HUMAN AIRWAY SMOOTH MUSCLE

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HUMAN AIRWAY SMOOTH MUSCLE

(HUMAAN BRONCHIAAL GLAD SPIERWEEFSEL)

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Contents

Part I

Introduction to the studies

Chapter 1

Airway smooth muscle function: Structural, electrophysiological, biochemical and mechanical aspects

Structure of airway smooth muscle

Airway smooth muscle is present in the airways from the trachea down to the smallest membranous bronchioles. In the trachea, smooth muscle is localised dorsally, were ramifying spindle-shaped muscle cells run in bundles in a transverse direction to connect the ends of the horse-shoe shaped cartilage plates and the annular ligaments. In the bronchi, airway smooth muscle inserts on the perichondrium and runs in helicoidal strands around the airway circumference. Spirally oriented bundles of airway smooth muscle are also present in the walls of the smaller non-cartilaginous bronchi, where the screw-like turns are more widely spaced and constitute a relatively large part of the volume of the airway wall[1]. Elastin and collagen surround the muscle bundles, and blood vessels and nerves are localised in these connective tissue spaces between the muscle bundles[2,3].

Ultrastructure of airway smooth muscle

Airway smooth muscle cells have an elongated spindle shape and a central cigar-like nucleus. Their cytoplasm contains the contractile proteins actin and myosin, as demonstrated by electron microscopy and biochemical studies. Myosin is only present in one fifth of the amount found in striated muscle, and the myosin filaments are roughly 20% longer than in striated muscle. Another difference is that each myosin filament is surrounded by 12-14 actin filaments in airway smooth muscle, and by 6 actin filaments in skeletal muscle. The actin filaments are attached to the muscle cell membrane in the so-called dense bands, and are also connected to similar structures in the cytoplasm, the dense bodies[3]. The actomyosin complex together with the dense bands and -bodies are thought to represent the equivalent of striated muscle sarcomeres, and form the contractile element[2]. Airway smooth muscle contains relatively few mitochondriae, and therefore produces only 10% of the amount of ATP produced by skeletal muscle[2]. Sarcoplasmic reticulum is also relatively sparse and is present as peripherally located saccular and tubular structures[4]. A Golgi apparatus can usually be identified. The cell membrane forms bottle-shaped micro inpocketings or caveolae, which may serve as a storage site for calcium ions[4].

Gap junctions

Where airway smooth muscle cells are apposed, small areas of tight junction can be seen that facilitate the propagation of electrical current by virtue of their low electrical impedance. These structures are called gap juctions[5,6], and are believed to be important because their number and size is variable in time and responds to various stimuli[7], which might influence airway smooth muscle contractile function in analogy to the changes in uterine smooth muscle during labor.

Electrophysiology of airway smooth muscle

Multi/single unit smooth muscle

Smooth muscle can be classified as multi-unit, single-unit or intermediate type, which refers to the density of innervation and electrical coupling between the cells. Multi-unit smooth muscle is characterised by a separate innervation of the muscle cells with few gap junctions. Multi-unit smooth muscle displays no spontaneous rythmical activities, does not develop action potentials after electrical or pharmalogical stimulation, and responds to a graded membrane depolarisation with a gradual contraction. Single-unit smooth muscle is sparsely innervated but has many gap junctions and functions as a syncytium. This type of smooth muscle has spontaneous rythmic oscillations of the membrane potential, and can develop action potentials upon depolarisation. Characteristically, multi-unit smooth muscle is present in e.g. large blood vessels; single unit in the intestine, ureters and uterus at term[3].

Airway smooth muscle is an intermediate type of smooth muscle, because it has relatively few gap junctions[2], and has no spontaneous electrical activity[8–10], but has a relatively sparse innervation[8].

Electrical activation of airway smooth muscle

Studies on the electrophysiology of airway smooth muscle have mainly been conducted on canine, bovine and guinea-pig tracheal smooth muscle. Tracheal muscle cells have a stable resting membrane potential of approximately -48 to -55 mV[9,10]. Pharmacological stimulation with contracting agonists or electrical stimulation causes a gradual depolarisation without action potentials[9–11]. A graded contraction begins when the membrane potential is elevated by more than 5 mV. In airway smooth muscle, this is a slow process[11].

Vagal nerve stimulation leads to a sustained, small potential change in bovine tracheal smooth muscle, called the excitatory junctional potential (EJP)[8]. The EJP represents a summary of the synaptic potentials produced by neurotransmitter release. The ensuing contraction is proportional to the degree of depolarisation, so in each muscle cell there is a graded response and not the 'all-or-none'

response that usually follows an action potential in other types of muscle cells[12].

Direct measurements of electrical activities of human airways have been made in a single study[13] where action potentials were recorded *in vivo* using an intrabronchial electrode. An increased electrical activity was found during asthmatic bronchospasm; the significance of this finding for airway smooth muscle function, however, needs further elucidation. Because small airway smooth muscle is embedded in the peripheral lung tissue and is intermingled with connective tissue, this is technically a difficult tissue to study. Thusfar, no electrophysiological studies on peripheral airway smooth muscle have been reported.

Several investigators have observed spontaneous electric activity and action potentials in airway smooth muscle[14,15]. These may, however, have resulted from metabolic depletion[16] or inappropriate dissection procedures.

Biochemistry of airway smooth muscle contraction

The increase in smooth muscle cell membrane potential that follows neurotransmitter release from nervous elements produces contractile element shortening via a highly complex series of biochemical events. This is schematically depicted in Figure 1. The actin and myosin protein filaments are arranged in parallel,

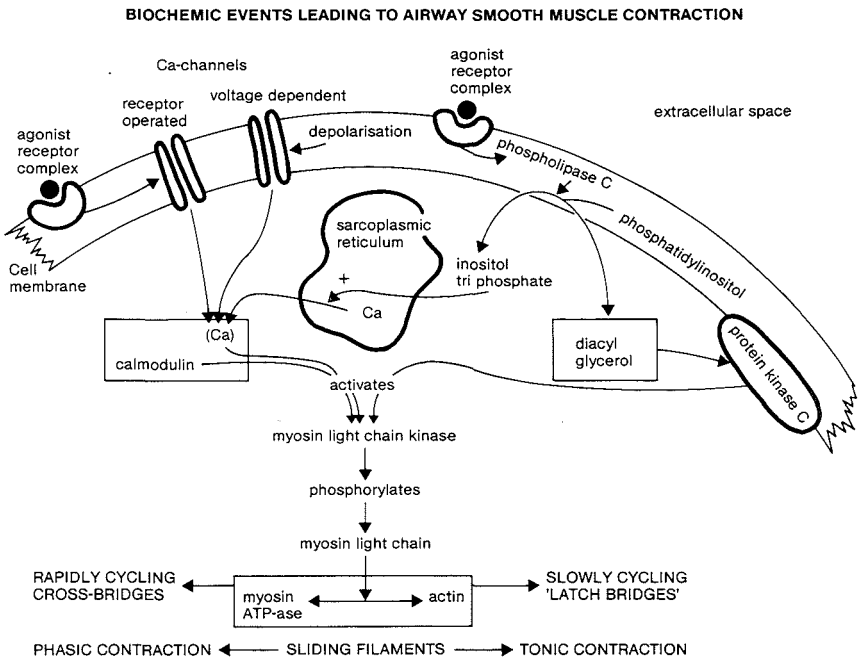

Figure 1. Schematic and simplified representation of the biochemical events that cause airway smooth muscle contraction.

each myosin filament being surrounded by 12-14 actin molecules[17]. Myosin consists of heavy chains (molecular weight, MW:200.000 Daltons) and two types of light chains (MW:20.000 and 17.000 D)[18]. Actin filaments are composed of two 42.000 D chains, wrapped in a helix. According to the sliding filament model, active transport of actin along myosin produces contraction of the smooth muscle cell. For this purpose, the myosin molecules have a globular end which possesses ATP-ase activity. Phosphorylation of the myosin light chains by myosin light chain kinase results in activation of this magnesium-dependent myosin ATP-ase[18,19]. Activated myosin ATP-ase hydrolyses ATP, which leads to flexion of the globular myosin head. Formation, breakdown and reformation of cross-bridges between the moving myosin molecules and actin filaments is called cross-bridge cycling, and results in shortening and stiffening of the contractile element and the smooth muscle cell[4,20].

The role of calcium

Activation of myosin light chain kinase is a Ca^{2+}-dependent process which starts when the intracellular [Ca^{2+}] increases from a basal concentration of 10^{-8} to 10^{-7}M to above 10^{-6}M[21]. The intracellular Ca^{2+}-concentration is much lower than the extracellular concentration, which is approximately 10^{-3}M. This gradient produces an inward Ca^{2+}-current when the cell membrane is made more permeable to Ca^{2+}. Penetration of Ca ions through the cell membrane is possible via the relatively ion-specific Ca channels. Ca channels are glycoprotein molecules in the cell membrane, which have an aqueous pore that opens to Ca ions in response to cell membrane depolarisation (voltage- or potential-dependent channels) or in response to stimulation of specific receptors (receptor-operated channels)[22]. Voltage- and receptor-operated channels are structurally closely related, but are activated seperately by their respective activators[23]. Furthermore, small amounts of calcium can enter the cell passively via a membrane leak and via sodium channels. This Ca entry is compensated by Ca efflux mechanisms and does not cause activation of the contractile apparatus[24]. Ca entry mobilises a larger pool of Ca ions, that is bound to the sarcoplasmic reticulum. This Ca-induced intracellular calcium release may also be important in cell activation [24,25].

When the intracellular [Ca^{2+}] exceeds 10^{-6}M, it binds to calmodulin, a 148-amino acid peptide (MW:16.500 D) that has four Ca binding sites[26]. Saturation of calmodulin with Ca activates the molecule; active calmodulin binds to myosin light chain kinase, thereby in turn activating this enzyme[17].

Contraction results when Ca is released from intracellular stores, such as the sarcoplasmic reticulum[27], and the inner surface of the cell membrane. The most active substances that release Ca intracellularly are Ca itself, inositol triphosphate and caffeine[27]. Various contractile agonists differ in their capacity to induce contraction by releasing intracellular calcium[4]: acetylcholine, for instance, contracts airway smooth muscle independent of Ca influx in various species[9,12,28], whereas histamine and serotonin are more dependent on extracellular calcium. The important role of intracellular calcium in human airway smooth muscle contraction to cholinergic stimulation has also been demonstrated[29,30].

Diacylglycerol/protein kinase C pathway

An alternative Ca-calmodulin independent path leading to smooth muscle contraction is the diacylglycerol/protein kinase C pathway[21]. Stimulation of a receptor produces activation of the membrane-associated enzyme protein kinase C, which converts phosphorylated phosphatidylinositol in the cell membrane into inositol triphosphate and diacylglycerol. Inositol triphosphate functions as a second messenger and releases Ca from intracellular stores, thus producing contraction. Diacylglycerol activates the enzyme protein kinase C by increasing the sensitivity of this enzyme to Ca, but not via an increase in intracellular Ca[21,31]. Activated protein kinase C in turn activates myosin light chain kinase. This leads to contraction, independent of Ca-calmodulin, in the presence of a basally low Ca concentration.

Sequence of biochemical processes

After receptor occupation by a contracting agonist, the rise in intracellular Ca has a transient nature. Within a few seconds, the Ca level in the cell falls to almost basal values, in spite of a persisting high Ca influx rate[21]. In this phase, the Ca-calmodulin-mediated response decreases, and the diacylglycerol pathway sustains the response when the stimulus is still present[2,32]. This part of the contraction response is highly effective, in that the response is sustained at a very low energy cost. The diacylglycerol/protein kinase C pathway probably activates slowly- or noncycling crossbridges, also called 'latch bridges'[3], that will be discussed later in this chapter.

Biochemistry of airway smooth muscle relaxation

Two mechanisms cause relaxation of airway smooth muscle, the first being related to the Ca-influx rate, the other to activation of adenylate cyclase.

Calcium clearing

The muscle cell membrane contains ionic exchange systems that can remove Ca out of the cell in exchange for magnesium or sodium ions[34]. The Ca-Na exchanger extrudes Ca ions driven by the electrochemical gradient of Na over the cell membrane, and is probably sensitive to alterations in membrane potential. The Ca-Mg ATPase is activated by the Ca-calmodulin complex[34] and by activated protein kinase A. The Ca influx is therefore a self-limiting process which elevates intracellular Ca only transiently. Ca can also be removed from the cytoplasm by sequestration in intracellular stores, such as the sarcoplasmic reticulum, which have ATPases that are presumably different from those on the cell membrane[4].

Cyclic AMP

The second relaxing mechanism, that acts more slowly, is activation of adenylate cyclase, which leads to the formation of cyclic AMP (cAMP). This cAMP activates protein kinases which remove Ca from the cytoplasm by stimulating ionic Ca efflux pumps in the smooth muscle cell membrane[17,34,35]. In addition, cAMP dependent protein kinases can inhibit the breakdown of cell membrane phosphatidyl inositol. This reduces the contraction that is mediated by diacylglycerol and inositol triphosphate[32]. The main physiologic activator of adenylate cyclase is stimulation of β-adrenergic receptors by circulating catecholamines[32]. Both relaxing mechanisms, acting via the Ca and cAMP messenger systems, are not independent but probably have many complex interactions[36].

Other biochemical events during relaxation

When the Ca influx into the muscle cell decreases, and the intracellular Ca level has returned to a basal level, dephosphorylation of the myosin light chains occurs. This process is catalysed by the enzyme myosin light chain phosphatase. The activity of this enzyme is independent of the intracellular calcium concentration, and its net effect on the phosphorylation state of the myosin light chains depends on the degree of activity of the counter-acting enzyme myosin light chain kinase[18]. Dephosphorylation of myosin light chains stops the cross-bridge cycling, which causes relaxation and elongation of the contractile elements, driven by passive elastic forces.

Airway smooth muscle mechanics

Isometric and isotonic functioning

Excitation of a smooth muscle preparation results in shortening and stiffening of the tissue. Dependent on experimental conditions, two modalities of action can be defined: isometric activity, where the muscle is maintained at a fixed length and upon stimulation develops force, and isotonic shortening, where the contractile elements contract and, hence, the muscle performs work against a (constant) load.

It is presently thought, that, *in vivo*, airway smooth muscle contracts quasi isotonically[1]. Isotonic shortening leads to airway narrowing. After the initial shortening, the muscle has to act against an increased load due to the elasticity of the non-contracting tissue surrounding the airways. This probably forces the airway smooth muscle to contract in a more isometric fashion with increased activation. The isometric stiffening of airway smooth muscle is the equivalent of tone development, which stabilises the airway.

Optimal length

The maximum isometric force or tension that airway smooth muscle can generate (T_{max}) is dependent on the initial length of the muscle, which determines the relative position of the myosin and actin filaments, according to a Frank-Starling type of curve. In canine trachea, the optimal length L_o is reached at a relatively small initial load of less than 10% of T_{max}[3]. The maximal tension is similar to that of skeletal muscle. This is remarkable because striated muscle has a much higher myosin content than airway smooth muscle[2].

It has been shown that airway smooth muscle, upon maximal activation, can shorten to even 10-20% of its optimal length, which is called 'supercontraction'[3], whereas skeletal muscle shortens at most to 65%. The mechanism that enables airway muscle to supercontract may be related to the different organisation and length of the contractile elements as compared to striated muscle, but remains to be elucidated. At initial lengths shorter than L_o, airway smooth muscle cannot be activated maximally[37]. This may be due to changed dimensions of the muscle, which impair activation of muscle cells that are localized centrally in the tissue. It is not known whether airway smooth muscle resting length *in vivo* is near L_o[1].

Latch bridges

The initial shortening of airway smooth muscle following electrical or pharmacological excitation is probably caused by the activity of normally cycling cross-bridges. The tension that develops later on in the activation process is believed to result from the action of 'latch bridges', which are activated a few seconds later than the normally cycling cross-bridges[3,33]. Latch bridges can efficiently sustain a contraction because of their low energy consumption[33,38,39]. The existence of latch bridges has first been demonstrated in vascular smooth muscle[40] and later in canine airway smooth muscle[33]. Their importance lies in their extreme efficiency, which results from a high load bearing capacity and a low energy consumption.

Elastic forces

Two types of passive elastic forces are important in the capacity of force maintenance of airway smooth muscle, as shown schematically in Figure 2. The 'series elastic component' can be defined as the non-contracting tissue that is in series with the contractile element[4]. Its stiffness during isometric contraction is proportionate to the number of cycling cross-bridges[3]. The 'parallel elastic component' represents the non-contracting structures that are parallel to the contractile element. Compression of these elements during muscle shortening may constraint the cycling of cross-bridges, and decrease the velocity of shortening.

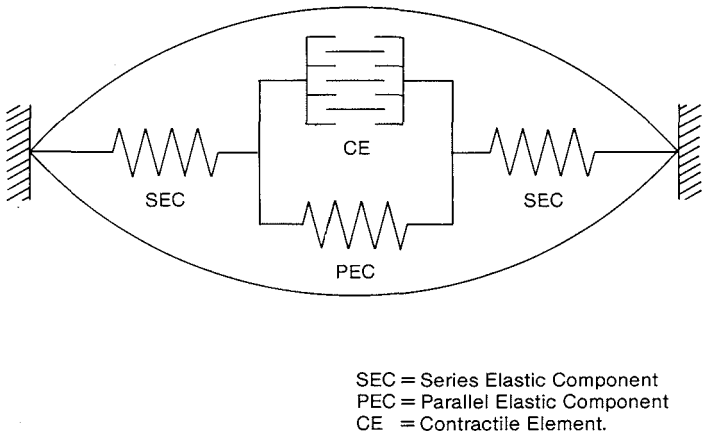

Figure 2. Schematic representation of the series – and parallel elastic components in the airway smooth muscle cell.

Force-velocity relationship

The rate of force development provides insight in the power that is produced by the muscle cells. Force-velocity relationships have only been examined in tracheal smooth muscle, because its measurement requires a pure muscle preparation with the cells oriented parallel to each other. Canine tracheal smooth muscle is a slow acting type of muscle, probably because of the slow rate at which energy-liberating reactions occur in this muscle[2,42]. The force that can be developed is, however, relatively high.

Relaxation

Relaxation of airway smooth muscle is also a slow process which is determined by the rate of inactivation of the contractile elements' cross-bridge cycling. This is in contrast to relaxation of striated muscle, where the load determines the relaxation rate. The difference may be related to the sparsity of the sarcoplasmic reticulum in airway smooth muscle, which may delay the Ca clearing of the cytoplasm. After relaxation, cross-bridges do not cycle, but may be attached, thereby providing muscle resistance to stress[43].

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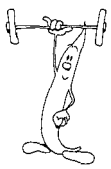

Chapter 2

Control of human airway smooth muscle: The autonomic nervous system

Introduction

The contractile state of airway smooth muscle under physiological conditions is controlled by the autonomic nervous system. This autonomic control is much more complex than previously thought. Not only a parasympathetic cholinergic system and a sympathetic adrenergic system, but also a non-adrenergic, non-cholinergic (NANC) system is present, which innervates airway smooth muscle and other structures in the lungs, and has inhibitory and excitatory components[1].

The NANC system probably acts via a spectrum of peptide neurotransmitters. It is likely that complex interactions occur between the different components of the autonomic innervation of the airways. Furthermore, it has been shown that nerves terminate on mast cells that are in close contact with muscle cells in human airways. This suggests that mast cell mediator release is to some extent also controlled by the autonomic innervation, and has a regulatory role[2].

Unravelling the roles of these various components under physiological and pathological conditions is very difficult. There are wide differences between species[3] and research on human tissue is still in its early years. Nevertheless, progress in the understanding of the autonomic innervation of human airways has been made[4].

Cholinergic innervation

Cholinergic excitatory nerves arise in the vagal nucleï in the brain stem and run in the vagus nerves to the lungs. Relatively few nerves supply smooth muscle and mucous glands in the central airways, where they enter from the adventitial side and synapse in ganglia in the tracheal and bronchial wall[4–7]. Postganglionic unmyelinated cholinergic axons run between the smooth muscle bundles, but do not penetrate between individual muscle cells[2,5,8] . Medium-sized bronchi (fourth to seventh generation) have a denser innervation than the trachea and second order bronchi, although close contacts between nerve endings and muscle cells are rare[2,8]. Cholinergic fibers are scanty in bronchioles and absent in alveolar walls[2,4]. This suggests that neural control is especially important in fourth to seventh order bronchi[2]. Activation of cholinergic efferents releases acetylcholine from the cholinergic nerve terminals, which stimulates muscarinic receptors of

the M_2 subtype on the muscle cells[9] and causes muscle contraction. Muscarinic receptor density decreases from central to peripheral airways[10], which confirms that the cholinergic system predominantly controls the central airways.

In the guinea pig and the cat, muscarinic receptors, presumably of the M_1-subtype, that are localized on the presynaptic membrane of postganglionic cholinergic fibers[11], inhibit the release of acetylcholine[12,13]. This may represent a negative feedback mechanism.

Adrenergic innervation

Although the sympathetic nervous system innervates various structures in the lungs, such as the blood vessels and mucous glands, virtually no direct sympathetic innervation of human airway smooth muscle can be demonstrated histologically. Functional experiments on human central airways have failed to show an adrenergic innervation[6,14–16]. Nevertheless, human airway smooth muscle has many adrenergic receptors on its cell membranes that are of the β_2 subtype[17,18]. These β_2 receptors increase in number from central to peripheral airways[10]. Stimulation of β_2 receptors produces muscle relaxation. Circulating adrenalin, produced by the adrenal glands, may act as an endogenous bronchodilator[19]. Although the basal adrenalin concentrations in plasma are much too low to have an effect on airway smooth muscle, the peak levels during stress may cause bronchodilatation. The physiologic role of the β_2 adrenoceptors on airway smooth muscle is not entirely clear; they certainly mediate the beneficial effect of β-agonists in asthma. In human lung, adrenergic fibers have been demonstrated that end in parasympathetic ganglia and that may modify ganglionic transmission[20], probably by stimulating α_2 receptors[21,22]. Furthermore, adrenergic nerve varicosities have been found in close proximity to cholinergic nerve endings, that may modulate acetylcholine release via stimulation of presynaptic β receptors[23].

The role of α adrenergic receptors on smooth muscle cells is obscure. They are mainly present in peripheral airways[10,24]. Their stimulation has no consistent effects on normal human airway smooth muscle[25], but α receptors may play a role in obstructive airway diseases[4,7,28].

Non-adrenergic, non-cholinergic innervation

The presence of this 'third nervous system' in the human lung has now been well documented[1]. The NANC system has inhibitory and excitatory components. NANC inhibitory nerves probably constitute the only direct inhibitory innervation of human airway smooth muscle[14,15,27–29]. NANC nerves reach the lungs via the vagus nerves and, like cholinergic nerves, synapse at ganglia in the tracheal and bronchial wall. The NANC inhibitory system is mainly present in central airways; NANC nerves become sparse in human peripheral airways[28,30–32]. There

is strong evidence that the neurotransmitters of this system are the 28-aminoacid polypeptide vasoactive intestinal peptide (VIP) and the related peptide histidine isoleucine (PHI)[30–35]. VIP has been shown to coexist with acetylcholine in parasympathetic nerve endings, and is therefore probably a co-transmitter of acetylcholine[4]. The elucidation of the importance of this system in humans awaits the development of specific blocking drugs.

