragen aan de linksverschuiving van de dosis werkingscurve. Hypertrofie van de gladde spier en verdikking van de wand van de luchtweg, die weer gedeeltelijk het gevolg is van spierhypertrofie, zullen de maximale respons verhogen. Geactiveerde granulocyten verhogen de gevoeligheid van de luchtwegen waarschijnlijk niet via sensitisatie van de gladde spier, maar ze leveren een potentiële bijdrage aan de bronchoconstrictie via leukotriënen die door eosinofielen worden vrijgemaakt. Het lijkt dat patienten met astma overgevoelig zijn voor directe stimulatie van de gladde spier door hypotone aërosolen en voor de vrijmaking van histamine, acetylcholine en neuropeptiden door hypertone zoutoplossingen. Afkoeling lijkt geen direct bronchoconstrictoire stimulus te zijn. Het lijkt meer aannemelijk dat afkoeling enerzijds het effect van andere constrictoire stimuli die ontstaan tijdens hyperventilatie van (koude) lucht verzwakt door de gevoeligheid van de gladde spier te verlagen en anderzijds deze constrictoire stimuli versterkt door de accumulatie van leukotriënen te faciliteren.

Vervolgens wordt besproken welke onzekerheden het extrapoleren van *in vitro* resultaten naar de *in vivo* situatie bemoeilijken. Op grond van deze onzekerheden wordt aangegeven hoe het gebruikte *in vitro* model zou kunnen worden veranderd zodat het beter aansluit op de *in vivo* situatie. Tenslotte worden een aantal punten geformuleerd die, vanwege de daarover bestaande onduidelijkheid, bij toekomstig onderzoek naar mechanismen van bronchiale hyperreactiviteit de bijzondere aandacht verdienen.

Dankwoord

Bijna nergens ter wereld blijkt het mogelijk om, zoals bij dit onderzoek het geval was, zeer regelmatig te beschikken over operatiemateriaal. Daarom wil ik in de eerste plaats de tientallen mensen bedanken die de afgelopen jaren, keer op keer, op het juiste moment, aan ons onderzoek hebben gedacht. Voor de belangeloze inzet van de longartsen, de secretaresses, de thoraxchirurgen, de OK-assistenten, de pathologen en de pathologie-assistenten van het Dijkzigt ziekenhuis, het Zuiderziekenhuis, het Ikazia ziekenhuis en het ziekenhuis Leyenburg, heb ik het grootste respect. Het maakte dit unieke onderzoek mogelijk.

Prof. Dr. Karel Kerrebijn, promotor, ben ik zeer erkentelijk voor de mogelijkheid om in zijn schat aan kennis en ervaring te delen. Hij zorgde voor een sfeer waarin hard werken ("We moeten nog éven naar dat stuk kijken"), ontspanning ("Zo, en nu is het tijd voor een bòrrel"), en een sappig verhaal elkaar plezierig afwisselden. Problemen ("Donders!") werden, een overvolle agenda ten spijt, binnen 24 uur ("Als je nou straks even ...") opgelost, de grote lijn nimmer uit het oog verloren.

Dr. Johan de Jongste, co-promotor, was de begeleider die menig AIO zich zou wensen. Hij liet me vrij waar het kon, stuurde mij bij als ik zwalkte of hallucineerde ("Volgens mij heb ik nu iets héél belangrijks ...."), en steunde me als het moeilijk was. Hij leerde me om de resultaten in verteerbare tekst ("hapklare brokken") om te zetten. Zijn oog voor detail en zijn allergie voor kromme zinnen en redeneringen hebben menige kapitale blunder voorkomen.

Rolien Raatgeep, analiste, heeft door dik en dun de experimenten met vaste hand uitgevoerd. Ze wist, dankzij haar uitmuntende geheugen, steeds het overzicht te bewaren en heeft mij voor vele protocollaire dwalingen behoed. Zélfs met haar been in het gips wist ze, via de telefoon, de reproduceerbaarheid van de experimenten op peil te houden.

Prof. Dr. I.L. Bonta, ben ik erkentelijk voor de gastvrijheid die wij op het instituut Farmacologie genoten en genieten. Zijn belangstelling voor niet-farmacologische zaken leidde tot menige interessante discussie.

Prof. Dr. J.H. Dijkman en Prof. Dr. C. Hilvering dank ik voor hun bereidheid het manuscript kritisch te lezen.

Prof. Dr. R. Pauwels en Dr. Guy Joos dank ik voor de prettige contacten die zich de afgelopen jaren tussen Rotterdam en Gent ontwikkelden.

Prof. Dr. F. Nijkamp en Prof. Dr. J. Zaagsma zorgden, dankzij hun grote kennis van de pulmonaire farmacologie, dat de bijeenkomsten van de projectgroep "Autonome regulatie en CARA" uitermate vruchtbaar waren.

I thank Dr. Roger Marthan, Université de Bordeaux, for his hospitality during my stay in "La capitale gastronomique de l'Europe". He introduced me to the ins and outs of "le muscle lys". I am delighted that he was prepared to come over for the occasion.