NANC excitatory nerves, containing subtance P and other tachykinins including neurokinin A, neuropeptide K, eledoisin-like peptide, and calcitonin gene-related peptide, have been identified in the airways of various animal species, and in humans[4]. Autoradiographic studies have localized substance P-receptors on human airway smooth muscle and submucosal glands[36]. The results of animal experiments on the effects of tachykinins on airway smooth muscle[37,38], vascular permeability[37], tracheal gland secretion[39] and mast cells have led to speculations on their role in obstructive airway diseases[32,40]. Their release upon sensory nerve activation suggests a possible involvement in local axon reflexes[4,32].

Ganglia

Ganglia of the parasympathetic system are present in the bronchial wall, localized external to the smooth muscle layer. They contain several types of neuronal bodies and have a complex structure, including cholinergic, adrenergic and NANC elements[20,30]. It seems likely that these components interact to integrate and coordinate the neural inputs to the airway smooth muscle. It has been shown that adrenergic stimulation of ferret and guinea pig paratracheal ganglia causes presynaptic inhibition of cholinergic neurotransmission[20]. This local ganglionic regulation process is still poorly understood; studies on human tissue are lacking.

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Chapter 3

Control of human airway smooth muscle: Inflammatory mediators

Introduction

During an inflammatory reaction, a large number of mediators is produced by inflammatory cells. Several mediators of inflammation are potent constrictors of human airways, and have other effects, e.g. stimulation of airway gland secretion, induction of capillary leakiness and chemotactic activity, that can contribute to airways obstruction. Because of their local release, mediators may reach levels that are high enough to cause smooth muscle contraction in the bronchial wall. Inflammatory mediators may therefore be important in the control of airway smooth muscle contraction, especially in inflammatory airways diseases such as chronic bronchitis and asthma. During the past decade, most research has focussed on the roles of histamine, prostanoids, leukotrienes and platelet activating factor, and these mediators will be considered in some detail in this chapter.

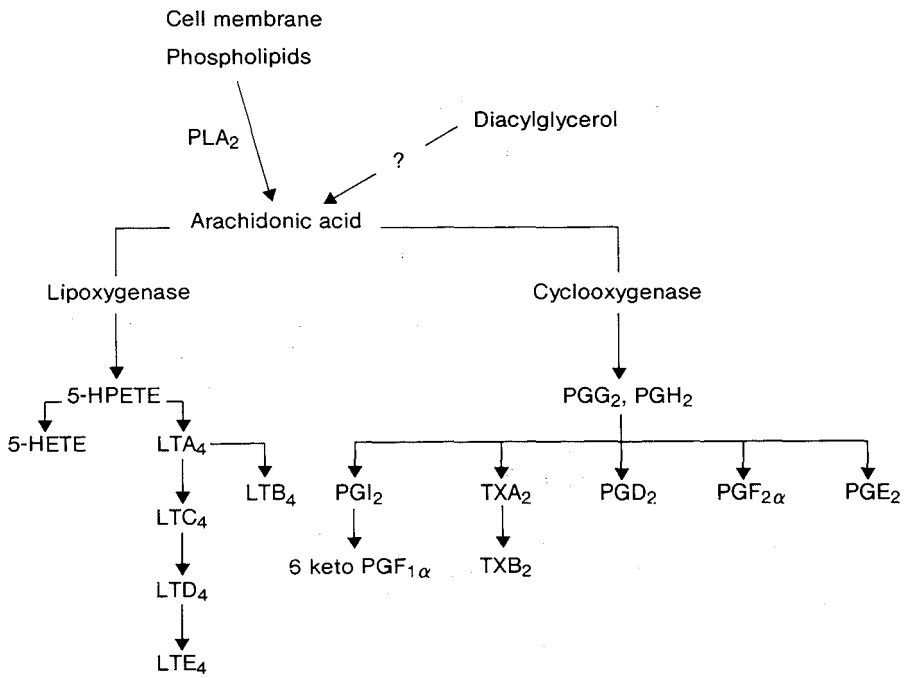
Histamine

Histamine is released from lung mast cells and causes airway smooth muscle contraction via stimulation of histamine receptors of the H_1 subtype[1–3]. The presence of H_2 receptors, which relax airway smooth muscle, has been demonstrated in monkey and rabbit trachea[4,5], but there is no valid evidence for relaxing H_2 receptors on human airway smooth muscle[6]. Histamine contracts airways with a potency and efficacy comparable to those of acetylcholine, but is more potent in peripheral airways than in central airways[4,7,8].

Products of arachidonic acid metabolism

Arachidonic acid is split from cell membrane phospholipids by the action of phospholipase A_2. Arachidonic acid can be converted to prostaglandins and thromboxanes via the cyclo-oxygenase pathway, and to leukotrienes via the lipoxygenase pathway (Figure).

SYNTHESIS OF LEUKOTRIENES AND PROSTAGLANDINS



Abbreviations: PL, phospholipase; HPETE, hydroxyperoxyeicosatetraenoic acid; HETE, hydroxy eicosatetraenoic acid; LT, leukotriene; PG, prostaglandin.

Figure 1. Metabolism of arachidonic acid.

Cyclooxygenase metabolites

Human lung tissue homogenates and lung mast cell suspensions produce cyclooxygenase metabolites spontaneously and after stimulation with calcium-ionophore, allergen or contracting agents like histamine or carbachol[9–12]. The main product is prostaglandin D_2 (PGD_2), followed by $PGF_{2\alpha}$, I_2, E_2 and thromboxane (Tx) A_2 and B_2[9–14]. *In vivo*, PGD_2, $PGF_{2\alpha}$ and TxA_2 are bronchoconstrictors in man[13,15–18], whereas PGE_2 produces inconsistent effects[15,19,20]. *In vitro*, PGD_2, $PGF_{2\alpha}$, and TxA_2 contract human airway smooth muscle preparations[3,14,21–25], with a potency and efficacy that are equal or less than those of histamine. PGE_2 has produced paradoxical effects *in vitro*, and is not very effective[21,22]. PGI_2 has no important direct effects on human airway smooth muscle[25], but may have a modulating role in the response to other agonists[26], or on neuromuscular transmission[27].

PG's are released by canine, guinea-pig and human lung after smooth muscle contraction by acetylcholine or histamine[25,28,29], which suggests that PG's modulate the effects of contracting agonists. Inhibition of this reactive PG synthesis, however, does not change the contractile response of human isolated airways[25,30,31]. Therefore, PG's do not seem to have an important direct role in the control of human airway muscle contraction.

In addition to their direct effects, prostaglandins and Tx analogues have a potentiating effect on cholinergic responsiveness in canine and guinea-pig central airways, either by increasing smooth muscle contractility or by enhancement of cholinergic neurotransmision[32–35]. PGE_1 and E_2 may inhibit cholinergic neurotransmission[32,36,37]. Cyclooxygenase metabolites may therefore also influence airway smooth muscle responses by indirect presynaptic actions. Finally, PG's may have an effect on muscle cell-to-cell coupling, by changing the number and size of gap junctions[39]. PGE_2 and PGI_2 increase the number of gap junctions in canine trachea, which could lead to an enhanced response to vagal stimulation[39]. *In vivo* studies on the effects of PG synthesis inhibition on human bronchial responsiveness to histamine, however, have failed to show important changes in histamine-induced bronchoconstriction[38].

Lipoxygenase metabolites

Leukotrienes (LT's), formerly known as SRS-A, are the lipoxygenase metabolites of arachidonic acid[40,41]. Several LT's are highly potent constrictors of human bronchus *in vivo* and *in vitro*[42,49]. LTC_4, D_4, E_4 and B_4 are produced by human lung mast cells, alveolar macrophages, lung parenchyma and bronchus, and by eosinophils, after stimulation with e.g. calcium-ionophore or allergen, and can be recovered from the sputum of patients with obstructive lung diseases[50,57].

Leukotrienes C_4 and D_4 are a 100-fold more potent than histamine in causing contraction of human airways *in vitro*[42,43] and a factor 1.000 – 10.000 more potent than histamine in causing bronchoconstriction in humans *in vivo*, whereas LTE_4 is only 10 – 40 fold more potent than histamine[47–49,58,59]. The maximal effect of LT's is lower than that of acetylcholine or histamine in human airways *in vitro*, but not *in vivo*. LT's may be more potent in peripheral than in central airways[60,61]. Apart from their effects on bronchial muscle, LT's can also contribute to airways obstruction by stimulating mucus secretion, inducing capillary leakiness, and attracting inflammatory cells[60,62,63]. LTB_4, that has only weak and indirect bronchoconstricting activity[64], has a strong chemotactic effect[53,65]. In guinea-pigs and cats, part of the response of peripheral airway smooth muscle to LT's seems the consequence of synthesis of cyclooxygenase products, especially TxB_2[66–71]. The effect of LT's on human airway smooth muscle is probably mainly a direct one[60]. Specific LT receptors have been demonstrated by radioligand binding studies and functional experiments on lung tissue from various animal species[72–76]. There is, however, no conclusive evidence for LT receptors on human airway muscle[60,63,77,78].

In addition to their direct contractile effect on airway smooth muscle, LT's may have other actions that indirectly could influence airway smooth muscle responses. LT's may inhibit the formation of gap junctions in canine trachea[39], which might reduce the contractile response to vagal stimulation. Furthermore, experiments on guinea-pigs have suggested that LT's may reduce the response to β-adrenoceptor stimulation, and increase the effect of histamine[79–81]. These effects of LT's have only been reported for experimental animals, so their relevance for human bronchial hyperresponsiveness is still uncertain. Other lipoxygenase products, e.g. hydroxyeicosatetraenoic acid (HETE) may also have an effect on airway smooth muscle; there are, however, insufficient data to support a possible role in human airways smooth muscle control.

Platelet activating factor

Recently there has been interest in the role of platelet activating factor (PAF) in the pathogenesis of asthma[82,83]. PAF is a phospholipid mediator that can be released by platelets, leukocytes, mast cells and macrophages[84]. Although PAF produces bronchoconstriction and hyperresponsiveness in guinea-pig and man *in vivo*[85,86], it has no direct effect on human airway smooth muscle *in vitro*[2]. It has been shown that PAF-induced airway obstruction in the guinea-pig *in vivo* is not due to muscle contraction[86]. Most evidence suggests that the actions of PAF on the lungs are mediated via its effects on microvascular permeability, its chemotactic potency and via the activation of inflammatory cells to produce other mediators, e.g. thromboxanes[87–89].

Other mediators of inflammation

Many more products of inflammatory cells are of interest for the pathophysiology of airways obstruction, e.g. major basic protein and eosinophilic cationic protein, superoxide radicals, proteolytic enzymes, bradykinin, adenosin and HETE's. Their effects on human airway smooth muscle, however, have not been examined in detail, or not at all. At present, there are insufficient data to support a direct action of most of these substances on human airway smooth muscle, although there may be important actions on other cell types in the lungs that may contribute to the development of obstructive lung disease[90,91].

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Chapter 4

Bronchial hyperresponsiveness

Introduction

Bronchial hyperresponsiveness is defined as an exaggerated bronchoconstrictor response to physical, pharmacological and chemical stimuli, such as inhaled histamine, sulphur dioxyde or cold, dry air, that do not have this effect in normal subjects[1,2]. Bronchial hyperresponsiveness is a characteristic of asthma, is common in chronic bronchitis with airflow limitation, and is sometimes present in patients with allergic upper airway disease[1,3]. There is a clear correlation between the degree of bronchial responsiveness and the severity of asthmatic symptoms[2], and hyperresponsiveness is regarded as a risk factor for the development of chronic airflow limitation[4].

In vivo, bronchial responsiveness is assessed by measuring a lung function index that reflects airway caliber (e.g. the forced expiratory volume in 1 sec, FEV_1, or the specific conductance, sGAW) before and after inhalation of a bronchoconstricting agonist such as histamine or methacholine. The agonist is administered repeatedly in doubling doses, and the test is continued until a certain degree of airflow limitation has occurred. From the measured values, the provocative dose (PD) of the agonist that produced a defined fall in FEV_1 or sGAW, is interpolated[5].

In many asthmatic patients, airway obstruction can be provoked to a degree that causes severe respiratory distress, even when starting the test with a normal baseline lung function. It is not possible, therefore, to measure a maximal effect, or a response plateau, *in vivo* in those subjects. In some normals, most subjects with chronic bronchitis and airflow limitation, and in patients with mild asthma, the induced bronchoconstriction reaches a plateau response after relatively high agonist doses[6–8]. In patients with chronic bronchitis, but not in most asthmatics, the degree of bronchial hyperresponsiveness is correlated with baseline airway caliber[9,10].

The different characteristics of the response to inhaled bronchoconstrictors in patients with asthma and chronic bronchitis have suggested that there may be fundamental differences in the pathophysiology of bronchial hyperresponsiveness between these two groups[3,6,11]. The mechanisms that cause either type of hyperresponsiveness are, however, not clear. This is reflected by the large number of hypotheses that try to explain bronchial hyperresponsiveness[1,2,12,13]. In this chapter, the various possible mechanisms of bronchial hyperresponsiveness will be reviewed.

Endogenous factors in bronchial hyperresponsiveness

Genetic factors

Family studies, studies in monozygotic twins, and animal experiments have indicated that bronchial hyperresponsiveness in asthma, chronic bronchitis and allergic upper airway disease is a heritable trait[14–17]. A considerable genetic heterogeneity seems present, and environmental factors, such as cigarette smoking, exposition to allergens, and viral infections, will determine the outcome in a given individual to an important extent[16]. It has been suggested that asthma and chronic bronchitis have a common genetic base: the 'Dutch Hypothesis'[18]. This concept awaits confirmation from long-term follow-up studies[19].

Autonomic dysbalance

The possibility of an abnormal control of airway caliber by the autonomic nervous system has drawn attention for many years[20,21]. An increased activity of the cholinergic system[22], impaired relaxation to, or secretion of circulating catecholamines[23–26] or an increased α-receptor-mediated bronchoconstriction[27,30], a decreased inhibition by the NAI system[31,32] or an enhanced activity of local reflexes with release of neuropeptides[33,34] have been put forward. Several of these theories are not yet supported by findings in humans, or do not explain the features of nonspecific bronchial hyperresponsiveness[21,35–37].

Inflammatory mediators

Asthmatics are hyperresponsive *in vivo* to a number of inflammatory mediators, including PGD_2, $PGF_{2\alpha}$ and several leukotrienes[38–45]. LTB_4 and PAF have been shown to induce bronchial hyperresponsiveness in experimental animals and humans, respectively[46,47] and LTC_4 enhances methacholine responsiveness in man[48]. The release of prostaglandins, leukotrienes, histamine, chemotactic factors and platelet activating factor during the course of an asthmatic attack has been documented in man[49–52].

In asthmatics, mediator releasing cells (basophils, neutrophils, eosinophils, macrophages and mast cells) may have an increased ability to release mediators and oxygen radicals[53–60]. Animal experiments have suggested, that different classes of mediators may augment the response to cholinergic nerve stimulation, modulate ganglionic transmission, enhance smooth muscle contractility, decrease β-receptor function or inactivate relaxing peptide transmitters[21,61–66]. Interaction of mediator classes further complicates this picture[64].

The inflammatory reaction that is caused by the released mediators will produce mucosal swelling due to extravasation of plasma and accumulation of inflammatory cells[67]. Increased mucosal thickness and epithelial disruption may further

contribute to bronchial hyperresponsiveness[68,69]. The direct and indirect effects of inflammatory mediators, their release in asthmatics, and their capacity to produce airway wall thickening make them likely candidates to play a role in the pathogenesis of bronchial hyperresponsiveness[70–76]. It is unlikely, however, that any single mediator will play a key role.

Airway epithelium

It has been speculated that an increased permeability of the bronchial epithelium, or a decreased production of smooth muscle relaxing factors by the epithelium, might cause bronchial hyperresponsiveness[77–81] . Epithelial damage results from exposure to cigarette smoke or viral infections, and has been demonstrated *in vivo* in man[82,83]. An intrinsically abnormal structure of the epithelium seems to be present in asthmatics[84,85]. It seems unlikely, however, that bronchial hyperresponsiveness in man is due to the increased permeability of airway epithelium[82,86]. In guinea-pigs and dogs, but not yet in man, bronchial epithelium has been shown to produce a muscle relaxing factor[77,87,88]. In man, the epithelium of central airways can produce lipoxygenase and cycloxygenase metabolites of arachidonic acid[89,90] that, however, have bronchoconstricting activities.

Abnormal airway mechanics

Airway smooth muscle contraction *in vivo* and *in vitro* is different in that the maximal shortening to 20% of the initial length that is possible *in vitro* certainly does not occur *in vivo*. In stead of trying to explain bronchial hyperresponsiveness, which can be predicted from the shortening capacity of normal airway smooth muscle cells, the question should be answered why normals do not respond or develop only a mild bronchoconstriction during bronchoprovocation testing[91,92]. The most likely explanation for this seems, that elastic forces from the surrounding structures prevent maximal muscle contraction *in vivo*. A decreased tethering effect of the lung tissue opposing the contractile force of airway smooth muscle could then lead to bronchial hyperresponsiveness[69].

Alternatively, if in hyperresponsive subjects the resting muscle length were more near optimal length compared to the resting length in normals, an increased contractility could result. It has been shown in dogs that hyperinflation, i.e. stretching of the muscle, increases bronchial responsiveness[93]. This suggests that, at resting lung volume, the muscle was not at its optimal length.

Finally, a given degree of smooth muscle contraction will produce an increased airflow resistance dependent on the thickness of the mucosa[69,94]. Small increases in mucosal thickness can therefore enhance the effect of muscle contraction considerably.

Abnormal airway smooth muscle cells

The non-specificity of bronchial hyperresponsiveness suggests an end organ abnormality. An intrinsic defect of airway smooth muscle may therefore underly bronchial hyperresponsiveness. This possibility will be discussed in the next chapter.

Exogenous factors in bronchial hyperresponsiveness

Allergy

A high percentage of asthmatics is allergic to airborne allergens. Allergic provocation leads to a direct, and often also a late asthmatic reaction, initiated by the release of mediators from mast cells and, perhaps, other cell types in the airways[95]. During late phase reactions, eosinophil numbers in the blood are elevated[96] and increased numbers of eosinophils are present in the airway lumen[97]. Activation of eosinophils may play a role in the pathogenesis of the late reaction by the release of toxic substances such as major basic protein[98].

There is also evidence of neutrophil activation during late phase allergic reactions[99]. In rabbits it has been shown that, for the development of a late phase reaction, neutrophils are already required at the time of the allergen challenge[100]. In humans, it is likely that the early reaction to allergen results from direct effects of mediators, whereas the late phase is the consequence of an inflammatory reaction of the bronchial mucosa[101]. In asthmatics, bronchial hyperresponsiveness increases shortly after the early reaction, and may remain elevated for days or even weeks[102,103]. The mechanism of this increase is therefore possibly related to the inflammatory reaction[104].

Infection

Viral respiratory tract infections can induce or worsen bronchial hyperresponsiveness for days or weeks[105–109]. Inflammation of the bronchial mucosa may play an important role in virus-induced hyperresponsiveness. It has been shown that influenza virus causes inflammation of the bronchial mucosa in normals without pulmonary symptoms[110].

If mediator release is exaggerated in asthmatics, this would give rise to a more severe inflammatory reaction in asthmatic airways, and this might cause an increased bronchial responsiveness as discussed previously[104,111]. It has been suggested that viral infections of small airways (bronchiolitis) may predispose to or predict future asthmatic symptoms[112].

It is not clear why in man bacterial infections usually do not lead to bronchial hyperresponsiveness, despite massive infiltration of the airways with neutrophils, and extensive epithelial damage. In guinea-pigs, bronchial hyperresponsiveness

has been evoked by administering haemophilus influenzae[113]. This effect might be due to hydroxyl radical production by alveolar macrophages, which attennuates β-receptor function[114].

Occupational stimuli, air pollution, ozone and cigarette smoke

Chronic exposure to noxious gases, such as toluene diisocyanate (TDI) or sulphur dioxyde, leads to bronchial hyperresponsiveness and symptoms of asthma. These effects are probably mediated by airway inflammation[115–117], although direct effects on airway smooth muscle may also play a role[118].

Administration of ozone to dogs, guinea-pigs or humans leads to *in vivo* bronchial hyperresponsiveness that lasts for several days[119–121]. This is associated with inflammation of the mucosa, and may be mediated by synthesis of arachidonic acid metabolites[122–124].

Cigarette smoking has been reported to increase bronchial responsiveness in some subjects, but not in others[125,126]. The effect is dose-related, and is also related to changes in baseline airway caliber[11]. Bronchial hyperresponsiveness in smokers differs from that in asthmatics because smokers do not respond to α-agonist provocation, are more reactive to histamine than to methacholine, and usually have a maximal response plateau in their response to inhaled histamine[11]. Inflammation is probably also an important determinant of smoking-induced hyperresponsiveness[127]. The degree of cigarette smoke-induced epithelial permeability does, however, not correlate with the degree of bronchial hyperresponsiveness[82,86]. Finally, smoke may produce bronchoconstriction via stimulation of vagal reflex activity and by stimulation of ganglionic nicotinic receptors[128].

Summary

From the literature it seems likely that endogenous and exogenous factors interact in an complex fashion to produce bronchial hyperresponsiveness. The various putative mechanisms that may lead to hyperresponsiveness are depicted schematically in the Figure.

An important feature in bronchial hyperresponsiveness is airway wall inflammation. Most exogenous factors seem to have their effect on bronchial responsiveness by inducing or enhancing airway wall inflammation. Most of the proposed 'intrinsic defects' might not be the cause, but the result of the inflammatory reaction, because mediators may modify the autonomic balance, smooth muscle function, and epithelial structure. Theoretical calculations and preliminary experimental data suggest that mechanical factors may well be important.

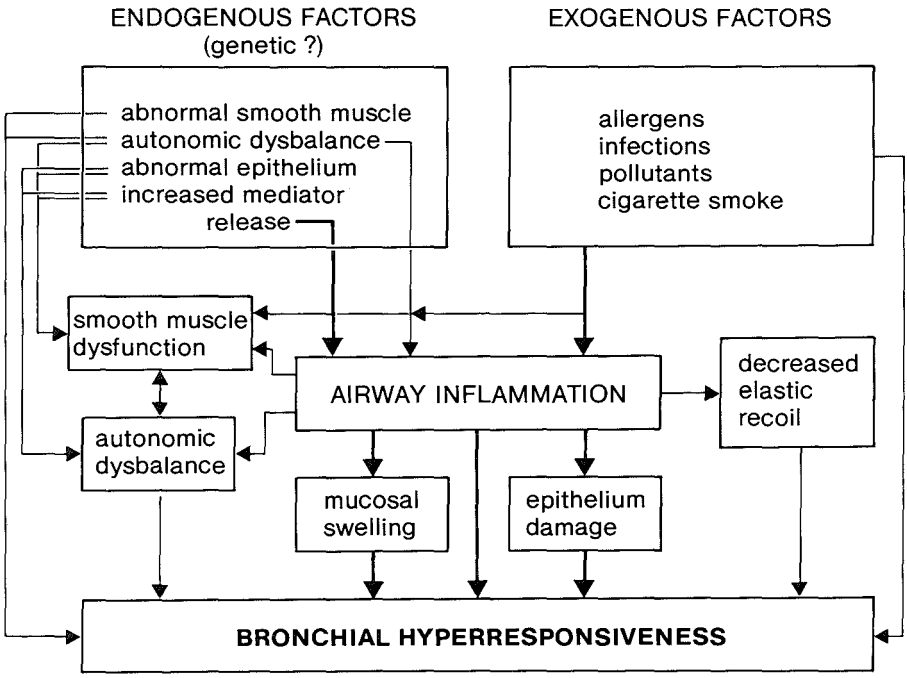

Figure 1. Mechanisms in bronchial hyperresponsiveness.

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Chapter 5

Airway smooth muscle and airway responsiveness Aims of the studies

Introduction

The function of airway smooth muscle in normal subjects is not evident. Possible physiological roles include maintenance of optimal regional ventilation/perfusion ratios, reduction of anatomic dead space, stabilisation of cartilaginous bronchi, defense against impurities and, less likely, squeezing mucus out of mucous glands and pulling open the alveoli next to the airways[1]. Any role of airway smooth muscle is necessarily limited, because an important degree of contraction will lead to airway narrowing and to an increased work of breathing. There is, however, no doubt that in asthma the acute bronchoconstriction following exposure to nonspecific or allergic stimuli is due to airway smooth muscle contraction. Most research on airway smooth muscle function has therefore concentrated on clarifying its role in bronchial hyperresponsiveness, airway obstruction and allergy.

From the foregoing chapters it can be concluded that many factors may be involved in the pathogenesis of the abnormal responsiveness of airway smooth muscle in patients with asthma or chronic bronchitis. One of these factors is an intrinsic abnormality of the airway smooth muscle cells. In order to examine this, several animal models have been developed, most of which have features in common with human allergic bronchoconstriction[2]. Because of important differences between species, and because there is no satisfactory animal model of spontaneous, non-allergic asthma, it is crucial to study human airway smooth muscle from subjects with and without airway hyperresponsiveness. The research on human lung tissue *in vitro* has been limited by the supply, and by difficulties in obtaining stable and reproducible responses of airway smooth muscle *in vitro*. Moreover, it is very difficult to obtain lung tissue from asthmatic subjects.

This chapter will give a summary of the research on airway smooth muscle hyperresponsiveness in experimental animals and man. From these data, the aims of the studies to be presented in parts II and III will be derived and briefly outlined.

Hyperresponsiveness as a smooth muscle abnormality

Airway smooth muscle cells can be hyperresponsive by being more sensitive

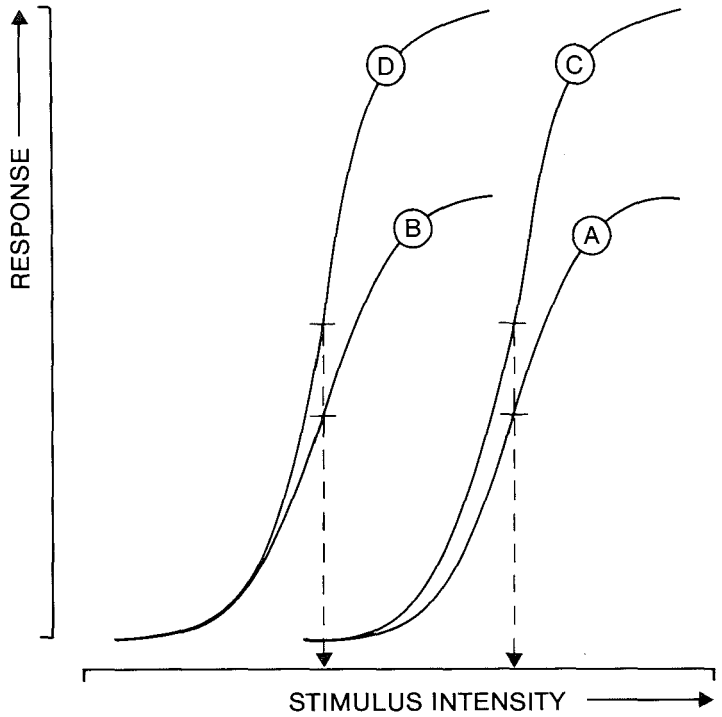

Figure 1. Types of smooth muscle hyperresponsiveness. Curve A: normal. Curve B: Increased sensitivity (deviation supersensitivity, prejunctional supersensitivity, type I); Curve C: Increased maximal response (non-deviation supersensitivity, postjunctional supersensitivity, type II); Curve D: combination of B and C.

to contracting agents, i.e. responding at lower agonist concentrations; by having a greater maximal response to 'normal' agonist concentrations, or by a combination of these two possibilities (Figure 1). The first possibility is a consequence of changes in agonist-receptor interaction, and has also been called 'deviation supersensitivity' 'prejunctional supersensitivity' 'or 'type I supersensitivity'. The second type of hyperresponsiveness is due to post-receptor changes in the excitation-contraction coupling or in the contractile apparatus, and has also been called 'non-deviation supersensitivity' 'postjunctional supersensitivity' or 'type II supersensitivity'[3,4]. Abnormalities of virtually all processes, described in chapter 2, that are involved in the contractile response of airway smooth muscle might produce hyperresponsiveness.

An increased sensitivity could result from increased numbers or affinities of receptors for contracting agonists or, conversely, from a decrease in the number or affinity of relaxing receptors[5]. Partial depolarisation of the cell membrane could also increase smooth muscle sensitivity[6].

Increased maximal responses of muscle cells might result from abnormalities in the excitation-contraction coupling, e.g. an enhanced release of Ca^7, or from an abnormal structure or quantity of the contractile proteins or the enzymes necessary for their activation[1]. Increased activity of cross-bridges, a decreased parallel elastic component or abnormal length-active tension relationships could also favour an increased contractile response[1]. When we consider not individual cells, but muscle tissue, several other putative mechanisms may cause an increased maximal response, such as an increased cell-to-cell coupling by gap junctions, which could lead to single unit behaviour[8]. The net effect of an increase in the number or size of gap junctions is not necessarily hyperresponsiveness, however, beacause this will depend on the balance between excitatory and inhibitory influences. Finally, an increased amount of muscle cells will lead to a higher maximal response. Muscle hypertrophy is a characteristic of human asthmatic bronchi[9].

Before these putative mechanisms can be examined, the question needs to be answered whether there is a primary smooth muscle abnormality in human bronchial hyperresponsiveness or airway obstruction.

The guinea-pig model

In guinea-pigs with spontaneous variations in histamine responsiveness of the airways, no relation was found between the histamine response *in vivo* and the histamine sensitivity of tracheal smooth muscle *in vitro*[10]. Several authors have reported that guinea-pigs that had been sensitized to egg-albumen have a decreased sensitivity and maximal contractility of their tracheal smooth muscle to histamine and methacholine *in vitro*[11,12]. Others, however, have reported an increased maximal responsiveness in this model[13].

The maximal effect of β-receptor stimulation in sensitized guinea-pig trachea appeared to be reduced[11] in a similar fashion as in the Haemophilus- inoculated guinea-pig[14]. There is some indirect evidence that in sensitized guinea-pig trachea the effect of stimulation of the NAI system is also less than in control tissue[15]. Measurement of the membrane potential of tracheal smooth muscle cells from sensitized guinea-pigs has shown that repeated allergen challenge causes a slight hyperpolarization of the cell membrane, that may be due to potentiation of the electrogenic sodium pump[6,16].

The dog model

The trachealis muscle of dogs that are allergic to Ascaris relaxes less to isoproterenol than non-allergic control tissue, and has an increased maximal response to methacholine[17]. These changes have been attributed to a basally low concentration of cAMP in the muscle cells[18]. In this same model, however, others have found no *in vivo* hyperresponsiveness to histamine, acetylcholine or

serotonin[19], whereas a selectively enhanced response of sensitized dog trachealis muscle to serotonin, but not to acetylcholine, has also been reported[20].

The tracheal muscle from dogs allergic to egg albumen is also hyperresponsive to histamine *in vitro*, both with respect to sensitivity and maximal effect[21]. In this model, subtle changes have been demonstrated in the mechanical response to electric field stimulation *in vitro*[22]. An increased velocity of shortening, myogenic responses after a quick stretch and an increased capacity to shorten have been reported, and these changes might be due to an increased activity of rapidly cycling cross-bridges[23].

Dogs sensitized to ragweed have hyperresponsiveness to histamine and methacholine *in vivo*[24]. *In vivo* studies on dogs allergic to ragweed have shown that a cyclo-oxygenase product, probably thromboxane, may be involved in the pathogenesis of allergic airway hyperresponsiveness[25].

The sheep model

Sheep that are allergic to Ascaris are hyperresponsive to H_1-receptor stimulation with histamine *in vivo*, probably because of a functional depression of relaxing H_2-receptors[26]. Isolated tracheal smooth muscle from this animal model, however, has no increased response to histamine and other contracting agonists[27]. Lung parenchymal strips of sheep allergic to Ascaris showed an increased response to histamine and other contracting agonists[27]. Lung parenchymal strips of sheep allergic to Ascaris showed and increased maximal response to a thromboxane analogue and to histamine, but not to $PGF_{2\alpha}$ and methacholine[27].

Studies on human airway smooth muscle

In 1951, Schild et al concluded that histamine was of major importance in human allergic asthmatic bronchoconstriction[28] on the basis of an experiment with bronchi from a single asthmatic. More than 30 years later, Dahlén et al did similar experiments on human lung tissue from two asthmatic patients and concluded that leukotrienes, but not histamine, were important in human allergic asthma[29]. Since then, functional studies on asthmatic human airways *in vitro* have produced evidence of an increased maximal contractility to histamine, LTC_4 and carbachol, and of a decreased sensitivity and maximal response to isoproterenol and other sympathomimetics[30–35]. There are however many discrepancies in the results of this relatively small number of reports, that clearly illustrate the confusion that has resulted from examining small numbers of asthmatic human airways *in vitro* with large differences in methodology and patient selection.

More work has been done on airway smooth muscle from better defined populations of non-asthmatic humans with and without bronchial hyperrespon-

siveness, who were operated for bronchial carcinoma. These studies have mainly concentrated on the sensitivity of isolated airways to constricting agents, and not on their maximal effect, because of a troublesome variability of maximal responses within- subjects. None of the studies has shown a significant correlation between the degree of bronchial hyperresponsiveness *in vivo* and the sensitivity of airway smooth muscle to histamine or methacholine *in vitro*[33,36–41]. Also, no differences were found in the relaxation responses of non-asthmatic airways to sympathomimetics or forskolin[35,41] and to stimulation of the NAI system[41]. Only small numbers of patients were studied, however. There are few reports on the responsiveness of passively *in vitro* sensitized non-asthmatic human airways. It has been shown that house-dust mite- or ragweed-induced contractions of sensitized human airways *in vitro* were due to release of prostaglandins, leukotrienes and histamine[42,43], but, unfortunately, the effects of the sensitization procedure on smooth muscle function has not been examined.

Aims of the studies

From the data in the literature we can conclude, that:

1. Animal models have not been very helpful, due to the many differences between species with respect to airway smooth muscle function and its control, and the effects of sensitization. Different investigators have produced contradictory results.
2. Calculations on the reproducibility of the *in vitro* studies on human airways are lacking, especially regarding the within- subjects variation. This makes interpretation of previous studies difficult. Moreover, most studies have been done using isometric transducers. This provides no insight in the shortening capacity of the muscle, which may be more relevant than force.
3. It is not possible to draw conclusions from studies on asthmatic human airways *in vitro*, because of the small number of patients and important methodological differences between the studies.
4. There has not been much interest in the relaxation responses of human isolated airways in relation to airway obstruction and hyperresponsiveness.
5. Structure-function correlation studies on human airways and studies on mechanics of human airway smooth muscle contraction are virtually lacking.

The studies presented in this thesis were undertaken to clarify the role of human airway smooth muscle and its autonomic control in airway obstruction and hyperresponsiveness. The following questions were addressed.

1. Is it possible to obtain reproducible and accurate measurements of the sensitivity and contractility of human isolated airway smooth muscle preparations to contracting and relaxing agonists?
2. Is airway smooth muscle function abnormal in patients with chronic bronchitis or asthma and bronchial hyperresponsiveness? Specifically: is the response to contracting agonists increased or the response to relaxing agonists decreased?

3. Is the function of human airway smooth muscle related to the degree of airway inflammation?
4. Are there abnormalities in the autonomic innervation of the airways in patients with asthma or chronic bronchitis and bronchial hyperresponsiveness?

For this purpose, we have developed a technique to measure the mechanical responses of human isolated airways reproducibly, with sufficient accuracy to detect differences between subjects. In part II, these methods and their reproducibility are described in detail. In part III, the results are reported of studies on human airway smooth muscle function in relation to clinical signs and symptoms of chronic bronchitis and airflow limitation, asthma, bronchial hyperresponsiveness and airway inflammation, followed by a summary and general discussion.

Note on terminology

The terms 'chronic obstructive bronchitis' and 'chronic obstructive pulmonary disease', or COPD, have been used depending on whether a study has been published in, or submitted to, a British or an American journal, respectively.

'Chronic bronchitis' is defined as proposed by the Medical Research Council[44] as a syndrome of chronic cough and sputum production on most days during at least three consecutive months each year, for more than two successive years. The term 'obstructive' indicates chronic airflow limitation, and is usually defined as an FEV_1/VC ratio lower than 70% or more than 1.64 standard deviations below mean predicted values[45]. 'Chronic obstructive pulmonary disease' is defined according to criteria of the American Thoracic Society, and is synonymous with chronic obstructive bronchitis[46].

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Part II

Measurement of human airway smooth muscle function *in vitro*

Chapter 6

Measurement of human small airway smooth muscle function *in vitro* with the bronchiolar strip preparation

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Summary

The technique by which human bronchiolar strips are prepared is described in detail. The biological viability of the preparations after storage at 4°C overnight and the reproducibility of contractile responses to KCl and methacholine were examined in lung tissue from six patients. Measurements were performed on the first and second day after surgical resection. On both days, most bronchioles showed an increase in contractility. The responses on the first day were not different from those on the second day. No significant changes in time were found for EC_{50} and slope of the methacholine concentration- response curves. The variability of responses between-patients was significantly larger than between-strips within-patients. The EC_{50} was the best reproducible parameter.

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

De Jongste JC, Van Strik R, Bonta IL, Kerrebijn KF. Measurement of human small airway smooth muscle function *in vitro* with the bronchiolar strip preparation. J Pharmacol Methods 1985;14:111-118. Reprinted with permission of Elsevier Science Publishing Company.

Introduction

Attempts to measure human airway smooth muscle function *in vitro* have been made by using slices of lung tissue[1], bronchial chains[2], tubes and spirals[3], and strips of lung parenchyma[4]. Lung tissue is usually obtained from autopsies up to 14 hours post mortem[5] and from thoracotomies performed because of bronchial

carcinoma[6]. We have developed a technique to prepare human bronchioles under standardised conditions using lung tissue obtained from thoracotomies to avoid the influence of hypoxia, which is present in autopsy tissue.

Little information is available on the reproducibility of contractile responses from different comparable sized airway preparations obtained from the same specimen of human lung tissue. We therefore compared within-patient variability of bronchiolar responsiveness and between-patient variability. Furthermore, we studied the reproducibility of contractile responses within a 55 h postoperative period including two episodes of storage in cooled buffer.

Methods

Human lung tissue was obtained from thoracotomies, usually performed because of lung cancer. Immediately after surgical resection, a specimen of macroscopically normal lung tissue, containing airways of 1-2 mm in diameter, was obtained. The tissue was immediately submerged in Krebs buffer at 0°C (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 was aerated with carbogen (95% O_2, 5% CO_2).

After rapid transport to the laboratory, the tissue was washed several times to remove the blood. In the cut surface, bronchioles with a diameter of 1-2 mm were identified and cannulated while the tissue remained in buffer. To ascertain that these were really airways, air was inflated gently into the cannula with a 5 ml syringe, to obtain tissue inflation. The cannulae were left in place to indicate the direction of the bronchiole, which facilitates its preparation. After cannulation, all bronchioles were cut from the tissue, leaving a rim of parenchyma in place to avoid damage. Care was taken not to squeeze or stretch the bronchiole. Subsequently, the bronchioles were placed over a stainless steel rod by positioning the cannula, which was still in place, end-to-end to the rod and then moving the airway segment over the rod. A small segment of the bronchiole was cut off for histological examination.

Under a binocular preparation microscope (magnification 20 x) the bronchioles were then prepared as free as possible from blood vessels, lymphatic tissue, and parenchyma using iris scissors. The tissue remained in fresh buffer. The bronchiolar tube was spiralised to obtain a strip measuring 20x2 mm. Utmost care was taken to avoid applying any force to the tissue as this is deleterious for its contractile function. Thin surgical silk threads were tied to both ends of the strip.

From most lung tissue specimens, at least 4 to 6 strips of similar size could be prepared. After completion, which takes about a quarter of an hour for each preparation, the strips were stored overnight in cooled (4°C) Krebs buffer containing penicillin ($3x10^{-5}$ g/l) and tobramycin ($5x10^{-3}$ g/l). Carbogen was bubbled through the buffer, and a perfusion pump changed the total bath volume every hour continuously in order to wash out substances possibly liberated during the preparation procedure and the narcotics (see below).

The following day, 20 hours postoperatively, the bronchioles were mounted in 10 ml double-jacketed organ baths containing Krebs buffer at 37°C, aerated with carbogen (pH 7.35, P_{CO2} 4.6 kPa, P_{O2} 71.8 kPa). One thread was fixed to a glass hook in the bottom of the organ bath and the other to a Grass FTO3D isometric force transducer, which could be moved vertically with a micrometer screw (Figure 1). The transducers were coupled via a carrier wave amplifier (Peekel instruments, The Netherlands) to a digital voltmeter and to a Rikadenki multichannel pen recorder. Before the measurements were started, the bronchioles were allowed to equilibrate for 2 h under an isometric tension of 500 mg, which was restored every 15 min when necessary. Preliminary experiments had shown that this resting tension resulted in optimal contractile force.

The bath fluid was changed every 15 min during this period. Pharmacological

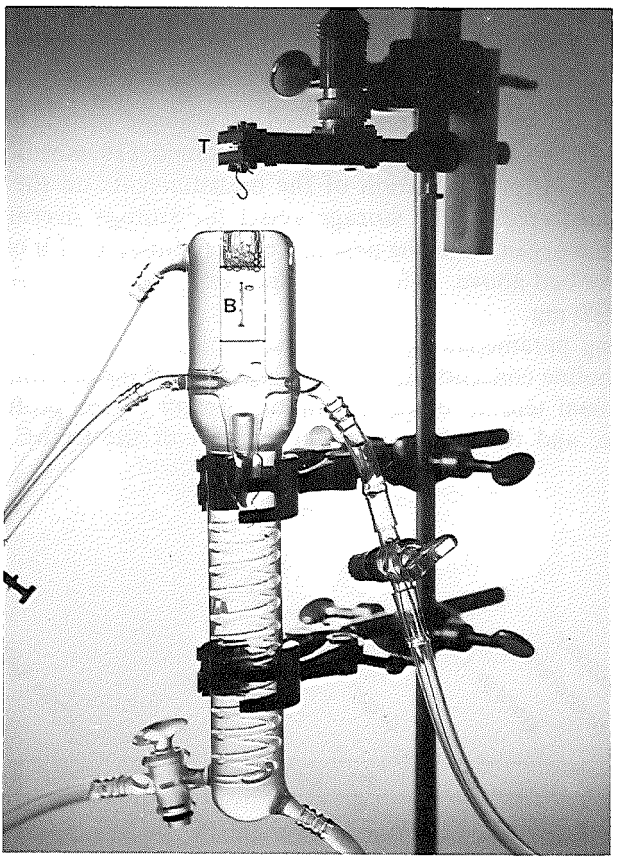

Figure 1. Bronchiolar strip (B) mounted in the organ bath. The strip is attached to an isometric transducer (T) which can be moved vertically with a micrometer screw.

stimulation was carried out by adding agonists in small (50 μl or less) volumes. In this way, cumulative concentration-response curves (CCRC) were obtained. After each complete curve, the bath fluid was changed four times with 2 min intervals.

Results were expressed as mg force per mg dry weight of the bronchiole and also as mg force per mg of protein present in the strip. For this purpose the bronchioles were dried for 48 h at room temperature and weighed after completion of all measurements. Subsequently the amount of protein in each strip was determined colorimetrically[7].

The viability of the bronchioles after storage and the reproducibility of responses thus obtained is illustrated by a study in which 18 bronchioles from 6 patients (three bronchioles from each patient) were stimulated repeatedly over a 55 h period. Lung tissue was obtained from six consecutive thoracotomies. The patients' ages ranged from 57 to 72 years; five had bronchial carcinoma and one had a bronchopleural fistula (patient 2). Premedication and anesthetics were the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane, and pancuronium. Each bronchiole was examined on the first and second day after thoracotomy. On both days, two complete methacholine CCRC (10^{-8} to 10^{-4}M) were made, preceded and followed by a single contraction evoked by adding high-potassium Krebs buffer that contained 118 mM KCl instead of NaCl (Figure 2). After completion of the measurements on the first day, the strips were transferred to the storage vessel for storage overnight and were remounted in the organ baths the next morning, followed by 2 h of equilibration at 37°C as described above. Before each drug exposure, the resting tension was readjusted to 500 mg.

The following parameters were measured: -log EC_{50}: the negative logarithm of the methacholine concentration that caused 50% of the maximal contraction; T_{max}: the maximal tension corrected for dry weight or for protein content of the bronchiole; and the slope of the linear part of the CCRC, expressed as

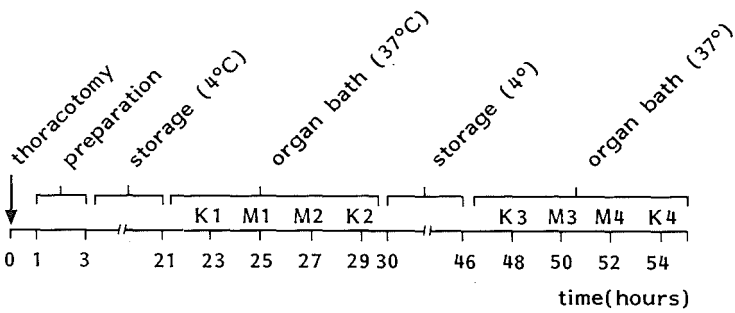

Figure 2. Design of the study. K = KCl response. M = Methacholine concentration-response curve.

Table 1. Statistical model of the various sources of variation

Source of variation	Degrees of freedom
1 Between patients	5
2 Between strips/within patients	12
3 Between days	1
4 Order of measurement per day	1
5 Days x measurement order	1
6 Days x patients	5
7 Measurement order x patients	5
8 Days x measurement order x patients	5
9 Residual variation	36
10 Total variation	71

Statistical model of possible sources of variation. The factors patients and strips/within patients were considered as random, whereas the factors days and measurement order per day were considered as fixed.

the increase in tension (as a percentage of maximal response) resulting from a tenfold increase in agonist concentration.

For both contractile agents, the various contributions to the total variation of 72 measurements were quantified through analysis of variance according to the statistical model shown in Table 1[8]. This model determined the testing procedure for evaluating the statistical significance of the mean squares by means of the F-test. Therefore the F-ratios (of two mean squares) were computed as follows: items 2, 6, 7 and 8 were compared to 9, 5 to 8, 4 to 7, 3 to 6 and 1 to 2. In addition, the net components of variance were calculated for between-patients and between-strips/within-patients variability as well as for residual variation. These net variance components were then transformed to relative standard deviations as a percentage of the overall mean.

Results

The values for each patient of -log EC_{50}, T_{max}, and slope are summarised in Table 2. Each figure represents the mean response of three bronchiolar strips from one patient. The contractility to KCl and methacholine increased significantly over the first postoperative day. On the second day, only the KCl-response increased, while methacholine T_{max} was constant and was comparable to the second measurement on the first day. No significant changes in time for -log EC_{50} and slope were found (Figure 3). Examination of CCRC after 48 h of storage without intervening exposure to KCl or methacholine indicated no difference from CCRC made on the first day.