Prof. Dr. R. van Strik en Dr. Theo Stijnen van het instituut Biostatistiek, dank ik voor hun bereidheid om mij de variantie analyse telkens nòg een keer uit te leggen.

Dr. Freek Zijlstra, Dr. Dicky van Heuven, Dr. Jan van Amsterdam, Drs. Arthur Voogd, Dr. Ton Bom, Dr. Joost van Hilten, Drs. Marien den Boer, Drs. Willem Bax, Shreekanth Tadipatri, Jan Heiligers, Mario Vermeer, Mw. Magda Busscher en de andere medewerkers van het instituut Farmacologie, creëerden samen een verrijkte omgeving waarvan onderlinge steun en met elkaar meedenken, maar ook ouwe-neelen, koffie-leuten, roddelen en hilariteit aangename bestanddelen vormden. Drs. Fred Beusenberg ("arty Fred") wil ik in het bijzonder bedanken voor het verzorgen van de tekeningen in dit proefschrift. Iets waartoe ik, gehinderd door enkele weerbarstige puntmutaties in mijn tekengenen, niet in staat was. Wendy Mol dank ik zeer voor haar bijdrage aan de experimenten beschreven in hoofdstuk 13.

Prof. Dr. Herman Neijens, Drs. Liesbeth van Essen, Drs. Hein Brackel, Drs. Anja Verberne, Drs. Anton Hulsmann, Drs. Peter Merkus, Drs. Frans Krouwels en de dames van de longfunctie, zorgden ervoor dat ik me ook in het Sophia Kinderziekenhuis prima thuis voelde. Marieke van Groen en Annelies de Reus van het stafsecretariaat, hebben mij als "kleine zelfstandige" altijd warm onthaald. Karin Wielsma, Jacqueline Augustijn en Riet Visser dank ik voor de goede regulatie van de geldstromen en Joop van Dijk voor zijn grafische ondersteuning.

Geen onderzoek vaart wel zonder de hulp van doorgewinterde glasblazers, instrumentenmakers en electrotechnici. Ik wil daarom Jan Ekas, Toon Hoegee, Meneer F. Schumacher, Eef Bresser, Leo Bekkering, Hans Thön, en Willem Boender van de Centrale Research Werkplaatsen bijzonder danken voor hun hulp bij het ontwerpen, vervaardigen, verbeteren en onderhouden van de meetopstelling.

Ook wil ik de mensen van de commissie Post-Doctorale Opleidingen en allen die aan dit, zo broodnodige, onderwijs meewerkten, danken voor hun inspanningen. Siegfried Marijnissen, wil ik speciaal bedanken voor de leerzame en aangename dagen bij Janssen Farmaceutica in Beersse, België.

Vele mensen van andere universiteiten hebben mij met raad en daad bijgestaan. Dr. Henk Kauffman, Drs. Ron van Amsterdam (Rijksuniversiteit Groningen), Prof. Dr. Flip Quanjer, Dr. Peter Sterk, Dr. Liesbeth Bel (Rijksuniversiteit Leiden) wil ik daarvoor in het bijzonder bedanken.

I would like to thank Prof. J. Hogg and Dr. Peter Paré (both St. Paul's Hospital, Vancouver, Canada), Dr. Sandra Anderson (Royal Prince Alfred Hospital, Camperdown, Australia), Dr Judy Black, Dr. Carol Armour (both University of Sydney, Sydney, Australia), Prof. T. Lee, Crawford Jacques, Steve O'Hickey (all Guys Hospital, London, United Kingdom), Dr. Bengt-Erik Skoogh, Dr. C-G Löfdahl (both Renströmska Sjukhuset, Göthenborg, Sweden), Dr. Nelly Frossard (Hôpital Cochin, Paris, France) and Dr. Mitsuru Munakata (Hokkaido University, Sapporo, Japan), for useful advice and stimulating discussions on our work.

Het Nederlands Astma Fonds dank ik niet alleen voor de subsidiëring van het onderzoek, maar ook voor de mogelijkheid om de resultaten ervan op congressen te presenteren.

Tenslotte wil ik mijn ouders en Jet bedanken voor hun nimmer aflatende steun en vertrouwen, ze hebben me echt àlle kans gegeven. Aan hun is dit proefschrift opgedragen.