The outcomes of the analyses of variance are displayed in Table 3 as F-ratios, being indicators of the statistical significance of the various contributions to the total variation, and relative standard deviations as a percentage of the overall mean.

Table 2. Methacholine and KCl-responses of six patients

	$-\log EC_{50}$				T_{max}								slope			
	M1	M2	M3	M4	M1	M2	M3	M4	K1	K2	K3	K4	M1	M2	M3	M4
Patient 1	6.10	6.08	6.05	5.91	257	331	270	244	208	214	221	185	42	41	36	36
Patient 2	5.62	5.54	5.27	5.28	78	83	75	81	130	170	169	159	72	65	69	68
Patient 3	6.42	6.49	6.57	6.47	172	214	205	223	83	121	45	162	58	47	42	61
Patient 4	6.35	6.46	6.91	6.50	150	192	173	181	91	180	84	150	45	43	43	47
Patient 5	5.50	5.93	5.95	5.87	40	69	99	114	47	101	58	112	54	45	46	47
Patient 6	6.61	6.47	6.58	6.43	101	113	140	143	62	110	76	102	50	51	49	52
Mean	6.10	6.16	6.22	6.08	133	167	160	164	103	149	109	145	53	49	48	52

Each figure represents the mean of three bronchiolar strips from one patient.

Abbreviations: M 1-4 = methacholine response 1-4 (see Figure 1); K 1-4 = KCl response 1-4 (see Figure 1); T_{max} = maximal contraction expressed as mg force per mg dry weight; slope = percent increase in contractile force resulting from a ten-fold increase in methacholine concentration.

Table 3. Results of analysis of variance

Sources of variation	F-RATIOS				
	-log EC_{50}	T_{max} (M) corr DW	T_{max} (M) corr prot	Slope (M)	T_{max} (K) corr DW
1 Between patients	8.45*	4.53*	4.93*	3.67*	5.50*
2 Between strips/within patients	6.45*	14.31*	9.15*	5.22*	5.10*
3 Between days	0.04	0.93	2.16	2.86	0.02
4 Order of measurement per day	0.72	21.66*	18.50*	0.03	7.67*
5 Days x measurement order	3.91	4.12	1.58	3.65	0.26
6 Days x patients	3.02*	2.68*	2.45	0.33	0.45
7 Measurement order x patients	0.92	0.27	0.60	0.57	3.96
8 Days x measurement order x patients	1.04	0.90	1.16	1.48	1.67
	NET COEFFICIENTS OF VARIATION[a]				
Between-patients	26%	171%	162%	69%	132%
Between strips/within patients	9%	80%	73%	36%	56%
Residual variation	4%	21%	24%	16%	25%

Abbreviations: T_{max} (M) corr DW = methacholine T_{max} corrected for dry weight of the preparation. T_{max} (M) corr prot = methacholine T_{max} corrected for the amount of protein in the preparation. Ohter abbreviations: see legend of Table 1.

* $p < 0.05$

[a] Coefficients of variation are expressed as percentage of the overall mean response and pertain to net components of variation for single measurements.

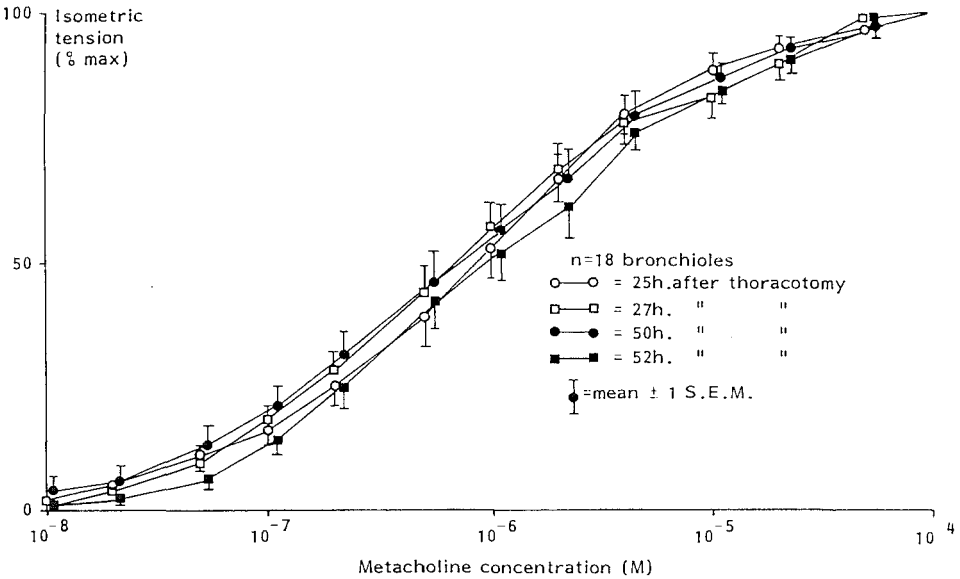

Figure 3. In vitro contractile responses to methacholine of human bronchioles (n = 18), repeatedly measured at 25, 27, 50 and 52 h after surgical resection. The bronchioles were stored at 4°C overnight twice (see Figure 1). The vertical axis depicts the contractile response to methacholine as a percentage of the maximal response in each preparation.

For all parameters, overall variability was determined to a large extent by between-patients differences. However, a considerable within-patient variability was present. Individual T_{max}- and -log EC_{50}-values differed slightly between the first and second pair of methacholine CCRC, but this difference showed no consistent direction. The precision of T_{max} measurements was not increased by correcting for the protein content of the bronchiole (Table 3).

Discussion

The study of human airways *in vitro* is of importance for answering questions related to properties and pharmacological modification of responses of airway smooth muscle[3,5,6,9–11]. Furthermore, bronchial responsiveness *in vivo* can be compared to *in vitro* airway responsiveness[12–15]. Bronchiolar strips have several advantages over other airway smooth muscle preparations. The responses of these strips are not influenced by vascular smooth muscle contractions, as is the case with parenchymal strips[4,16,17,18]. Bronchioles have a relatively homogenous structure compared to larger bronchi, so that irregularly distributed cartilage plates cannot influence smooth muscle function. Finally, almost all lung tissue specimens removed surgically contain enough macroscopically normal bronchioles to allow multiple measurements, which is often not the case with respect

to bronchi. Reproducible measurements of -log EC_{50}, and, to a lesser degree, of T_{max} and slope of methacholine CCRC can be obtained within 55 h post-operatively without a loss in sensitivity or contractility. T_{max} to KCl and methacholine showed a significant increase on the first day, and, for KCl only, on the second day postoperatively; -log EC_{50} and slope remained constant with time. This indicated a vertical shift of the CCRC. The increase in T_{max} is most likely not due to a change in muscarinic receptor function, as KCl induces a contraction without receptor activation. The increase in T_{max} in time could also be due to a gradual fall in baseline tone. This was apparently not the case, as the resting tension was consistently adjusted to 500 mg before commencing each curve. After cooling, responsiveness was unchanged and this confirmed earlier observations[6].

Differences between patients could be shown for -log EC_{50}, T_{max} and slope. All these parameters showed significant additional between-patients variability, which is therefore not merely a reflection of between-strips/within-patients variation (Table 3).

The relatively large within-patients variability of maximal contractile force in strips can have several causes. The most probable explanation of this variability seems to be that it is mainly caused by the mechanical forces applied to different bronchioles during the operative removal and the preparation procedure. Slight differences in preparative technique, for instance the angle of spiral cutting, can have important consequences. However, real differences may also be present. Because automation of the delicate preparation procedure seems virtually impossible, it is important that the cutting of the bronchioles is always done by the same experienced person.

We have tried to improve the reproducibiliby of T_{max} by correcting contractile force for the amount of protein in the bronchiolar strip. This, however, resulted in a similar coefficient of variation (Table 2). Armour et al, likewise, did not find any benefit from correction for smooth muscle volume, determined histometrically[14], so further attempts to relate contractile force of bronchiolar strips to the amount of protein or muscle seem of little value compared to correction for the weight.

Our results show that it is possible to demonstrate significant differences between-patients by measuring bronchiolar responses *in vitro*. The sensitivity (-log EC_{50}) can be accurately measured and is the best reproducible parameter. The contractility (T_{max}) and slope showed a much larger variability; multiple measurements were necessary because of a relatively large within-patient variability and one should be very careful in interpreting differences between patients. Whether the present results, using methacholine and KCl, have general validity needs to be established in further studies with other contracting agents.

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Chapter 7

Human small airway smooth muscle responses *in vitro*: actions and interactions of methacholine, histamine and leukotriene C_4

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Summary

The *in vitro* contractile responses to methacholine, histamine and leukotriene C_4 (LTC_4) were measured in human bronchiolar strips obtained after surgery. LTC_4 was approximately 100 times more potent than both methacholine and histamine (mean -log EC_{50} values were 8.01, 6.18 and 5.87 respectively). All three agonists produced a similar maximum contractile response (T_{max}) and no difference was found in the time course of methacholine, histamine and LTC_4-induced responses. When methacholine, histamine and LTC_4 were applied in succession on a single airway preparation, no interactions between these agonists were demonstrated for -log EC_{50}. However, interactions were found concerning T_{max}. The -log EC_{50} could be measured accurately and reproducibly and showed a relatively small within-patients variation (coefficients of variation 4-5%). In contrast, T_{max} showed a considerable within-patients variation (coefficients of variation 47-91%), which limits its usefulness when small numbers of airway preparations are studied.

Key words: human airway smooth muscle, bronchiolar strip, methacholine, histamine, leukotriene C_4.

De Jongste JC, Mons H, Van Strik R, Kerrebijn KF. Human small airway smooth muscle responses *in vitro*: actions and interactions of methacholine, histamine and leukotriene C_4. European J Pharmacol 1986;125:29-35. Reprinted with permission of Elsevier Science Publishing Company.

Introduction

Recently, a number of studies has been published in which *in vitro* findings concerning human airway tissues were related to *in vivo* parameters, such as bronchial hyperresponsiveness, chronic bronchitis and airway inflammation[1–7]. Various types of airway preparations were used by the different authors and it is often not clear how accurately single responses represent the contractile properties of a given tissue sample. In a previous study, we calculated the between- and within-patients variation of methacholine responses of human bronchiolar strips. We found that this preparation was stable for 55 h post thoracotomy and that prolonged storage at 4°C did not influence contractile properties[8]. We used the same technique in the present study to analyse the responses to methacholine, histamine and leukotriene C_4 (LTC_4) and estimated the relative contributions of various sources of error (i.e. between-patients and between-strips/within-patients) to the total variation of sensitivity (expressed as -log EC_{50}) and maximum contraction (T_{max}).

For this type of study, ideally only one agonist should be applied to a single airway preparation and one single response curve should be made unless tachyphylaxis or interactions are studied. However, due to the limited amount of human lung tissue that can be obtained for pharmacological experiments, it is advantageous to apply several agonists to one airway preparation. This is only justified if the agonists do not interact. Until now, indirect evidence for interactions between cholinergic agonists, histamine and leukotrienes is only available for dog and guinea pig airways[9–11]. To examine the feasibility of testing the effects of methacholine, histamine and LTC_4 on a single bronchiolar strip, we applied these agents in succession to each of 90 preparations from 30 patients and evaluated the possible occurrence of interactions.

Methods

Human lung tissue was obtained from thoracotomies performed because of carcinoma. Within a few minutes after resection, a piece of macroscopically normal lung tissue was submerged 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 was aerated with 95% O_2, 5% CO_2. After rapid transport to the laboratory, bronchioles (approximately 10 to 13th generation) were carefully dissected free from parenchyma and blood vessels as described previously[8]. The tissue was stored overnight at 4°C in a slow, continuous flow of aerated Krebs-Henseleit buffer. We and others previously have shown that this storage procedure does not influence tissue sensitivity and contractile function[8–12]. The extensive washing of the tissue will remove anesthetics (pentothal, atropine, fentanyl, halothane, pancuronium and O_2/N_2O) and substances liberated during the dissection. Measurements were performed on the first day following thoracotomy. Three bronchiolar strips from each patient were mounted

in 10 ml double-jacketed organ baths containing aerated buffer at 37°C. They were allowed to equilibrate for 2 h under an isometric tension of 500 mg which was adjusted every 15 min when necessary. Preliminary experiments had shown that a resting tension of 500 mg resulted in optimal contractility. The contractile responses were measured using Grass FT03D isometric force transducers coupled to a Rikadenki pen recorder and a digital voltmeter via a carrier wave amplifier (Peekel instruments).

After baseline stabilisation, all strips were pretreated with methacholine (10^{-8} to 10^{-4} M increasing in 10-fold concentration steps followed by washing) in order to ensure a stable contractility during the rest of the day[8,13]. Cumulative concentration-response curves (CCRC) were made by adding agonists in small volumes (less than 50 μl). The tissue was washed 4 times with 2 min intervals after completion of each CCRC and subsequently the bath fluid was changed every 15 min until a stable baseline was obtained. Successive CCRC were separated by 2 h intervals. Before starting a new measurement, the baseline tone was readjusted to 500 mg whenever necessary. The following parameters were derived from each CCRC: -log EC_{50}: the negative logarithm of the concentration of agonist which produced 50% of maximal contraction, and T_{max}: maximal tension, expressed as mg isometric force per mg dry weight of the bronchiole. The dry weight was determined after drying for 48 h at room temperature.

The following drugs were used: methacholine hydrobromide, histamine hydrochloride (Janssen Pharmaceuticals, Belgium) and synthetic LTC_4, (a kind gift of Dr. J. Rokach, Merck Frosst Laboratories, Canada). All agonist solutions were prepared freshly on the day of the experiment and kept on ice. Three bronchioles from one patient were studied simultaneously. Each strip received methacholine (10^{-8} to 10^{-4} M), histamine (10^{-8} to 10^{-4} M) and LTC_4 (10^{-10} to 10^{-7} M). The order in which these substances were applied was determined by randomised 3x3 latin squares. All 3x3 latin squares can be reduced to only two possible configurations (Figure 1) by interchanging the rows, which is justified because strip selection can be considered as random. In this study we included 15 experiments according to the first latin square (group 1) and 15 according to the second (group 2), both randomised over 3 strips as rows.

Statistical analysis

The mean -log EC_{50} values for each agonist were compared between the two groups and within-patients by Student's t-test (two tailed), since -log EC_{50} values could be considered as normally distributed. The average T_{max} values were compared by distribution-free tests because of apparent skewness of the distribution; paired Wilcoxons signed rank test (two tailed) for comparison of within-patients responses and Mann-Whitney U-test (two tailed) for comparison of responses for each agonist between the 2 groups.

We estimated the various components of the total variation of the 270 CCRC through analysis of variance according to the statistical model shown in Table 1[14]. This model determined the testing procedure for evaluation of the statistical significance of the mean squares by means of the F-test. Assuming that patient

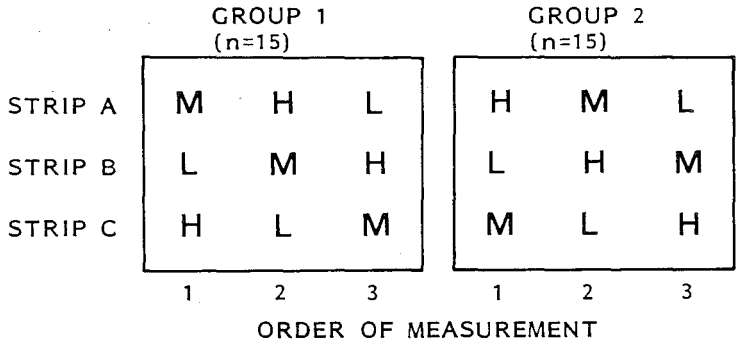

Figure 1. Design of the study. Three bronchioles (strip A, B and C) from each patient were stimulated with methacholine (M), histamine (H) and leukotriene C_4 (L). The sequence of agonists was determined by randomised latin squares. All latin squares could be reduced to one of the two configurations shown by changing the rows, because strip selection can be considered as random.

Table 1. Statistical model of the various sources of variation

Sources of variation	Degrees of freedom
1 Between patients	14
2 Between strips/within patients	30
3 Order of measurement	2
4 Between agonists	2
5 Measurement order x patients	28
6 Agonists x patients	28
7 Residual variation	30
8 Total variation	134

Sources of variation when 3 agonists are tested on 3 preparations according to a 3x3 latin square within each patient, for n = 15 patients (1 group). The factors 1 and 2 were considered as random, 3 and 4 as fixed.

and strip selection were random factors, and measurement order and agonists were fixed factors, the F-ratios (of two mean squares) were computed as follows: item 1 was compared to 2, items 2, 5 and 6 to 7, item 3 to 5 and item 4 to 6. Components of variance were calculated for between-patients and between-strips/within-patients variability as well as for residual variation. These variance components were transformed to relative standard deviations (coëfficients of variation) as a percentage of the overall means.

Results

The mean CCRC's for methacholine, histamine and LTC_4 are shown in Figure 2A-C; the corresponding -log EC_{50} values are given in Table 2. LTC_4 was 100 times more potent than both methacholine and histamine. Methacholine was slightly more potent than histamine. The -log EC_{50} values were 6.18 ± 0.06 and 5.88 ± 0.04 respectively, $P < 0.001$ (mean $\pm$ S.E.M., $n = 30$). Figure 2A-C shows that the order of measurement had no influence on -log EC_{50} and that the responses in both groups were almost identical. It can be seen from Figure 2C that the mean LTC_4-CCRC did not reach their plateau within the 10^{-9} to 10^{-7} M concentration range. A plateau, defined as a less than 5% increase in tension after the final concentration step, was only reached in 33 out of the 90 bronchioles.

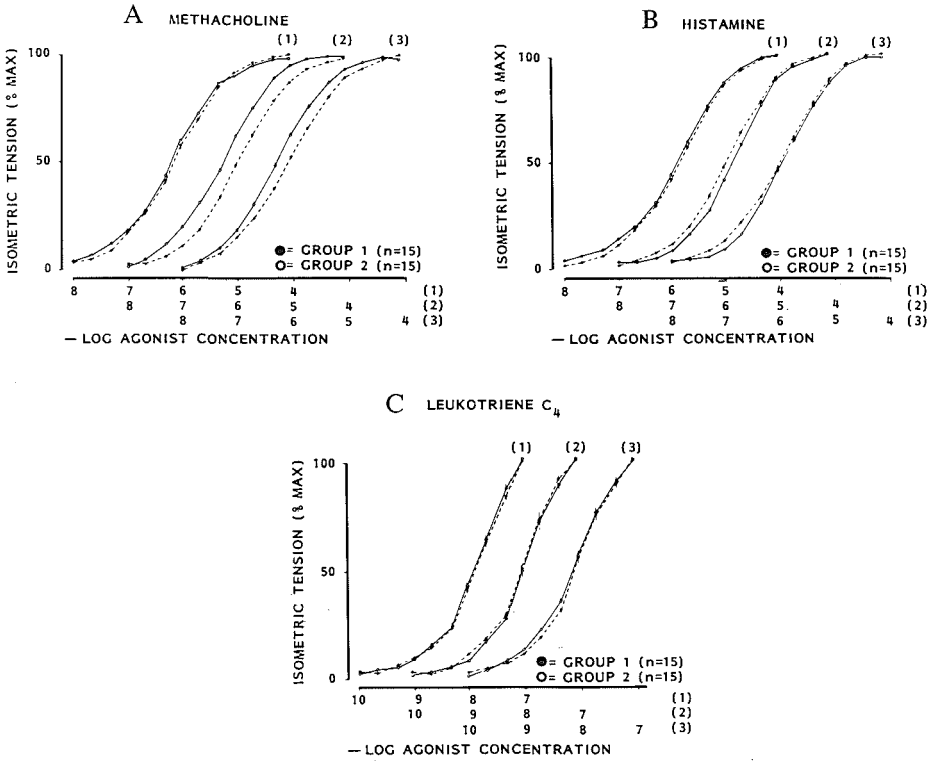

Figure 2. Mean concentration-response curves for methacholine, histamine and leukotriene C_4. Results are expressed as a percentage of maximal contraction in each curve versus the agonist concentration (-log M) and are the mean $\pm$ S.E.M. ($n = 15$). Responses in groups 1 and 2 are shown separately for each agonist, and are given for the curves from the three separate preparations as indicated by the numbers in brackets (= measurement order). Note the arrangement of the horizontal axis: three different calibrations correspond to the respective CCRC (indicated by the numbers in brackets). Filled circles, dashed lines = group 1; open circles, continuous lines = group 2.

Table 2. -log EC_{50} values for methacholine, histamine and LTC_4 in group 1 and 2

	-log EC_{50}					
	HISTAMINE		METHACHOLINE		LEUKOTRIENE C_4	
	group 1	group 2	group 1	group 2	group 1	group 2
Curve 1	5.86 ± 0.07	5.88 ± 0.08	6.09 ± 0.08	6.23 ± 0.12	7.89 ± 0.06	7.91 ± 0.06
Curve 2	5.92 ± 0.09	5.84 ± 0.06	6.05 ± 0.11	6.28 ± 0.10	8.03 ± 0.07	8.00 ± 0.07
Curve 3	5.92 ± 0.09	5.85 ± 0.08	6.10 ± 0.10	6.31 ± 0.09	8.09 ± 0.05	8.11 ± 0.06

Results are expressed as mean ± S.E.M., n = 15 in each group. No significant differences were found between curves 1, 2, and 3 and between corresponding measurements in the two groups for each agonist.

Table 3. Analysis of variance

Item no.[a]	Sources of variation	F-ratios[b]			
		-log EC_{50} (group 1)	-log EC_{50} (group 2)	T_{max} (group 1)	T_{max} (group 2)
1	Between patients	2.71*	5.54***	4.92**	1.84
2	Between strips/within patients	3.17**	4.54***	5.70***	14.32***
3	Order of measurement	1.54	2.70	17.82	31.02*
4	Between-agonists	482.04***	472.05***	3.23	12.22
5	Measurement order x patients	1.54	1.83	2.59**	1.48
6	Agonists x patients	3.74***	7.22***	1.77	1.14
		Coefficients of variation[c]			
1	Between-patients	8%	10%	105%	124%
2	Between strips/within patients	5%	4%	47%	91%
7	Residual variation	3%	2%	20%	20%

[a] Items are numbered according to the statistical model shown in Table 1.
[b] Results of analysis of variance, expressed as F-ratios, calculated seperately for -log EC_{50} and T_{max} in both groups. The F-ratios were computed as follows: item 1 was compared to 2, items 2, 5 and 6 to 7 (residual variation), item 3 to 5 and item 4 to 6.
[c] Coëfficients of variation are expressed as a percentage of the overall means.
Significances: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

These 33 bronchioles were distributed equally in both groups. In a small number of preparations we extended the LTC_4-CCRC to 10^{-6}M, which always resulted in a plateau. However, the limited amount of LTC_4 that was available made it impossible to complete all LTC_4 curves. The submaximal values were nevertheless included in the statistical calculations for reasons of completeness.

The results of the analysis of variance, expressed as F-ratios, are shown in Table 3. The -log EC_{50} measurements for the three agonists showed a significant between-patients variability, which was greater than the between-strips/within-

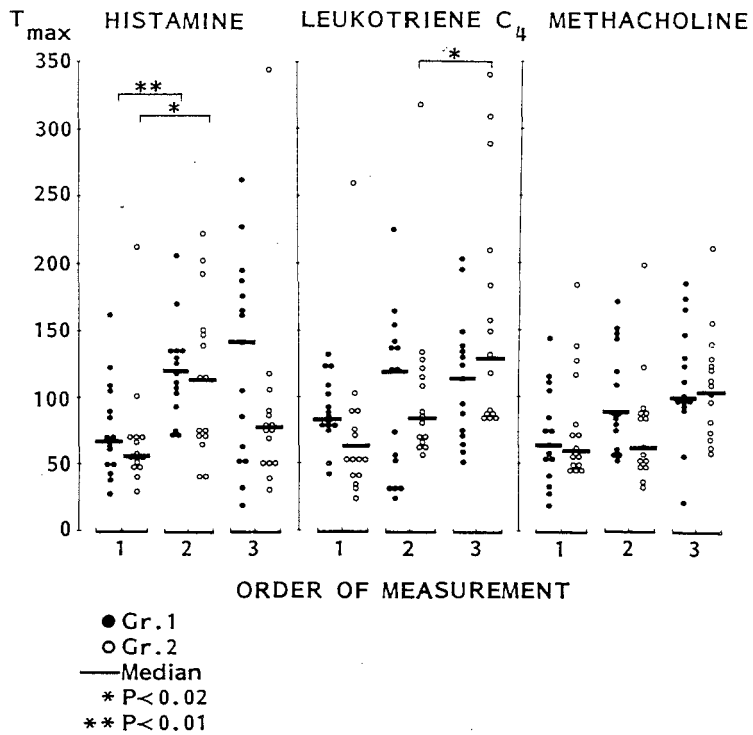

Figure 3. Individual values for maximum contractile force (T_{max}) for histamine, leukotriene C_4 and methacholine in groups 1 and 2, according to measurement order (numbered 1, 2 and 3 in the graphs). Median values are indicated by the horizontal bars. Filled circles = group 1, closed circles = group 2.
Horizontal bar: median. * $P < 0.02$; * * $P < 0.01$

patients variability. The latter also contributed significantly to the total variation. The small coefficients of variation indicate a high reproducibility of -log EC_{50} in both groups. The T_{max} showed a significant between-patients variation compared to between-strips/within-patients variation only in group 1. No significant between-patients variation in T_{max} was found in group 2 due to a relatively large between-strips/within-patients variation in some patients in this group. Coefficients of variation were large for T_{max}, with a considerable residual variation. The mean values of T_{max} are not useful because of a non-normal distribution. The individual T_{max} values are shown in Figure 3, and the median values are indicated. Despite the wide range, there were significant differences between the first and second histamine responses in both groups, between second and third methacholine responses in group 2 and between first and second LTC_4 responses in group 1. No significant differences were found between corresponding values for group 1 and 2, or in the maximal forces in the first curves, i.e. the maximum contractile response produced by methacholine, histamine and LTC_4

was similar. Contraction and relaxation time following washout of LTC_4 followed a time course which was comparable to that of the other two agonists.

Discussion

Our findings show that the sensitivity of human bronchioles *in vitro* to methacholine, histamine and LTC_4 can reproducibly and accurately be measured with minimal within-patients variation. The maximal contractile force however showed a much larger variability, and the within-patients variation was almost as large as the between-patients variation. The F-test may be somewhat unreliable due to the non-normal distribution of T_{max} values. However, this test is fairly robust against departures from normal distribution in balanced designs such as this one. In addition, we found a non-specific tendency for contractility to increase with time as appears from Figure 3. This may be the result of an increase in function of the contractile apparatus beyond the receptor level. Another possible explanation is that an inhibitory substance, for instance a prostaglandin (PG), is produced by the preparation initially, and that this production decreases with time. This speculation is supported by preliminary data from our laboratory, indicating that PGE_2 and 6-keto-PGF_1, the stable product of PGI, are produced by human bronchioles in the organ bath and depress smooth muscle tone. However, Brink et al[12] and Finney et al[15] have found no effect of indomethacin on methacholine and carbachol responses of human bronchi.

Finally, a fall in baseline tone could influence T_{max}, but this was apparently not the case as the baseline was stable in most preparations and was always adjusted to 500 mg before the start of each curve.

The opposite finding by Black et al of a decrease in carbachol contractility upon repetitive stimulation[16] might be related to differences in preparative or storage techniques: we stored the bronchioles in a continuous flow of cooled gassed buffer[8], while in the study by Black et al the tissue was left overnight in a sealed storage vessel with previously gassed buffer at 4°C, which might influence the viability of the preparation, e.g. due to the action of proteolytic enzymes.

Our observations on the magnitude and time course of LTC_4 responses are not in agreement with previous observations by Ghelani et al and Hanna et al, who concluded that LTC_4 is only a partial agonist, the mechanism of action of which was not closely linked to the contractile apparatus, because of a delayed onset of action and slowly developing response[17,18]. This discrepancy may have resulted from the use of different airway preparations (bronchioli versus bronchi) and different sources of the agonist (synthetic LTC_4 versus rat or guinea-pig LTC_4).

The inclusion of submaximal LTC_4-responses in the statistical calculations has introduced a bias in variance and LTC_4-mean values. However, because a plateau was invariably reached in a number of preparations when the LTC_4-CCRC was extended to 10^{-6} M, and because the first LTC_4-CCRC of both groups

were similar (Figure 2C), it can be speculated that this bias has not essentially influenced the interpretation of our results.

The statistical evaluation indicated that -log EC_{50} values, measured consecutively on one preparation, are equivalent to single measurements, because no influence of the order of measurement could be demonstrated and identical mean -log EC_{50} values were found for first, second and third curves in both groups for all three agonists (Table 3). This increases the amount of reliable data three-fold, which is of advantage when studying small numbers of preparations. The same is not true for T_{max}. Apart from an apparent non-specific increase in contractility with time, the outcomes of the analysis of variance point to the presence of interactions between the agonists. Although this type of study can only demonstrate the presence of interactions without precisely defining them, it seems that LTC_4 T_{max} is increased after histamine pretreatment (Figure 3, right panel). Despite a wide range, this increase was significant in group 1 and nearly so in group 2, suggesting that histamine induces an increase in airway smooth muscle responsiveness to leukotrienes.

In conclusion we found that methacholine, histamine and LTC_4 cause dose-dependent contraction of human bronchioles. LTC_4 was 100 times more potent than methacholine and histamine; the three agents induced maximum contractions of similar magnitude. When these substances were applied in succession to a single preparation, no interactions occurred regarding the -log EC_{50}, and this finding can be used to increase the information that can be obtained from a limited number of airway preparations. T_{max} showed a nonspecific increase during the day and, in addition, interactions occurred when agonists were added in succession. Therefore only single T_{max} measurements should be taken into account. These appeared to be of limited value in human bronchioles due to the large within-patients variation. We recommend that -log EC_{50} should be considered mainly as a measure of human small airway function *in vitro* when small numbers of airway preparations are studied.

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Chapter 8

Comparison of isometric and isotonic responses of human small airway smooth muscle *in vitro*

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Summary

The difference between isometric and isotonic responses of human small airway smooth muscle to a number of pharmacological agonists was studied. The isotonically measured sensitivity to methacholine was 1.4 times less than the isometrically measured value ($p < 0.05$) and similar small discrepancies were found for histamine, leukotriene C_4, prostaglandin $F_{2\alpha}$, isoproterenol and theophylline. Maximal isometric force and isotonic shortening after methacholine were linearly related ($p < 0.01$). The between-methods difference is relatively small. Because the difference was of similar magnitude and in the same direction in all tissues studied, it is of little practical importance for conventional pharmacological experiments.

Key words: airway smooth muscle, human bronchiolar strip, isotonic, isometric.

De Jongste JC, Mons H, Van Strik R, Bonta IL, Kerrebijn KF. Comparison of isometric and isotonic responses of human small airway smooth muscle *in vitro*. J. Pharmacol Methods 1987;17:165-171. Reprinted with permission of Elsevier Science Publishing Company.

Introduction

It has been common practice to use isometric force transducers to perform *in vitro* pharmacological experiments on airway smooth muscle. However, airway smooth muscle *in vivo* probably contracts semi-isotonically, causing airway narrowing. This seems especially true for small airways (bronchioles) as their walls lack cartilage, which could hinder isotonic functioning.

There are numerous reports that compare isometric and isotonic responses of various types of striated and smooth muscle, and differences in contraction kinetics have been shown. However, in pharmacological studies measurements are usually performed when a contraction has reached its plateau, and it has not been determined to what extent such concentration-response curves produced isometrically and isotonically differ. The present study was undertaken to quantitate the possible difference between isometric and isotonic measurements of human small airway smooth muscle responses to pharmacological stimulation.

Methods

Human lung tissue was obtained at surgery for lung cancer. Macroscopically normal tissue specimens were collected immediately after surgical resection and submerged 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) aerated with 95% O_2 and 5% CO_2. After rapid transport to the laboratory, bronchiolar strips were prepared and, after storage overnight, cumulative concentration-response curves (CCRC) were made according to a technique described previously[1]. From each of 10 patients, 6 bronchiolar strips were studied simultaneously: 3 isometrically using Grass FTO3D force transducers and 3 isotonically using Harvard model 386 heart-smooth muscle transducers. Isometric CCRC always started from 500 mg initial tension, and isotonic measurements were performed employing a 500 mg load because preliminary findings indicated that this load produced optimal contractions. From each CCRC the following parameters were derived: -log EC_{20} and -log EC_{50}: the concentration of agonist that produced 20% and 50% of the maximal effect; T_{max} for isometric measurements: the maximal contractile force expressed as mg isometric force per mg of dry weight of the bronchiole (determined after 48 h of drying at room temperature) and S_{max} for isotonic curves: the maximal shortening expressed in μm (because all strips were approximately 20 mm long correction for length was not carried out). All strips were primed by adding methacholine (10^{-8} to 10^{-4} M) in 10-fold concentration steps followed by washout, because this ensured a stable function for the rest of the day. After return to baseline, a second methacholine CCRC was generated in all strips. Finally, a third CCRC was made using histamine (10^{-8} to 10^{-4} M), Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (10^{-8} to 10^{-5} M), leukotriene C_4 (LTC_4) (10^{-10} to 10^{-7} M), isoproterenol (10^{-9} to 10^{-5} M) or theophylline (10^{-5} to $2x10^{-3}$ M) on one isotonically and one isometrically functioning strip. For each of the latter 5 agonists, 5 paired observations were obtained; each of these drugs was applied to only one pair of strips from a given tissue sample. Relaxing agents were applied after raising baseline tone with the methacholine EC_{50} of that preparation. After relaxation with either theophylline ($2x10^{-3}$ M) or isoproterenol (10^{-5} M), 'zero tone' or 'maximal length' was determined in Ca-free buffer containing EDTA ($4x10^{-3}$ M) and isoproterenol (10^{-5} M). The maximal response of both isometric and isotonic measurements

for relaxing agonists was expressed as a percentage of the difference between methacholine EC_{50}-induced contraction and maximal relaxation.

Drugs

Methacholine hydrobromide, histamine dihydrochloride and d,l-isoproterenol were obtained from Janssen (Belgium); $PGF_{2\alpha}$ and EDTA from Sigma; theophylline monohydrate from Brocacef; and synthetic LTC_4 was a gift from Dr. J. Rokach, Merck Frosst Laboratories (Canada).

Statistical methods

For all agonists, the values of -log EC_{20} and -log EC_{50} for isotonic and isometric measurements were compared by paired t-tests (two tailed). The linear correlation of S_{max} and T_{max} was determined for each agonist separately. Methacholine triplicate measurements were analysed by three-way analysis of variance. For the other agonists, pairs of measurements were analyzed by two-way analysis of variance. The level of significance was $\alpha = 0.05$.

Results

All airway smooth muscle preparations responded in a dose-dependent fashion to all agonists. Mean values for -log EC_{50} and -log EC_{20} are given in Table 1. The mean methacholine CCRC resulting from isometric and isotonic measurements are shown in Figure 1. A small but significant difference was present

Table 1. Sensitivity of human bronchiolar strips measured isotonically and isometrically

Agonist[a]	method	–log EC_{50}	–log EC_{20}
Methacholine	isotonic	5.97 ± 0.06*	6.64 ± 0.07
	isometric	6.12 ± 0.08*	6.76 ± 0.06
LTC_4	isotonic	7.92 ± 0.12	8.62 ± 0.29
	isometric	8.08 ± 0.10	8.70 ± 0.17
$PGF_{2\alpha}$	isotonic	5.46 ± 0.09	5.87 ± 0.18
	isometric	5.66 ± 0.12	6.12 ± 0.13
Histamine	isotonic	5.37 ± 0.12	6.03 ± 0.23
	isometric	5.56 ± 0.13	6.02 ± 0.15
d,l Isoproterenol	isotonic	7.00 ± 0.24	7.56 ± 0.20
	isometric	7.50 ± 0.33	7.94 ± 0.29
Theophylline	isotonic	3.55 ± 0.12	3.95 ± 0.13
	isometric	3.69 ± 0.12	4.35 ± 0.20

[a] n = 10 paired triplicate measurements for methacholine; for the other agonists, n = 5 paired measurements.

* $p < 0.05$ for comparison between methods.

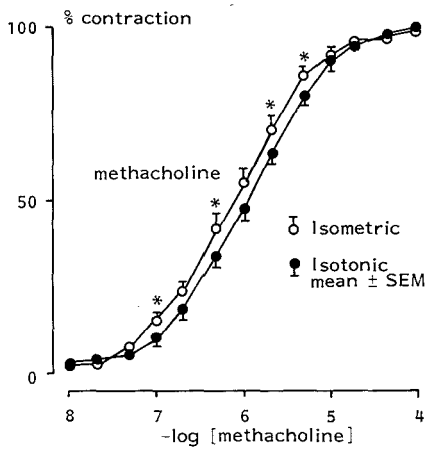

Figure 1. Mean methacholine concentration-response curves of 60 bronchiolar strips from 10 patients. Triplicate measurements were performed isometrically (open circles) and isotonically (closed circles). The vertical axis depicts the percentage of maximal response for each strip. Mean -log EC_{50} values were significantly different ($p < 0.05$). *: $p < 0.05$.

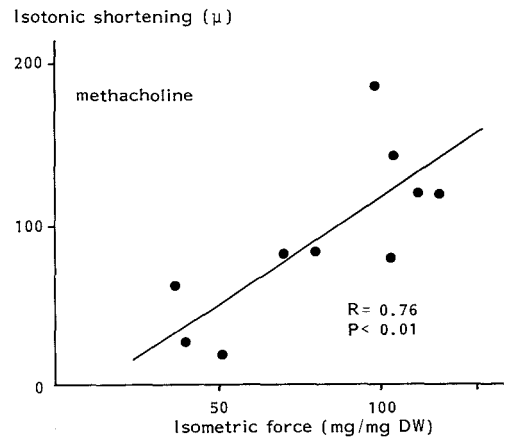

Figure 2. Relation between methacholine (10^{-4} M)-induced maximal isometric force (T_{max}) expressed as mg of force per mg of dry weight (DW) of the bronchiole, and maximal isotonic shortening (S_{max}), expressed as μm of absolute shortening, measured in 60 bronchiolar strips from 10 patients (each dot represents the mean of 3 isometric and 3 isotonic responses within a patient).

in -log EC_{50} of the two curves ($p < 0.05$) corresponding with a potency difference of approximately 1.4. Analysis of variance showed that this difference was in the same direction and of similar magnitude in all tissues studied, and that

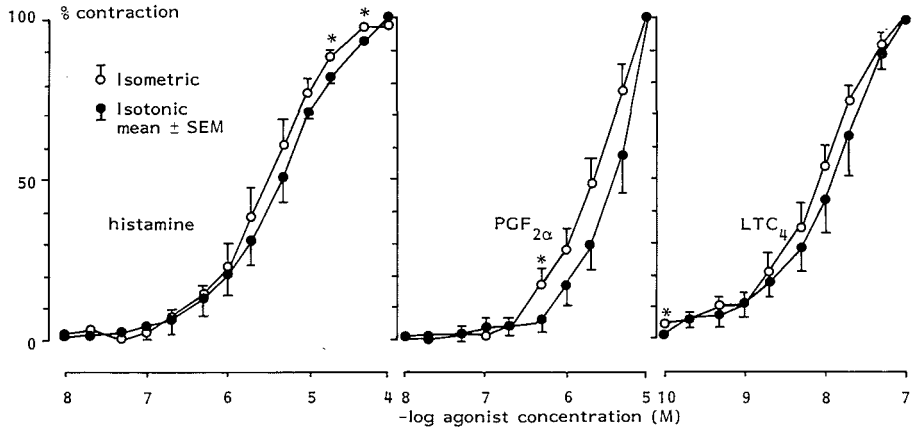

Figure 3. Mean isometric (open circles) and isotonic (closed circles) concentration-response curves for histamine, $PGF_{2\alpha}$ and LTC_4 (n = five pairs of bronchiolar strips from five different patients for each agonist). No significant difference was found between isometric and isotonic -log EC_{50} (* : $p<0.05$).

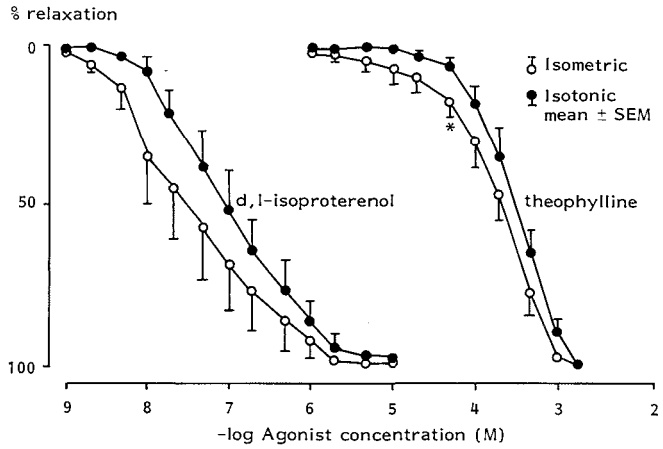

Figure 4. Mean isometric (open circles) and isotonic (closed circles) concentration-response curves for d,l-isoproterenol and theophylline (n = five pairs of bronchiolar strips from five different patients for each agonist). There was no significant difference between isometric and isotonic -log EC_{50} (* : $p<0.05$).

no significant within-patients/within-methods variation existed. For -log EC_{20} methacholine the difference between methods did not reach significance. A significant ($p<0.01$) relation was found between mean T_{max} and S_{max} values of methacholine responses (Figure 2). In Figure 3, the mean CCRC for LTC_4, $PGF_{2\alpha}$ and histamine are shown. Although no significant differences in -log

EC_{50} and -log EC_{20} were found by paired t-test, and two-way analysis of variance indicated no significant contribution of the between-methods variation for each agonist separately, the mean isotonic curves always tended to be shifted towards higher agonist concentrations compared to the isometric curves. The statistical significance of this consistent tendency could not be assessed because several pairs of strips from the same tissue samples were used to examine the effects of the various agonists (see: Methods). Similar results were obtained with the relaxing agents isoproterenol and theophylline (Figure 4). Maximal relaxation (percentage reversal of methacholine-induced tone) in the isometric and isotonic mode were $98{,}8 \pm 4{,}9\%$ and $86{,}0 \pm 6{,}6\%$, respectively, for isoproterenol and $81{,}4 \pm 3{,}7\%$ and $77{,}4 \pm 3{,}8\%$, respectively, for theophylline (mean $\pm$ SEM, $p > 0.05$).

Discussion

The present results indicate that a small, but significant, difference exists between isometric and isotonic measurements of pharmacological responses of human small airway smooth muscle *in vitro*. The sensitivity to methacholine, measured isometrically, was increased by a factor 1.4 compared to the isotonically determined sensitivity. A similar trend was observed for the responses to histamine, LTC_4 and $PGF_{2\alpha}$ as well as for the relaxant agents isoproterenol and theophylline, although no level of significance was reached, probably due to the limited number of observations.

Differences in isometric and isotonic responses have been examined in detail in various types of striated and smooth muscle among which tracheal smooth muscle[2–5]. These studies were mainly concerned with length-tension relationships and contraction kinetics, and have shown that isometric responses develop more rapidly and usually occur at a higher stimulus level than isotonic responses. It has been suggested that isotonic responses represent incomplete activation of the contractile apparatus due to the concurrent shortening and thickening of the muscle, which might hinder diffusion of the agonist and cause changes in the arrangement of the contractile elements ('folding'), which can also impair optimal functioning[4,5]. However, it has not been determined how important these physiological considerations are for the outcome of conventional pharmacological experiments. This is especially relevant for the type of study that links *in vitro* data to clinical data, such as bronchial responsiveness to inhaled methacholine[6,7]. Several studies have failed to demonstrate a relation between isometric *in vitro* responses and *in vivo* airway responsiveness and it is possible that such a relation would have been found had *in vitro* responses been measured isotonically instead of isometrically. The present findings suggest that this probably is not the case, because the difference between both methods is small and consistent between different lung tissue specimens. The magnitude of the between-methods discrepancy may become larger when initial tension or length is increased[3,5]. However, pharmalogical experiments are commonly performed starting from 'optimal'

tension or length as we have done. Our results are unexpected with regard to the relative positions of the isometric and isotonic curves. Michelson and Shelkovnikov showed that isotonic curves were shifted to a varying degree towards lower agonist concentrations compared to isometric curves[4], whereas we found the reverse. This means that in human small airway smooth muscle some tension develops before shortening occurs, which suggests that intrinsic mechanical resistance has to be overcome.

In conclusion, isometric and isotonic pharmacological responses of human small airway smooth muscle show significant but small differences in sensitivity to methacholine and probably also to a number of other agonists. Maximal force and shortening are linearly related. The observed discrepancies seem of little practical importance for conventional pharmacological experiments.

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Chapter 9

Non-neural components in the reponse of fresh human airways to electric field stimulation *in vitro*

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Summary

Fresh human bronchi, obtained at thoracotomy and maintained at 37°C, were studied *in vitro* to investigate their response to electric field stimulation (EFS). We found complex responses that were not only composed of a rapid initial nerve-mediated cholinergic contraction and a non-adrenergic nerve-mediated relaxation, but, in 80% of preparations, also of a tonic contraction with a sustained time course. This sustained phase was not blocked by the nervous conductance blocker tetrodotoxin (TTX), and was therefore not neurally mediated. Controlled transient cooling to 4°C in the organ bath reduced this sustained phase selectively for several hours. The leukotriene-antagonist FPL-55712, dexamethasone, which inhibits phospholipase A_2, and the anti-asthma drug disodium cromoglycate, all reduced the sustained phase significantly. In 20% of strips, an additional TTX-resistant contraction was seen directly following the cholinergic phase. This contraction could be inhibited by indomethacin. A similar small peak sometimes appeared after selective blocking of either the cholinergic or the sustained phases. Experiments in which the epithelium was removed from the strips suggested that this indomethacin-sensitive response, but not the sustained phase, was dependent on the presence of epithelium. These results show that EFS of fresh human bronchi stimulated cholinergic and non-adrenergic inhibitory nerves, and gave rise to a partly epithelium-dependent synthesis of arachidonic acid metabolites, which caused contractile responses that interfered with the neurally mediated responses.

Key words: human airway smooth muscle, bronchial strip, electric field stimulation, cholinergic nerves, non-adrenergic inhibitory nerves, arachidonic acid metabolites, leukotrienes, prostaglandins.

De Jongste JC, Mons H, Bonta IL, Kerrebijn KF. Non-neural components in the response of fresh human airways to electric field stimulation *in vitro*. J Appl Physiol 1987, in press. Reprinted with permission of the American Physiological Society.