Curriculum Vitae

Roberto Jongejan was born on december the 6th, 1960, in Buenos Aires, Argentina. He attended primary school in Madrid, Spain, and in Sassenheim and finished his secondary school (VWO) in Leiden in 1978. Pending his admission to medical school (in The Netherlands places for medical school are assigned by lot) he studied Technology of Food Processing at the Agricultural University in Wageningen. In 1980 he started his medical studies at the university of Ghent, Belgium. After drawing a lucky number in 1981, he continued his medical training at the medical faculty of the University of Amsterdam, for which he was awarded a grant from Shell. In 1985 he spent 4 months as a research fellow at the Netherlands Institute for Brain Research (head: Prof. D.F. Swaab). From 1985 to 1986 he was a teaching assistant at the department of Biochemistry (head: Prof. E.J. de Haan) and the departments of General Practice (head: Prof. H. Lamberts), Medical Psychology (head: Dr. L.J. Krol) and Social Medicine (head: Prof. H.J.J. Leenen), all at the University of Amsterdam. After his doctoral degree in 1986, he started working on a research project supported by the Netherlands Asthma Foundation at the department of Pediatric Respiratory Medicine (head: Prof. K.F. Kerrebijn) of the Sophia Children's Hospital. The experiments for this project, which are described in this thesis, were performed at the department of Pharmacology (head: Prof. I.L. Bonta) of the Erasmus University Rotterdam. Between 1987 and 1990 he followed courses in statistics, english, drug research and development, epidemiology and immunology. Since 1984 he has also been working as a stand-by steward with KLM, Royal Dutch Airlines.

He shares his life with Juliette Janssen, historian-to-be.

List of Publications

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List of Abbreviations

A23187	calcium ionophore
AMP	adenosine-3',5'-monophosphate
ANOVA	analysis of variance
ASM	airway smooth muscle
ATP	adenosine triphosphate
B	regression coefficient for covariate
BAL	bronchoalveolar lavage
BCS	baseline contractile state
BMDP	biomedical computer programs
c-AMP	cyclic adenosine-3',5'-monophosphate
CCRC	cumulative concentration response curve
CI	confidence interval
Con A	concanavalin A
COPD	chronic obstructive pulmonary disease
D	dalton
EC_{10}	effective concentration that causes 10% of the maximal effect
EC_{20}	effective concentration that causes 20% of the maximal effect
EC_{50}	effective concentration that causes 50% of the maximal effect
EDTA	ethylene diamine tetra-acetic acid
EF_{50}	effective frequency that causes 50% of the maximal effect
EFS	electric field stimulation
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetracetic acid
E_{max}	maximal effect
EpDRF	epithelium-derived relaxing factor
FEV_1	forced expiratory volume in 1 second
FMLP	formyl-methionyl-isoleucyl-phenylalanine
FPL 55712	LTC_4/D_4 receptor antagonist
g	gram or gravity, i.e. the unit of force exerted upon a body during acceleration and deceleration
HETE	hydroxy-eicosatetraenoic acid
Hz	herz
H_1	type 1 histamine receptor
IgE	immunoglobulin E
IL	interleukin
IP_3	inositol 1,4,5-triphosphate
IVC	inspiratory vital capacity
kPa	kiloPascal
L	liter
LAR	late ashmatic reaction
L_{max}	length at which the airway muscle develops maximal active tension

LT	leukotriene
M	molar
MACR	maximum active contractile range
MANOVA	multivariate analysis of variance
MAX_{Raw}	calculated maximal increase in R_{aw} at maximal airway narrowing in the presence of methacholine (10^{-4} M)
Mch	methacholine
ms	millisecond
MWU	Mann-Whitney U
NANC	non-adrenergic non-cholinergic
NDGA	nordihydroguaiaretic acid
NEP	neutral metalloendopeptidase
NK	neurokinin
NS	not significant
osM	osmolar
P	the chance that a relation within a set of data or a difference between groups of data has the observed magnitude under the hypothesis that in reality no relation or difference exists
PAF	platelet activating factor
PBS	phosphate buffered saline
PC_{20}	provocative concentration that causes a 20% reduction in FEV_1
PC_{3R}	concentration of methacholine that causes a threefold increase in R_{aw}
PC_{6R}	concentration of methacholine that causes a sixfold increase in R_{aw}
Pco_2	carbon dioxide partial pressure
PD_{20}	provocative dose that causes a 20% reduction in FEV_1
PG	prostaglandin
pH	negative logarithm of hydrogen ion concentration
PHI	peptide histidine isoleucine
PHM	peptide histidine methionine
PMA	phorbol 12-myristate 13-acetate
PMP	proportion of the airway perimeter occupied by smooth muscle (histology)
PMS	proportion of smooth muscle shortening
Po_2	oxygen partial pressure
P_w	the proportion that the airway wall occupies in the total area of the circle formed by the outermost layer of airway smooth muscle in a histological section
R_{aw}	airway resistance
r_s	Spearman's rank correlation coefficient
SEM	standard error of the mean
S_{max}	maximal shortening of an airway segment in mm
$S_{max\%}$	maximal shortening of an airway segment as a % of the highest response at any preload

SOD	superoxide dismutase
SP	substance P
SPSS	statistical package for the social sciences
STZ	serum treated zymosan
TTX	tetrodotoxin
Tx	thromboxane
U44619	9.11-dideoxy-11α,9-epoxymethano-$PGF_{2\alpha}$
V	volt
VC	vital capacity
VIP	vasoactive intestinal peptide
wt	weight
ZAG	zymosan activated granulocytes