Introduction

It has been shown that human bronchus responds to electric field stimulation (EFS) *in vitro* with a fast, cholinergic nerve-mediated contraction followed by a slow relaxation that results from stimulation of non-adrenergic inhibitory (NAI) nerves[1-5]. Previous studies were performed on human lung tissue, obtained at thoracotomy or autopsy. It has been common practice to collect tissue specimens in cold buffer. It cannot be excluded that prolonged hypoxia (in the case of autopsy) or cooling change the responsiveness of the tissue to EFS *in vitro*. In order to elucidate this, we studied the responses to EFS of human bronchi which were collected with minimal hypoxic delay and were maintained at 30-37°C after surgical removal. We found that fresh human bronchus that had not been cooled exhibited not only the biphasic nerve-mediated contraction/relaxation responses, but also, in 80%, a large, sustained contraction phase. In 20% of all preparations studied, another smaller contractile phase was present with its peak immediately following the cholinergic response. We examined the frequency-response characteristics, the effect of controlled cooling and of epithelium removal on the response to EFS, and applied various blocking drugs in order to characterise the nature of these additional contractile phases.

Methods

Human lung tissue was obtained from 40 patients at thoracotomies performed because of carcinoma. Within a few minutes after surgical removal, a piece of macroscopically normal lung tissue was submerged in 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) gassed with carbogen (95% O_2, 5% CO_2) at 37°C. After rapid transfer to the laboratory, cartilaginous bronchi (approx. 5th to 7th generation) were dissected free from parenchyma and blood vessels and were cut helically at a 45° pitch to obtain bronchial strips measuring 20 x 2 mm, as we have described previously[6], while the tissue remained in oxygenated warm buffer. One to 6 bronchial strips were dissected from each specimen. Bronchial strips were suspended in 10 ml double-jacketed organ baths containing Krebs-Henseleit buffer, aerated with carbogen, at 37°C (pH: 7.35, P_{CO2}: 4.6 kPa, P_{O2}: 71.8 kPa). The time from surgical resection until mounting in the organ bath was less than 1 h. Contractile responses were measured isotonically using high-precision angular displacement transducers[7] and employing an isotonic load of 1 gram. Field stimulation took place via platinum plate electrodes measuring 40 x 5 mm which were glued to the organ bath wall on both sides parallel to the bronchial strip (electrode distance 15 mm).

Electrical stimuli were delivered by a custom made tissue stimulator which produced voltage-constant rectangular pulses of alternating polarity.

Bronchial strips were equilibrated for 1-2 h in the organ bath while the buffer was changed every 15 min. When a stable baseline was obtained, EFS was carried

out, employing 30 sec tetani of supramaximal voltage (50V) and a short pulse duration (0,3 ms) at a frequency of 30 Hz. These stimulus parameters caused optimal contractions and relaxations, which were inhibited by the nerve conductance blocker tetrodotoxin (TTX, 3 μg/ml) indicating that nerves were stimulated selectively (preliminary findings). After completion of the experiments, a number of strips was maximally relaxed in calcium-free buffer containing d,l-isoproterenol (10^{-4} M) and EDTA ($4x10^{-3}$ M). The difference between baseline and maximal relaxation was considered to reflect active baseline tone: the part of the intrinsic tone that is caused by spontaneous smooth muscle activity.

Frequency-response curves were made by applying 30 sec. pulse trains (50 V, 0.3 msec) with increasing frequencies (1 to 100 Hz), separated by 30 min intervals.

The effect of controlled transient cooling of the tissue was studied in two ways. First, bronchi were exposed in the organ bath to a temperature of 4°C for 30 min. and the responses to EFS before and 1 h after cooling were compared. Secondly, strips were stimulated at hourly intervals during two 8-hour periods before and after storage in aerated cold buffer overnight (12 h at 4°C). This was done to evaluate the duration of the effect of cold, and furthermore to examine possible tachyphylaxis of the response to EFS. Cooling was accomplished by changing the thermostat setting of the circulation pump that perfused the organ baths' double jackets. Changing the temperature of the organ bath content took 20 min.

The effect of pharmacological blockers was tested by adding blocking drugs to the organ bath content when strips had reached a stable baseline and had a reproducible response to EFS. Drug incubations lasted for 20 min, except in the case of dexamethasone (2 h) because of the mode of action of this drug, which entails protein synthesis[8]. Control experiments were done in which EFS was repeated under identical conditions, but without a blocker being present. Only one blocking drug was tested on a given strip.

To estimate a possible role of the airway epithelium in the response of bronchial strips to EFS, we removed the epithelium from the strips by gently rubbing the mucosal surface with a small piece of cotton gauze. The effectiveness of this procedure was confirmed by histological examination. We compared the responses of paired strips with and without epithelium.

Data analysis

The relatively high intrinsic variability of the absolute contractile responses of human airways[6] makes normalisation imperative. Therefore, we expressed all responses of a given strip as a percentage of its cholinergic contraction to maximal EFS. All data are presented as mean values $\pm$ S.E.M. The significance of the differences between means was determined by Student's t-test for paired or unpaired data, as appropriate (two tailed, $\alpha = 0.05$).

Drugs

The following drugs were used: histamine dihydrochloride, methacholine hy-

drobromide and d.l-isoproterenol sulphate (Janssen), TTX and EDTA (Sigma), atropine sulphate (Brocacef), mepyramine maleate and methysergide maleate (Rhône-Poulenc), hexamethonium bromide (Fluka), timolol maleate (Merck, Sharp & Dhôme) disodium cromoglycate (DSCG, Fisons), indomethacin and dexamethasone (Duchefa) and the leukotriene antagonist FPL 55712 (a gift from Fisons).

All drug solutions were freshly prepared daily in 0,9% NaCl or, in the case of indomethacin and FPL 55712, in methanol. Dilutions were chosen so that drugs could be added to the organ baths in volumes of 5 to 50 μl. In preliminary experiments, equivalent amounts of methanol had no effect on baseline tone or airway smooth muscle function.

Results

Effects of EFS on fresh airways maintained at 37°C

After EFS, 78 out of 98 bronchial strips from 40 patients (80%) exhibited responses that consisted of a rapid initial contraction followed by a sustained, tonic contraction (Figure 1a), which reached a maximum approximately 10 min after onset of EFS. The mean amplitude of this sustained phase was $56{,}2 \pm 5.0$ % of the initial response. In 35 preparations, both contractile phases were separated by a relaxation below baseline (Figure 1b). In 9 of the 78 strips with a sustained contractile phase, another smaller contraction could be seen that had its peak approximately 1 min. after onset of EFS (Figure 1c). This small peak was also present in 11 strips without a sustained phase (Figure 1d). The mean amplitude of this contractile peak was 62.1 ± 9.6 % of the cholinergic response. The remaining 9 strips showed either only the initial rapid contraction ($n = 3$) or a biphasic contraction-relaxation response ($n = 6$) without a sustained contraction (Figure 1e). Following elevation of baseline tone by histamine 10^{-6}M a biphasic contraction-relaxation response to EFS was seen in the 3 preparations that initially showed no relaxation.

These findings contrasted with our previous (unpublished) observations in 39 bronchi from 32 subjects, that had been collected at surgery in ice-cold buffer. All these strips responded to EFS with biphasic contraction-relaxation responses, but a sustained contraction was found in only 5 (13%).

Thus, it appeared that the initial response to EFS of fresh human airways, that had not been cooled previously, was composed of a rapid initial contractile phase (100%), a second, smaller contraction (20%), a relaxation phase (45%) and a tonic sustained contraction (80%), numbered according to peak latency after EFS (Figure 2). Phases 1 and 3 corresponded to the nerve-mediated cholinergic and NAI response described previously[1–5,9]. Nerve mediation was apparent from the inhibition of both phases by the nervous conductance blocker TTX (3×10^{-6} g/ml). The non-adrenergic nature of the relaxation phase 3 was confirmed because it was not inhibited by the β-receptor blocker timolol 10^{-6}M. Atropine (1.2×10^{-6}M) blocked phase 1 completely (Figure 3)(Table 1). Neither TTX nor atropine affected the sustained phase 4 and phase 2 (Figure 3).

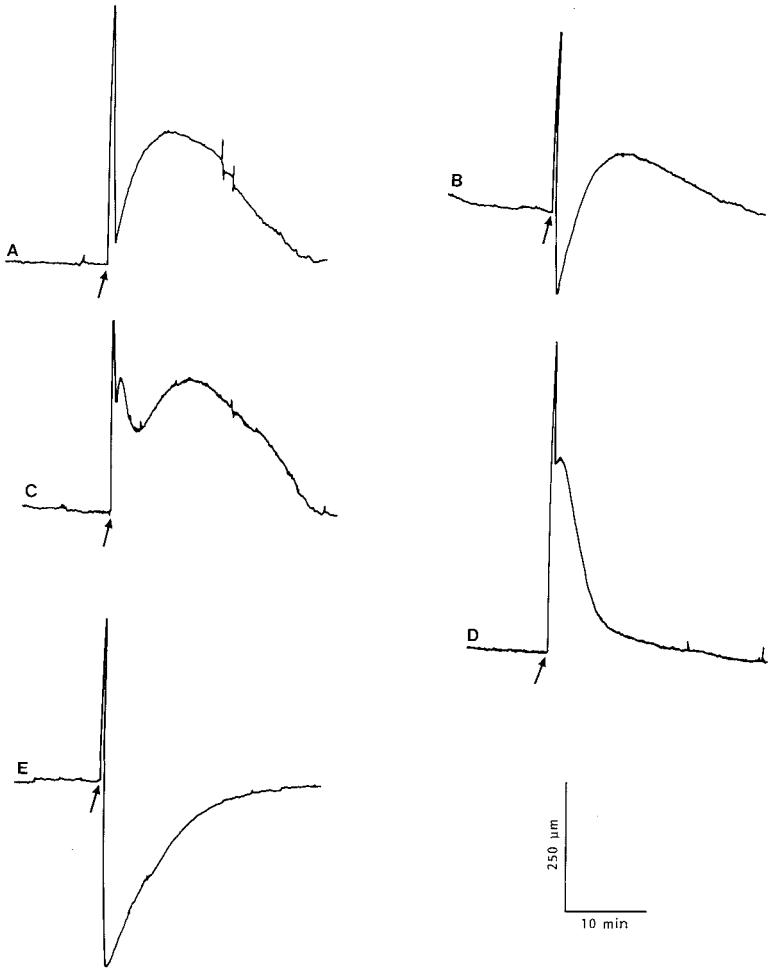

Figure 1 Representative response patterns of fresh human bronchi, maintained at 37°C after resection, to electric field stimulation *in vitro*. Arrows indicate the onset of a 30 sec pulse train (30 Hz, 0.3 msec, 50 V). A: initial rapid cholinergic contraction (phase 1) followed by a sustained contraction (phase 4). B: phases 1 and 4, separated by a deflection below the baseline, that is due to stimulation of non-adrenergic inhibitory nerves (phase 3). C: phases 1, 2 and 4. D: phase 1, only followed by phase 2. E: phase 1 and phase 3. Response pattern A and B were seen initially in 70%, C in 9%, D in 11% and E in 9% of all preparations (n = 98 strips from 40 patients).

We have attempted to relate the presence or amplitude of phase 2 and 4 responses to clinical variables such as chronic bronchitis or airflow limitation. No correlations were found, however.

We also analysed phase 4 responses as a % of maximal cholinergic contraction of 30 strips (3 strips from each of 10 tissue specimens) by means of analysis

Table 1. Effects of cooling and pharmacological blockers upon the 4 phases of the response of isolated fresh human bronchus to electric field stimulation

Drugs (concentration)	Number of patients	Phase 1	Phase 2	Phase 3	Phase 4
Cold (30 min, 4° C)	5	B 100 (5)	N.D.	-79.6 ± 19.6 (5)	64.1 ± 10.1 (5)
		A 130 ± 11.6		-55.9 ± 17.5	11.5 ± 5.7 ***
Atropine ($1.2x10^{-6}$ M)	5	B 100 (5)	N.D.	N.D.	41.9 ± 18.3 (5)
		A 0 ± 0***			47.5 ± 16.2
FPL-55712 ($11.5x10^{-6}$ M)	7	B 100 (7)	13.8 ± 13.8 (5)	-16.7 ± 7.5 (5)	37.7 ± 9.7 (7)
		A 105.7 ± 8.3	28.2 ± 11.8	-11.2 ± 5.1	3.3 ± 2.9***
Dexamethasone (10^{-4} M)	5	B 100 (5)	N.D.	N.D.	38.7 ± 8.5 (5)
		A 93.8 ± 9.0			6.5 ± 2.5*
Disodiumcromoglycate (10^{-4} M)	8	B 100 (8)	N.D.	- 9.4 ± 4.3 (5)	61.0 ± 12.6 (8)
		A 93.3 ± 4.3		-27.3 ± 5.7*	25.1 ± 6.1**
Indomethacin ($6x10^{-6}$ M)	10	B 100 (10)	68.2 ± 11.1 (6)	- 8.4 ± 2.9 (5)	32.0 ± 10.8 (9)
		A 80.5 ± 4.2***	10.2 ± 4.7***	-30.5 ± 8.9*	40.9 ± 10.0
Mepyramine ($2.8x10^{-6}$ M)	4	B 100 (4)	N.D.	N.D.	52.3 ± 12.6 (4)
		A 101.8 ± 12.1			60.4 ± 29.0
Methysergide ($2x10^{-6}$ M)	3	B 100 (3)	N.D.	N.D.	88.8 ± 22.9 (3)
		A 87.2 ± 6.7			79.5 ± 17.5
Hexamethonium ($1.4x10^{-5}$ M)	3	B 100 (3)	N.D.	N.D.	86.8 ± 22.0 (3)
		A 101.4 ± 1.4			81.3 ± 18.9
Control	23	B 100 (23)	57.1 ± 11.3 (5)	-21.5 ± 6.3 (10)	49.8 ± 6.4 (20)
		A 103.3 ± 2.3	48.6 ± 11.2	-13.6 ± 2.3	52.2 ± 6.6

Abbreviations: B, before; A, after; ND, not done.
All responses are expressed as a percentage of the cholinergic response to maximal EFS before incubation.
Means ± S.E.M. are given. Numbers between brackets refer to the number of strips in which that phase was present.
Statistical significances: *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ (paired Students' t-test, two tailed).

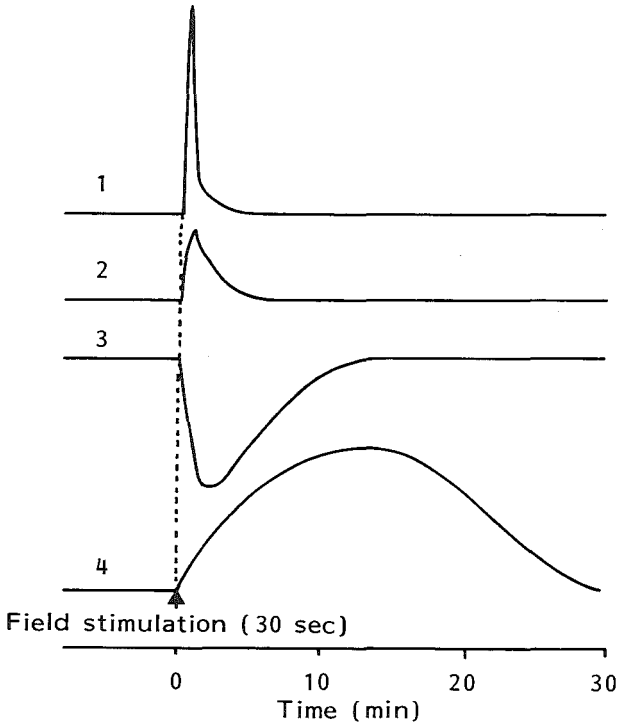

Figure 2. Schematic representation of time course, peak latency and amplitudes of the 4 phases that constitute the response of fresh human bronchus to electric field stimulation (EFS) *in vitro*. Phases are numbered according to peak latency after EFS; 1: cholinergic nerve-mediated, rapid contraction. 3: non-adrenergic inhibitory nerve-mediated relaxation. 2 and 4: non-neural contraction.

of variance and the F test. It appeared that the between-subject variation did not contribute significantly to the total variation in phase 4 responses ($p > 0.10$).

Characterisation of the sustained phase

Frequency- and voltage-response curves were performed on airways that had a phase 1 and phase 4 response (4 patients). Frequency-response curves showed that phase 1 was maximal at 20 to 50 Hz, and that phase 4 gradually reached a maximum at 50 to 100 Hz (Figure 4). Voltage-response experiments on 3 strips are shown in Figure 5. Phase 1 was already maximal at 30 V, whereas phase 4 still increased between 40 and 50 V in some strips (50 V was the maximal voltage that our tissue stimulator could deliver). These tracings illustrate that the sustained response can interfere with NAI relaxations, which leads to an apparent amplitude decrease of both phases 3 and 4 (Figure 5).

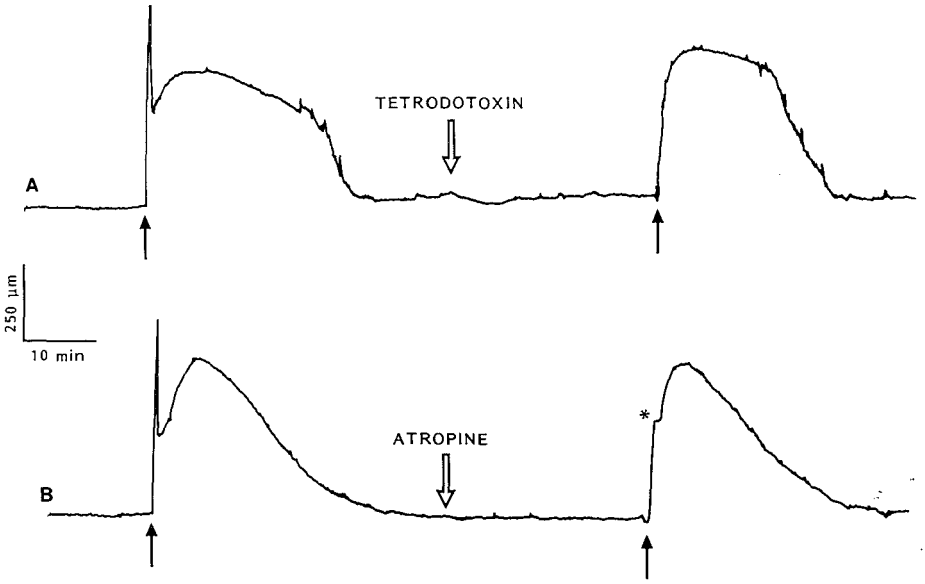

Figure 3. Effect of tetrodotoxin ($3x10^{-6}$ g/ml) and of atropine ($1.2x10^{-6}$ M) on the response of 2 fresh human bronchial strips from 2 different patients to electric field stimulation *in vitro*. Small arrows indicate the onset of a 30 sec pulse train (30 Hz, 0.3 msec, 50 V). Note the appearance of a phase 2 (indicated by the asterisk) after inhibition of phase 1 by atropine; phase 4 is not influenced by TTX or atropine.

Effect of transient cooling

Five strips (5 patients) were exposed in the organ bath to a temperature of 4°C for 30 min. After rewarming, EFS responses showed a significant reduction of phase 4 ($p < 0.005$) compared to pre-cooling values (Table 1). Furthermore, phases 1 and 3 tended to be increased and decreased, respectively, after cooling. This was probably the consequence of a reduction of spontaneous muscle tone during the cooling.

The responses to repeated EFS during an 8 h period before and after cooling overnight were studied on 9 strips (5 subjects), 6 of which had a phase 4 response. The mean results are depicted in Figure 6. On the day of surgery, phases 1 and 4 were relatively stable and showed no tachyphylaxis. On the second day, phase 1 was initially reduced to $82.3 \pm 8.8\%$ of the maximal cholinergic response on the previous day, but returned to the pre-cooling level within 3 h. Phase 4 was reduced much more from $61.5 \pm 20.9\%$ to $11.2 \pm 4.8\%$, and increased slowly to $46.3 \pm 6.1\%$ after 7 h, which was considerably below the maximum of the preceding day (Figure 6).

Effects of drugs

Because the slow time course and TTX resistance of phase 4 suggested that

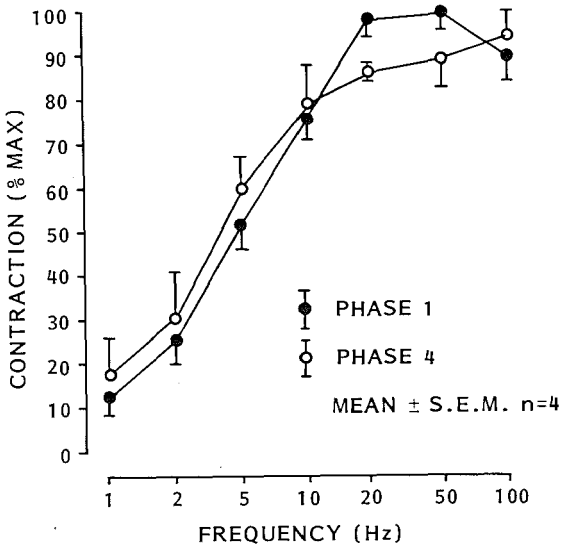

Figure 4. Frequency-response curves of phases 1 (closed circles) and 4 (open circles). Electric field stimulation (30 sec of 50 V, 0.3 msec pulses) was applied on 4 bronchial strips (4 patients) at intervals of 30 min. Responses are expressed as a % of the maximal amplitude of that phase in a given strip.

this phase was caused by endogenous release or synthesis of a contracting substance, we examined the effects of a number of pharmacological 'blockers'. The mean results of these experiments are given in Table 1. FPL-55712, a leukotriene antagonist, caused a selective pronounced reduction of phase 4 from $37.7 \pm 9.7\%$ to $3.3 \pm 2.9\%$ ($P < 0.001$), but had no significant effect on phases 1, 2 and 3 (7 strips from 7 patients). A small phase 2 response, that was not visible initially, appeared in 4 of the 10 strips after treatment with FPL-55712. In another strip that initially had a prominent phase 2 response, no change in phase 2 was observed after FPL-55712. Dexamethasone, that interferes with arachidonic acid metabolism by inhibiting phospholipase (PL)A_2, also caused a selective inhibition of phase 4 ($P < 0.05$) in 5 strips (5 patients) with phase 1 and 4 responses. The anti-asthma drug DSCG, that inhibits mediator release by an essentially unknown mechanism, reduced phase 4 moderately but significantly from $61.0 \pm 12.6\%$ to $25.1 \pm 6.1\%$ ($P < 0.005$, 8 strips from 8 patients). In these strips, that also had phase 3 relaxation responses, the decrease of phase 4 resulted in a more negative phase 3, as could be expected from the experiments shown in Figure 5. The cyclooxygenase inhibitor indomethacin, the antihistamine mepyramine, the serotonin antagonist methysergide and the ganglionic blocker hexamethonium had no effect on the sustained contraction (Table 1).

Because of its peak latency, the contractile phase 2 reminded to the so-called 'rebound contraction', which is characteristic for the response of ferret and guinea-

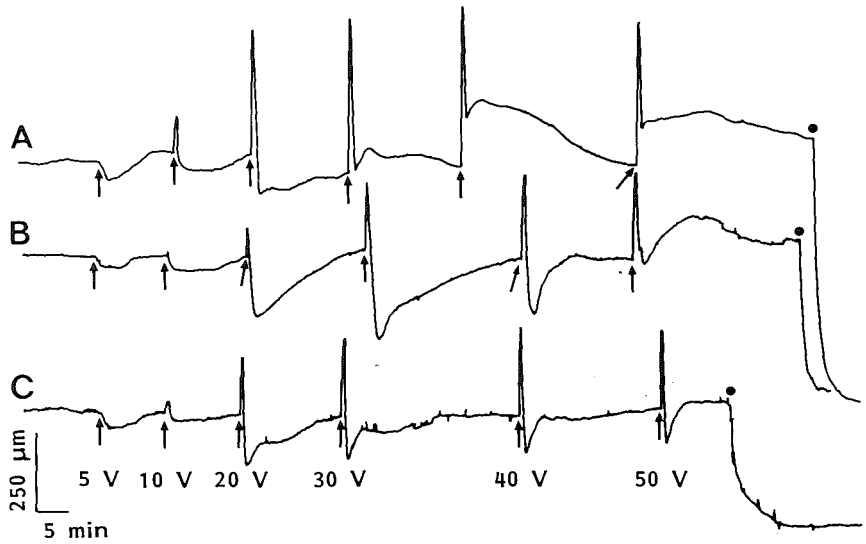

Figure 5. Repetitive field stimulation of three human bronchi with increasing voltages (5 to 50 V, 0,3 msec, 30 Hz for 30 sec). Arrows indicate the onset of field stimulation. At the lowest voltage the relaxation phase 3 appears first, followed by the cholinergic contraction phase 1 at 10 V. At 20 V, phase 4 is visible as a hump interfering with the relaxation phase (especially clear in panel A, third response) and with increasing voltage the sustained contraction phase 4 appears above the baseline (panels A and B). In some cases a phase 4 response which does not appear as a second contraction (panel C) seems to be present; it then only distorts the appearance of the relaxation phase 3. The phase 2 response is only visible in panel B (sixth response, small peak following phase 1). At the end of each experiment preparations were maximally relaxed (dots indicate addition of calcium-free buffer containing EDTA and isoproterenol) to estimate active spontaneous baseline tone (see: methods). It appears that the presence of a relaxation response in preparations with considerable spontaneous tone can be masked by the phase 4 sustained contraction (panel B).

pig intestinal tract to EFS[10–12]. This rebound contraction has been reported to be indomethacin sensitive. Therefore, we examined the effect of indomethacin on strips that had a prominent phase 2 response. Indomethacin reduced phase 2 significantly from $68.2 \pm 11.1\%$ to $10.2 \pm 4.7\%$ ($P < 0.005$, 6 strips from 6 subjects), and concomitantly caused a significant reduction of phase 1 and an increase of phase 3 ($P < 0.005$ and $P < 0.05$, respectively). This was most likely due to the partial superposition of the phases 1, 2 and 3, so that changes in phase 2 also caused changes in phases 1 and 3. Although no significant effect of indomethacin on phase 4 was found, we observed that indomethacin treatment of 2 strips that initally only had a phase 1 and 2 response, resulted in development of a phase 4 response (Figure 7).

Effect of epithelium removal

Because bronchial epithelium is a potential source of contracting substances[13],

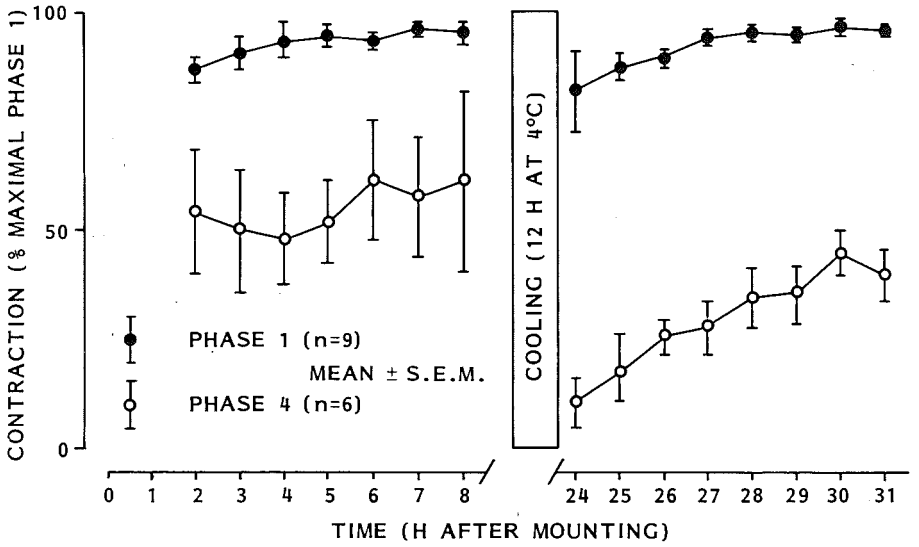

Figure 6. Effect of repeated electric field stimulation (30 sec trains of 50 V, 0.3 msec, 30 Hz pulses) on the amplitudes of the cholinergic phase 1 (closed circles) and the sustained phase 4 (open circles) during two periods of 8 h at 37°C, before and after storage overnight for 12 h at 4°C. Each point represents the mean ± S.E.M. of 9 (phase 1) or 6 (phase 4) separate experiments. Contractions are expressed as a percentage of the maximal cholinergic (phase 1) response of a given strip. It is shown that cooling had only a minor and transient effect on phase 1, but profoundly depressed the amplitude of phase 4, that did not return to pre-cooling values even after 8 h.

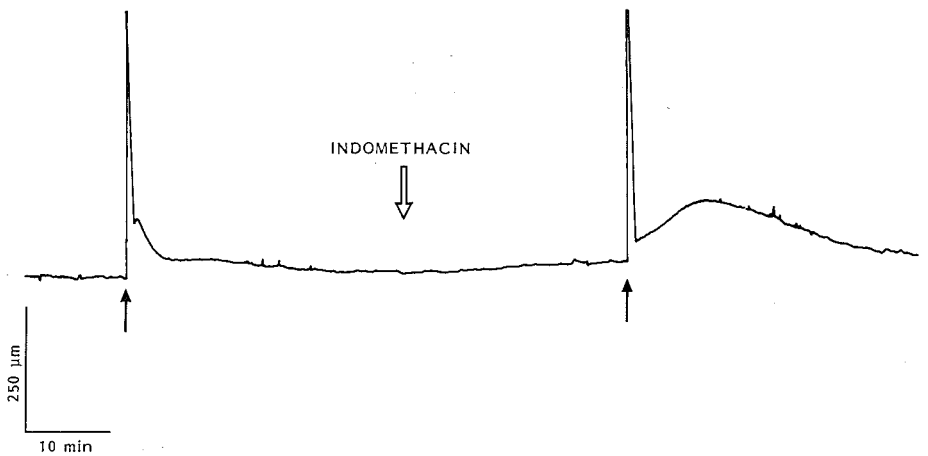

Figure 7. Tracing of one of two bronchial strips that initially showed a phase 1 and 2 response, but developed a sustained phase 4 after treatment with indomethacin. Arrows indicate onset of a 30 sec train of 0.3 msec, 50 V pulses at 30 Hz.

Table 2. Effect of removing the epithelium on the response of isolated human bronchus to electric field stimulation.

	After 2 h		After 4 h	
	epithelium present	epithelium removed	epithelium present	epithelium removed
Phase 1	100	100	115.0 ± 13.9	112.7 ± 8.8
Phase 2	27.4 ± 20.1*	8.6 ± 5.6*	41.3 ± 16.0*	4.4 ± 2.7*
Phase 3	6.7 ± 5.1	13.2 ± 8.1	2.4 ± 2.1	8.0 ± 3.7
Phase 4	35.3 ± 18.8	15.8 ± 8.6	33.0 ± 14.2	23.0 ± 9.9

EFS responses were elicited 2 and 4 h after mounting the bronchial strips in the organ baths. All values are expressed as a % of the initial cholinergic response to maximal EFS. Means ± S.E.M. of 7 paired experiments (7 patients) are given.
*$p < 0.05$ (paired Student's t-test, two tailed), comparison of paired strips with and without epithelium.

we examined the effect of epithelium removal on the EFS response in paired strips with and without epithelium (14 strips from 7 patients). Responses to EFS were elicited 2 and 4 h after mounting the strips in the organ bath. Of the 7 intact strips, 6 had a phase 4 and 3 a phase 2 response. Of the 7 denuded strips, 4 had a phase 4 and 3 a phase 2 response. These phase 2 responses had a significantly lower amplitude than in the intact strips (Table 2). Paired analysis showed no significant differences, both at 2 and 4 h after mounting, in the amplitudes of phase 4.

Discussion

In this study, we present evidence that EFS of isolated fresh human bronchi, that were maintained at 37°C, not only produced a nerve-mediated cholinergic contraction and a NAI relaxation response, but also gave contractile responses that were the result of endogenous release of contracting substances. Two distinct non-neural response components were defined, one with a sustained time course that was present in 80% and the other, with its peak 1 min after onset of EFS, in at least 20% of all airways that we have examined. We numbered these phases 1-4 according to their peak latency after EFS. The tonic phase 4 was blocked by FPL-55712, which acts as a leukotriene receptor antagonist in the concentration we have used[14,15]. Phase 2 was inhibited by the cyclo-oxygenase inhibitor indomethacin. This suggested that phase 2 and 4 resulted from synthesis of cyclo-oxygenase metabolites (prostaglandins or thromboxanes), and LT-like substances, respectively. This is further supported by the observation that indomethacin not only inhibited phase 2, but in some tissues also induced phase 4 responses (Figure 7), which suggested deviation of the substrate (arachidonic acid) from the cyclo-oxygenase to the lipoxygenase pathway.

Mobilisation of arachidonic acid from the cell membrane by activation of

PLA_2 is an early step in the synthesis of both prostaglandins and leukotrienes. Therefore, the significant inhibition of phase 4 by dexamethasone may have resulted from the PLA_2 inhibitory action of the corticosteroid. It seems likely that the effect of DSCG on the sustained phase 4 reflects the capacity of DSCG to prevent mediator release. This may be mediated via an effect on calcium influx[16,17].

We have shown that the sustained phase 4 was selectively attenuated by transient cooling, an effect that lasted for several hours (Figure 6). This explains why sustained responses have not been found more often, as in most previous studies lung tissue was allowed to cool prior to organ bath experiments. The mechanism by which cold depresses these responses is not clear; it may be speculated that cold depresses the membrane excitability or the mediator generating capacity of certain cells in the bronchial strips. However, *in vitro* experiments have shown that temperature-induced membrane changes follow a much faster time course than the changes we have observed[18].

The release of AA-metabolites by human and animal lung tissue has been documented extensively. It can be stimulated by addition of specific antigen, calcium ionophore, histamine, carbachol or by mechanical stimulation and also occurs spontaneously[19–25]. However, the release of AA metabolites by human bronchus after field stimulation has not been described before. It remains to be determined which cells produce these AA metabolites. The most likely candidates are the mast cell[26,27], which can release mediators after field stimulation *in vitro* (J. Bienenstock, personal communication) or the bronchial epithelium, which is an active site of AA metabolism[13]. Both cell types are present in the bronchial strip.

We examined the possible role of the bronchial epithelium in a series of experiments on paired strips with and without epithelium (Table 2). Phase 2 contractions were significantly reduced in strips without epithelium. This suggests that, after EFS, the epithelium synthetises the prostaglandin-like substance that leads to the contractile response phase 2. No difference was found, however, between the sustained phase 4 responses of intact and denuded strips, which indicates that the LT-like substance that is responsible for the sustained phase 4 contractions was not produced by the epithelium.

Our findings show a number of similarities to results of smooth muscle research in experimental animals. Sustained responses similar to our phase 4 after EFS have been reported by Ördög and coworkers in guinea pig trachea[28], without demonstration of the underlying mechanism. Lundberg et al[29] have reported tonic responses of guinea pig main- and hilar bronchus, but not of trachea, to EFS, that were blocked by a substance P antagonist. In previous unpublished experiments, we have never observed the sustained responses of guinea pig trachea to EFS described by Ördög. The sustained contraction of guinea-pig bronchi to EFS[29] is, however, easily reproducible but different from the human phase 4 reponses because it has a different time course, is not cold-sensitive and can not be blocked by FPL-55712 (unpublished observations).

In a recent paper, Nielsen-Kudsk et al[30] reported biphasic contractile responses of guinea-pig trachea to high potassium (K^+) concentrations, with a time course that closely mimicked the phase 4 response of the human bronchi. This tonic response was Ca^{++}-influx dependent and coincided with the production of inhibitory prostaglandins[30]. These findings may, however, be fundamentally different from what we found in the human tissue, because K^+ will depolarize airway smooth muscle cells, which leads to Ca^{++}-influx by opening of voltage-dependent Ca^{++} channels, whereas EFS pulses of 0.3 msec will certainly not depolarize the muscle cells because of their strong current rectifying properties and large electric time- and space constants[31]. The K^+ effect, therefore, may largely be a direct one, whereas EFS acts indirectly via mediator synthesis to produce a sustained response. Thus, although in experiments on animal airways sustained contractile responses have been found, these may not share a common mechanism with the human bronchial phase 4 response. In several airways that initially only had a phase 1 and phase 4 response, FPL-55712 not only blocked phase 4 but also revealed a phase 2, that may have been masked by a prominent phase 4 initially. This effect of FPL-55712 suggests that phase 2 responses may have occurred more often than in the 20% of all preparations in which they were seen initially. Like the 'rebound contraction' of ferret and guinea pig gastrointestinal smooth muscle, phase 2 was indomethacin sensitive[10]. It can be speculated, therefore, that comparable mechanisms underly the human phase 2 response and the 'rebound contraction' in tissues that have a common embryological origin. There are, however, arguments against this: the rebound contraction was considered to be initiated by cessation of EFS and to occur only during active NAI relaxation, whereas phase 2 had its onset at the beginning of EFS, and was not dependent on the presence of an active relaxation response.

The present finding of AA-metabolite synthesis by human bronchi after EFS *in vitro* stresses the importance of standardizing procedures and baseline conditions in the study of neural response patterns of human airways. If only the nerve-mediated responses are to be examined, inhibitors of AA metabolism seem necessary to avoid these interfering response phases 2 and 4, that may otherwise distort the neurally mediated responses to an unknown degree.

LT's are thought to be important in the pathogenesis of obstructive lung disease[32–34]. Experimental data suggest that LT's are at least partly responsible for the bronchoconstriction that follows allergen challenge in asthmatic patients[22]. The time course of our sustained response, which is probably the consequence of LT synthesis, is reminiscent of the course of the early phase of the *in vivo* bronchoconstriction after allergen challenge[35] and is also similar to the time course of the bronchoconstriction that follows exercise. Furthermore, the clinically effective anti-asthmatic drugs dexamethasone and DSCG blocked the phase 4 sustained response *in vitro*. Corticosteroids protect against late allergic bronchial responses and also attenuate the immediate bronchoconstriction that follows allergen challenge in asthmatics[36,37]. Both DSCG and, to a lesser degree, corticosteroids protect against exercise-induced bronchoconstriction[38]. It can

therefore be speculated that the presently described sustained *in vitro* responses share a common mechanism with the bronchoconstriction produced by allergen challenge or excercise *in vivo*. It may be worthwile to define the nature of these *in vitro* observations more precisely.

In conclusion, two mechanisms produce the response of fresh human bronchus which has not been cooled to field stimulation: selective stimulation of post-ganglionic cholinergic and non-adrenergic nerves in the bronchial wall, which produces a fast initial contraction (phase 1) followed by a slow relaxation (phase 3), and stimulation of mediator releasing cells to produce AA-metabolites which cause a second rapid (phase 2) and third sustained (phase 4) contraction. The latter process appeared selectively sensitive to transient cooling to 4°C, which abolished these responses for several hours.

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Part III

Human airway smooth muscle function in health and disease

Chapter 10

Increased *in vitro* histamine responses in human small airway smooth muscle from patients with chronic obstructive pulmonary disease

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Summary

We tested the hypothesis that abnormal responses of airway smooth muscle contribute to the pathogenesis of airway obstruction in chronic obstructive pulmonary disease (COPD). For this purpose, lung tissue from 10 patients with and 10 patients without COPD was obtained during thoracotomies. Lung function was measured preoperatively. The *in vitro* responses of isolated bronchioles were measured using histamine, leukotriene (LT) C_4 and methacholine as contracting agents, and the results of the *in vitro* measurements were compared between patients with and without COPD. Histamine efficacy (maximal isometric force, T_{max}) *in vitro* of bronchioles from patients with COPD was significantly greater than the histamine T_{max} of the bronchioles from patients without COPD ($P < 0.01$). This difference was probably not due to histamine tachyphylaxis or the production of relaxing prostaglandins by airways without COPD, as neither mechanism could be detected in separate experiments on airways without COPD. No differences were found between *in vitro* bronchiolar responses to LTC_4 and methacholine in patients with and patients without COPD. Increased histamine responses of small airways may be one of the determinants of airway obstruction in COPD.

Key words: human airway smooth muscle, histamine, methacholine, leukotriene C_4, chronic obstructive pulmonary disease, bronchial hyperresponsiveness.

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Introduction

Increased bronchial responsiveness to nonallergic stimuli is common in patients with chronic obstructive pulmonary disease (COPD)[1]. Although baseline airway caliber is considered as an important determinant of this increased responsiveness[2,3], additional mechanisms may be involved. A number of recent studies have compared *in vivo* bronchial responsiveness in COPD with *in vitro* responses of isolated human airway smooth muscle (ASM). These studies have failed to show a relation between *in vivo* and *in vitro* bronchial responsiveness[4–11]. *In vitro*, human ASM appeared to exhibit a rather constant sensitivity to histamine or methacholine, regardless of the degree of bronchial responsiveness *in vivo*[5–11]. However, a possible role of ASM in bronchial hyperresponsiveness in patients with COPD has not been excluded. Data on absolute responses (isometric force development or isotonic shortening) as a measure of efficacy are scanty and show no differences between responses of ASM from subjects with and without bronchial hyperresponsiveness[5,6]. This may, however, be due to the large variability of these absolute responses. An increased efficacy of ASM responses might well cause *in vivo* bronchial hyperresponsiveness because small increases in ASM shortening can lead to large increases in airways resistance[12]. Another flaw of previous studies is that airways of different generations have been studied together[5–11], which will have introduced an extra source of variability because of differences in contractile properties of central and peripheral ASM. The present study was undertaken to test the hypothesis that abnormal responses of ASM contribute to the pathogenesis of COPD. We used established *in vitro* techniques with known reproducibility[13,14] to measure the sensitivity and efficacy of human small airway ASM responses to contractile agonists (histamine, leukotriene (LT) C_4 and methacholine) and used only airways of similar caliber (tenth to thirteenth generation) in order to limit the variability that will result from taking central and peripheral airways together. *In vitro* ASM responses from airways obtained from patients who had no chronic respiratory disease were compared with responses of airways from patients with COPD.

Methods

Patients

Twenty patients who were scheduled for pneumonectomy or lobectomy were included in the study. Lung resection was indicated because of bronchial carcinoma. Preoperatively, all patients underwent lung function studies, including measurements of inspiratory vital capacity (VC) and forced expiratory volume in 1 sec (FEV_1). To assess reversibility of air-flow limitation, the effect of nebulised isoproterenol on baseline airway caliber was determined. According to the criteria proposed by the American Thoracic Society, patients were included in the group with COPD when there was a history of at least 3 yr of chronic or recurrent cough with expectoration (at least 3 months each year) together with air-flow

limitation, defined as a FEV_1/VC of less than 70%. Patients were classified as without COPD when there had been no respiratory symptoms (apart from the recent symptoms caused by malignancy) in the past and when lung function studies were within normal limits. The smoking habits and recently taken medication were recorded. Patients were regarded as non-smokers when they had ceased smoking at least 5 yr previously. The study protocol was approved by the ethical committee of the University Hospital.

In vitro measurements

Immediately after surgical resection, a piece of macroscopically normal lung tissue was submerged 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 25, glucose 5.55) previously gassed with carbogen (5% CO_2, 95% O_2). The hypoxic delay was kept to a minimum (usually well within 15 min). After rapid transportation to the laboratory, bronchioles (approximately tenth to thirteenth generation) were carefully dissected as described in detail before[13] and were cut helically at a 45-degree pitch to obtain thin bronchiolar strips measuring approximately 2 x 20 mm. The preparations were stored overnight in a slow, continuous flow of aerated, cooled (4°C) Krebs-Henseleit buffer containing penicillin ($3x10^{-5}$ g/L) and tobramycin ($5x10^{-3}$ g/L). This procedure should provide a thorough washout of narcotics (thiopentone, fentanyl, pancuronium, atropine, O_2/N_2O, halothane) and of substances possibly liberated during the dissection. We formerly demonstrated that our storage procedure did not influence the contractile function of the preparations[13].

The next morning, bronchiolar strips were mounted in 10 ml organ baths containing Krebs-Henseleit buffer of 37°C, aerated with carbogen (pH: 7.35; P_{CO2}: 4.6 kPa; P_{O2}: 71.8 kPa).

Isometric force was measured using force-displacement transducers (Model FTO3D; Grass Instruments, Quincy, MA), which were connected via a carrier wave amplifier (Peekel instruments, The Netherlands) to a Rikadenki pen recorder and a digital volt meter. After 2 h of equilibration with changing of the bath fluid every 15 min, a resting tension of 500 mg was applied; preliminary experiments had shown that this tension resulted in optimal isometric force development by bronchiolar strips. Cumulative concentration-response curves (CCRC) were made by adding agonists in small (less than 50 μl) volumes. Methacholine hydrobromide and histamine hydrochloride (Janssen pharmaceuticals, Belgium) were dissolved in saline. Synthetic LTC_4 (a gift of dr. J. Rokach, Merck Frosst Laboratories, Canada) was kept on stock in saline at -70°C and was diluted before the experiments. Drug solutions were freshly prepared daily and kept on ice for the duration of the experiments.

The CCRC were made using the following concentration ranges: histamine, 10^{-8} to 10^{-4} M; methacholine, 10^{-8} to 10^{-4} M; LTC_4, 10^{-10} to 10^{-7} M. Before the measurements were started, each strip was pretreated with methacholine (10^{-8} to 10^{-4} M in 10-fold concentration steps followed by washout). This was

done because we previously found that the first response of a given strip was always considerably lower than all subsequent responses, which then showed a good reproducibility over a period of at least 55 h[13]. After a maximal response was reached, drugs were washed out by changing the bath fluid 4 times at 2 min intervals, and thereafter every 15 min until return to baseline. Most preparations had a stable baseline tension throughout the day; incidentally, preparations required minor adjustments in order to restore a 500 mg resting tension before starting a new CCRC. Successive CCRC were separated by 2 h intervals.

From a CCRC, the following parameters were derived: EC_{50}, (the molar concentration of an agonist that causes 50% of its maximal effect on a given strip) as a measure of sensitivity, and T_{max} (the maximal isometric force expressed in milligrams of force per milligram of dry weight of the bronchiole), as a measure of efficacy. Dry weight was determined after completion of an experiment by weighing the bronchiole after 48 h drying in ambient air at room temperature. Three bronchioles from each patient were studied and the three different agonists were applied to each preparation in a sequence determined by randomised (3x3) latin squares. In this way, triple CCRC for each agonist were obtained in all patients. We previously showed that this procedure did not result in interactions between agonists with regard to the sensitivity of the tissue[14]. Therefore, the geometric mean of triplicate (logarithmically transformed) EC_{50} measurements was calculated for each agonist in each patient. Because we found that T_{max} was influenced by subsequent exposition of bronchiolar strips to the various agonists[14], only the first CCRC of each strip was used to calculate T_{max}.

In a separate set of experiments, we examined whether histamine tachyphylaxis and/or the production of endogenous prostaglandins modulate the bronchiolar response to histamine. Three sequential histamine CCRC were carried out with an interval of 1 h between curves on two bronchioles from each of 7 patients without COPD, indomethacin ($3x10^{-6}$ M), a prostaglandin synthesis inhibitor, being present in 1 of the 2 organ baths. Also, on a third strip from each of the 7 tissue specimens, two successive non-cumulative histamine curves (NCCRC) were made.

Statistical methods

All data of the two patient groups were compared using Student's t-test (two tailed) as within the groups the data were normally distributed. Successive histamine CCRC, histamine CCRC with and without indomethacin as well as histamine CCRC and NCCRC were compared using Student's paired t-tests (two tailed, $\alpha = 0.05$).

Results

Clinical and lung function data are given in Table 1. Twenty patients were divided in 2 groups according to the absence or presence of COPD. All patients with

Table 1. Patient characteristics

Subject no.	Age (yr)	Sex	VC (% pred)	FEV_1/VC (%)	ΔFEV_1[a] (%)	Current Cigarette smoking[b]	Medication[c]
Patients without COPD							
1	63	M	90	78	–	–	–
2	74	M	79	70	4	–	–
3	58	M	100	70	0	+	–
4	55	M	102	77	0	–	–
5	53	M	119	73	–	–	–
6	58	M	94	78	–	++	–
7	49	M	110	90	0	+	–
8	45	M	117	73	–	++	–
9	69	M	108	75	–	pipe	–
10	67	M	116	76	–	–	–
mean ± SEM	59 ± 3		104 ± 4	75 ± 2			
Patients with COPD							
11	69	M	100	53	10	+	ster
12	55	M	94	67	0	++	–
13	68	M	91	38	–	–	–
14	76	M	78	49	–	++	ipr
15	57	M	113	60	6	–	–
16	69	M	89	59	2	+	–
17	56	M	115	45	4	++	–
18	64	M	56	65	12	++	–
19	63	M	95	44	–	–	ster, ipr, sal
20	61	M	83	45	0	++	ster, ipr
mean ± SEM	64 ± 2		91 ± 5	53 ± 5			

Abbreviations: VC, vital capacity; FEV_1, forced expiratory volume in 1 sec;

[a] ΔFEV_1: % increase in baseline FEV_1 after inhalation of isoproterenol.

[b] Cigarette smoking: minus sign = no smoking for 5 yr of more; plus sign = current smoking 20 cigarettes per day or less; double plus sign = more than 25 cigarettes per day for more than 1 yr.

[c] Medication regularly used before the operation: ster = corticosteroids (prednisolone or dexamethasone); ipr = ipratropiumbromide; sal = salbutamol.

COPD had a history of recurrent or chronic cough with expectoration of (usually non-purulent) sputum and had had airflow limitation for more than 3 yr. None had a history suggestive of asthma. The mean age of the two groups was similar. Current cigarette smoking was more frequent in patients with COPD. Heavy smoking, defined as smoking more than 25 cigarettes per day for more than 1 yr, was reported by 2 subjects without and 5 with COPD. The lung function measurements indicated that the baseline VC was not significantly different between the 2 groups, although it tended to be lower in the COPD group. By definition, all patients with COPD had airflow limitation (FEV_1/VC ratio below 70%). In 7 patients with COPD, the effect of nebulised isoproterenol on baseline FEV_1 was determined. Most patients showed only minor improvement.

In vitro studies

Histamine, LTC_4 and methacholine caused concentration-dependent responses in all bronchioles. The mean CCRC for histamine, methacholine and LTC_4 are shown in Figures 1, 2 and 3, respectively; the -log EC_{50} and T_{max} values for each patient separately are given in Table 2. From Figure 1 it can be seen that histamine efficacy was significantly greater in patients with COPD from $5x10^{-7}$ M to 10^{-4} M. Histamine T_{max} was twofold higher in the patients with COPD than in those without ($P<0.01$), but methacholine and LTC_4 efficacy were similar (Figures 2 and 3). The EC_{50} values were comparable to our previous results in a random group of patients[14] in that LTC_4 (-log EC_{50}: 8.09 ± 0.07 and 7.95 ± 0.06 in airways without and with COPD, respectively) was 100 times more potent than both histamine and methacholine, and that methacholine (-log EC_{50}: 6.24 ± 0.14 and 6.05 ± 0.12 in airways without and with COPD, respectively) was slightly more potent than histamine (-log EC_{50}: 5.85 ± 0.08 and 5.85 ± 0.11 in airways without and with COPD, respectively). No differences in EC_{50} were found between the 2 patient groups. There was no consistent effect of smoking on *in vitro* responses. The use of medication was not associated with changes in *in vitro* responses. The results of the experiments on histamine tachyphylaxis and the effect of prostaglandin synthesis inhibition are shown in Table 3. No tachyphylaxis to histamine was found in experiments with and without indomethacin. In accordance with our earlier findings[14], most bronchioles

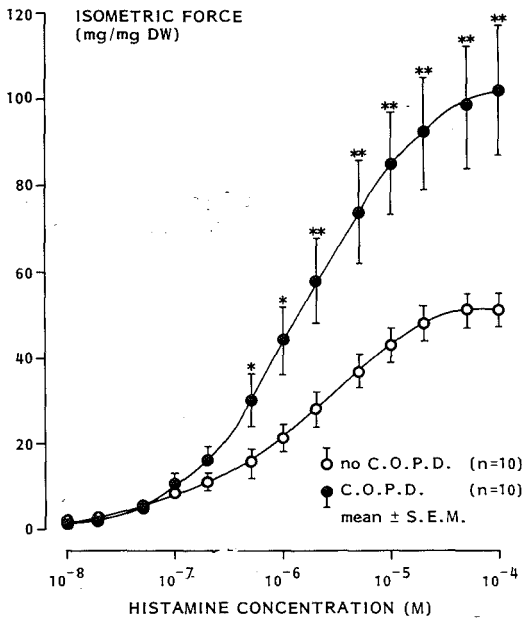

Figure 1. Mean histamine concentration-response curves of airway smooth muscle preparations from 10 patients with COPD (filled circles) and 10 patients without COPD (open circles). * $p<0.05$ * * $p<0.01$. The vertical axis depicts isometric force, expressed in mg force per mg dry weight (DW) of the airway preparation.

showed some increase of T_{max} with time. There were no differences in EC_{50} and T_{max} of CCRC with or without indomethacin and NCCRC.

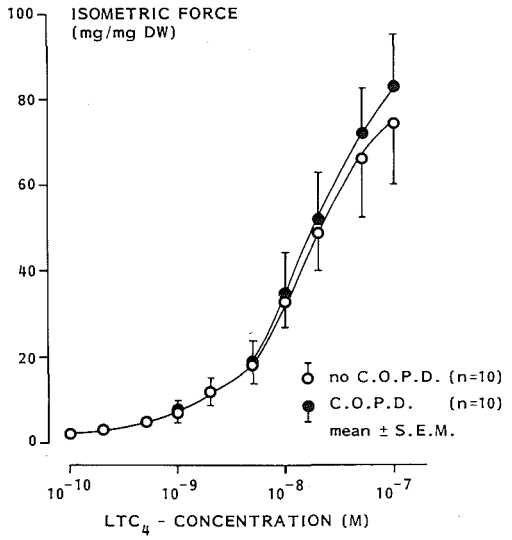

Figure 2. Mean LTC_4 concentration-response curves of airway smooth muscle preparations from 10 patients with COPD (closed circles) and 10 patients without COPD (open circles). DW = dry weight.

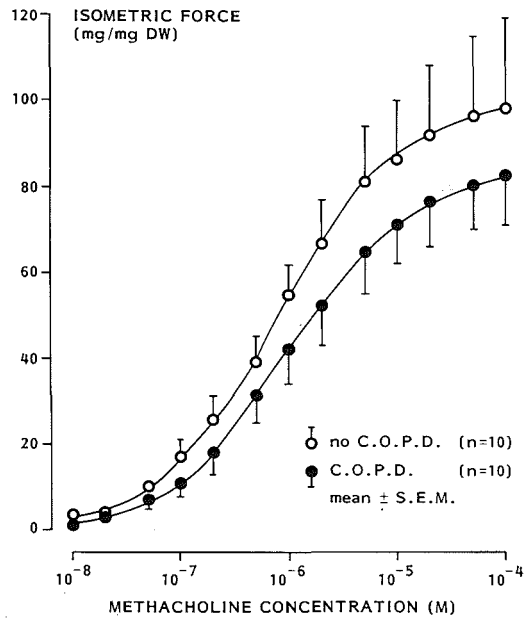

Figure 3. Mean methacholine concentration-response curves of airway smooth muscle preparations from 10 patients with COPD (closed circles) and 10 patients without COPD (open circles). DW = dry weight.

Table 2. *In vitro* responses of bronchiolar strips

Subject no.	$-\log EC_{50}$[a]			T_{max} (mg force/mg dry weight)		
	Histamine	LTC_4	Methacholine	Histamine	LTC_4	Methacholine
Patients without COPD						
1	5.71	8.22	6.65	59	114	132
2	5.96	7.86	6.13	48	40	84
3	5.37	7.87	5.65	37	32	108
4	6.03	7.84	6.04	34	46	103
5	5.95	8.35	6.53	54	71	52
6	6.11	8.18	6.20	70	73	92
7	6.07	8.15	6.78	67	56	24
8	6.10	8.42	6.81	48	45	53
9	5.68	8.21	5.77	44	79	90
10	5.55	7.83	5.81	55	185	260
mean ± S.E.M.	5.85 ± 0.08	8.09 ± 0.07	6.24 ± 0.14	2 ± 4*	74 ± 14	100 ± 20
Patients with COPD						
11	6.18	8.22	6.24	121	121	79
12	6.19	8.02	6.58	104	104	75
13	6.03	8.04	5.70	89	75	123
14	5.88	7.95	6.16	107	63	124
15	5.84	8.15	6.31	54	138	73
16	5.51	7.79	6.05	57	60	55
17	6.01	8.06	6.00	53	61	12
18	5.84	7.68	5.78	212	71	76
19	5.96	7.89	6.39	99	124	90
20	5.04	7.67	5.24	124	17	113
mean ± S.E.M.	5.85 ± 0.11	7.95 ± 0.06	6.05 ± 0.12	102 ± 15*	83 ± 12	82 ± 11

[a] $-\log EC_{50}$ values are the mean of 3 measurements (see: Methods)
* $p < 0.01$ (comparison of non-COPD with COPD).

Table 3. Sequential histamine responses of human bronchioles

	first response	second response	third response
$-\log EC_{50}$[a]			
CCRC, without indomethacin	5.98 ± 0.13	5.95 ± 0.13	5.87 ± 0.14
CCRC, with indomethacin	6.13 ± 0.16	5.90 ± 0.11	5.86 ± 0.10
NCCRC	5.87 ± 0.09	5.87 ± 0.06	
T_{max}[b]			
CCRC, without indomethacin	100	112 ± 6%	104 ± 5%
CCRC, with indomethacin	100	93 ± 6%	120 ± 14%
NCCRC	100	135 ± 21%	

Definition of abbreviations: CCRC = cumulative concentration – response curves; NCCRC = noncumulative curves; T_{max} = maximal isometric force expressed in milligrams of force per milligram of dry weight.
[a] All values are mean ± S.E.M. of 7 experiments.
[b] T_{max} is expressed as % of the first CCRC T_{max} in each strip (mean ± SEM, n = 7).

Discussion

The present results suggest that the histamine efficacy in ASM of patients with COPD is greater than that in ASM of patients without COPD. This was not found in earlier studies[5,7,9,11]. Furthermore, our findings show that smoking does not influence *in vitro* ASM responsiveness in subjects with or without COPD. No significant differences were found regarding the *in vitro* responses to methacholine, which is in agreement with previous studies[6,8,10], and to LTC_4, which has not been reported before. By selection, both patient groups differed significantly in baseline airway caliber, which could be responsible for the altered histamine efficacy. This seems unlikely, however, because FEV_1/VC and T_{max} were not linearly correlated in the COPD group.

A number of methodologic factors may be responsible for the discrepancy between our results and those of other investigators. Measuring abnormalities in efficacy requires a strictly standardized technique, such as applied by us; otherwise the within-subjects variability will be so large that it would be virtually impossible to detect differences between patients. Although in our hands this variability is still considerable[13], the present results show differences that exceed the expected random variation.

Furthermore, our results regarding histamine responses may be determined by the fact that we studied bronchioles only, whereas other investigators have also or exclusively used larger airways. From functional and autoradiographic studies it is known that histaminergic and cholinergic responses and receptor numbers in central and peripheral airways differ, histamine being more effective in peripheral airways[15–19]. Therefore, an increased histamine efficacy *in vitro* may be limited to the small airways and will not be detected when central airways are also included. This is supported by the findings by Roberts et al[9] and Cerrina et al[11], who studied larger airways only, using a technique similar to ours. These investigators did not find differences in histamine responses of airways from patients with and without chronic bronchitis, although T_{max} values were only mentioned in the study by Roberts et al[9].

It can be argued that human ASM functions isotonically *in vivo* and that our method measured isometric force, which may not be entirely relevant. However, we have shown that isometric and isotonic responses of human ASM to various agonists (including histamine, LTC_4 and methacholine) show only a very small and consistent difference with respect to EC_{50} and that maximal shortening and force are linearly related[20]. This makes it unlikely that different results would have been obtained had responses been measured isotonically.

Others have compared the results of *in vivo* bronchial challenges with *in vitro* responsiveness of airways and were unable to demonstrate a relation between the two[5–11]. This may not be surprising because bronchial responsiveness *in vivo* is variable in time and is modified by a number of internal factors (baseline airway caliber, vagal nerve activity, infection, smoking, antigen provocation)[21], which probably do not act by changing ASM function. Such a comparison is further complicated by the fact that *in vivo* challenges almost never produce

complete dose-response curves. Sensitivity could be determined *in vivo* by measuring the threshold doses, even admitting that measurement errors are a problem. Incomplete curves do not allow accurate measurements of efficacy. Receptor theories should therefore not be used to explain *in vivo* data.

An increased specific histamine efficacy of isolated human ASM has been described once before in a single bronchus from an asthmatic patient[22]. Although the few reports that exist on *in vitro* responses of human asthmatic airways do not confirm this finding[9,11,23,24], this may well be due to large differences in the applied techniques. Asthma and COPD or chronic bronchitis may be different expressions of the same genetic abnormality[25]. If this hypothesis is true, it would be logical to find similar abnormalities in ASM from asthmatics and 'bronchitics', which supports the idea that this abnormality has genetic determinants.

Our results do not allow conclusions regarding the mechanism that produces the difference in histamine efficacy. The similarity of the *in vitro* responses to both methacholine and LTC_4 in patients with and without COPD argues against nonspecific mechanisms such as ASM hyperplasia or an increased cell-to-cell coupling of the smooth muscle cells[26]. From the present findings it cannot be determined whether the histamine response is increased in subjects with COPD or suppressed in those without. Several possible explanations for the observed difference can therefore be put forward. Histamine may give rise to the production of arachidonic acid metabolites by cells in the bronchiolar preparation, e.g., the airway epithelial cells. It has been shown that human respiratory epithelium is active in producing arachidonic acid metabolites and that stimulation of muscle contraction in human bronchus causes the selective synthesis of the relaxant prostaglandin (PG)E[27,28]; preliminary results from our laboratory have shown that the bronchiolar strip can release PGE_2 and prostacyclin spontaneously into the organ bath. Inhibitory prostaglandins may depress the ASM histamine response to a lesser degree in patients with COPD. Our findings indicate, however, that indomethacin does not enhance the response of human small airways to histamine, which is in accordance with earlier findings by Brink et al on central human airways[29]. It seems, therefore, that inhibitory prostaglandins are not the cause of the difference in histamine efficacy.

Alternatively, histamine tachyphylaxis in ASM from patients without COPD could explain a decreased response in the course of a CCRC. Selective histamine tachyphylaxis, probably caused by endogenous PG synthesis, has been demonstrated in isolated canine ASM[30]. We found, however, that histamine tachyphylaxis did not occur during 3 successive CCRC on human small airway preparations, and that cumulative and noncumulative CRC were not different. Thus, it seems unlikely that the observed difference is a consequence of histamine tachyphylaxis in airways without COPD.

Finally, the difference may be caused by an altered histamine receptor activation-contraction coupling, or non-COPD ASM may have less histamine receptors, which would result in a sub-optimal activation by a given histamine concentration. These two possibilities cannot be excluded at this moment. Further

studies should be done to confirm our findings and to investigate the various mechanisms that may be involved.

In summary, we found a significantly increased efficacy of histamine in bronchioles from patients with COPD compared with subjects without COPD. *In vitro* responses to LTC_4 and methacholine were not different. We speculate that an increased histamine response contributes to the pathogenesis of airflow limitation in COPD.

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Chapter 11

Relaxation responses of airway smooth muscle from subjects with and without chronic bronchitis and airflow limitation

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Summary

Airflow limitation and bronchial hyperresponsiveness in chronic bronchitis may result from an increased contractility or a decreased relaxability of airway smooth muscle (ASM). To elucidate this, we obtained ASM from 10 subjects with and 10 without chronic obstructive bronchitis at thoracotomies, and measured contractile and relaxation responses to the cholinergic agonist methacholine, to l-isoproterenol, which stimulates β-adrenoceptors, and forskolin, which relaxes ASM via stimulation of intracellular cAMP production. Furthermore, we applied electric field stimulation (EFS) in order to stimulate non-adrenergic inhibitory (NAI) nerves in the bronchial wall, the main direct inhibitory nerve supply to human ASM. Methacholine was equipotent in bronchiolar ASM from subjects with and without chronic obstructive bronchitis, but its maximal effect in bronchitic ASM was significantly lower than in normal ASM ($p < 0.05$). The mean relaxation responses to l-isoproterenol and forskolin were not different in the two groups, but 3 of the 10 patients with chronic obstructive bronchitis had a maximal relaxation response to l-isoproterenol that was significantly smaller than the control values obtained in normal ASM. EFS relaxed airways from subjects with and without chronic obstructive bronchitis to a similar degree. NAI nerve-mediated responses were relatively ineffective compared to l-isoproterenol- and forskolin- induced relaxations. The present findings suggest that airflow limitation and hyperresponsiveness in chronic bronchitis are not due to abnormal (cholinergic) contractile-, or (adrenergic) relaxation responses of bronchiolar ASM, nor to an impaired functioning of the NAI system. A decreased β-receptor function, that is not related to the use of sympathomimetic bronchodilator drugs, may however exist in a subgroup of bronchitics.

Key words: human airway smooth muscle, bronchial strip, methacholine, isoproterenol, forskolin, electric field stimulation, non-adrenergic inhibitory system, chronic bronchitis, chronic airflow limitation.

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Introduction

Airflow limitation and an increased bronchial responsiveness to nonallergic stimuli are frequent findings in chronic bronchitis[1]. Possible mechanisms that underly both the obstruction and the hyperresponsiveness include an increased contractility or a decreased relaxability of airway smooth muscle (ASM). The sensitivity and maximal contractile response to pharmacological stimulation of isolated ASM from subjects with or without chronic bronchitis, airflow limitation and bronchial hyperresponsiveness have been compared[2–7]. The results of these studies indicated that ASM from subjects with airflow limitation or hyperresponsiveness is not hypersensitive to contracting agonists such as histamine or methacholine. The maximal response is more difficult to assess accurately *in vitro* than the sensitivity, due to a relatively large intrinsic variability of the maximal contractions of human ASM[8]. We have recently reported increased maximal responses to histamine, but not methacholine and leukotriene C_4, in small airways smooth muscle from subjects with chronic bronchitis and airflow limitation[7]. This finding does not explain, however, why *in vivo* some subjects are hyperresponsive to a wide variety of stimuli. Hypothetically, this could be due to defective counteracting mechanisms. Bronchodilatation is probably mediated by circulating catecholamines that stimulate a homogenous population of β_2 adrenergic receptors on ASM[9,10]. There is also direct inhibitory innervation of ASM, which is non-adrenergic[9]. Few studies have compared the relaxability of human airways in relation to airflow limitation or bronchial hyperresponsiveness[5,6,11–13]. Animal experiments have suggested that β-adrenoceptor numbers and function[14,15] and non-adrenergic inhibitory (NAI) nerve-mediated relaxations[16] are reduced in experimental bronchial hyperresponsiveness. Decreased β-adrenergic responses of ASM from patients with severe airflow limitation and hyperresponsiveness have been found by Cerrina et al[6] but not by Taylor et al[5]. Studies on ASM from subjects with asthma have produced contradictory results[11–13]. NAI nerve-mediated relaxation responses of airways from subjects with and without airflow limitation and hyperresponsiveness were compared in only two reports, suggesting that NAI function is normal in non-asthmatic hyperresponsiveness, but may be reduced in asthma[5,13]. The small number of observations and the lack of data on intraindividual reproducibility of *in vitro* findings make it impossible to draw conclusions from the available data. In the present study, we measured relaxation responses of isolated ASM from subjects

with and without chronic bronchitis and airflow limitation, using the β-agonist l-isoproterenol and forskolin, a diterpene, which stimulates cyclic adenosine monophosphate (cAMP) production independent of receptors. This was done to differentiate between defective β-receptor function and possible defects in intracellular cAMP generation, which both may cause reduced relaxation responses after β-receptor stimulation[14]. Furthermore, we measured the relaxation responses to electric field stimulation (EFS), which activates NAI nerves in the bronchial wall.

Methods

Lung tissue was obtained from 20 subjects who underwent surgery for bronchial malignancies. Prior to thoracotomy, the medical history was taken, and patients underwent a physical examination, bronchoscopy and lung function measurements, including the inspiratory vital capacity (IVC), the forced expiratory volume in 1 sec (FEV_1) before and after 10 mg of inhaled isoproterenol and the total lung capacity (TLC), determined with the helium dilution method. Chronic obstructive bronchitis was diagnosed according to criteria proposed by the Medical Research Council[17] when subjects reported chronic cough and sputum production for at least two years, and during 3 or more months each year, and in case of airflow limitation, defined as an FEV_1/VC ratio that was 2 standard deviations or more below predicted mean values (reference values were from reference[18]). Control subjects had no history or symptoms of chronic bronchitis or asthma, and a normal lung function.

In vitro studies

Immediately after surgical resection, a macroscopically normal piece of lung was removed from the resected specimen and transported to the laboratory in Krebs-Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.55) aerated with 95% O_2 and 5% CO_2. Tubular segments of bronchioles (10th to 13th generation, internal diameter 0.8 to 1.2 mm, length 2mm) were carefully dissected free from adhering parenchyma and blood vessels, using a modification of the technique described previously[19] and were mounted between two stainless steel wires in 10 ml organ baths. Furthermore, cartilaginous airways (5th to 7th generation) were dissected and cut spirally to obtain bronchial strips measuring 20 x 2 mm. Bronchiolar segments were stored overnight in a slow flow of cold (4°C) aerated buffer[19].

The next day, the segments were equilibrated for 2 h at 37°C while the buffer was changed every 15 min. We have previously documented that bronchiolar ASM function does not change after this storage procedure[19]. Isotonic responses to pharmacological stimulation were recorded with high-precision angular position transducers[20], using an isotonic load of 0.5 g. This load stretched the segments to their optimal length, as determined in preliminary experiments. All segments were primed with methacholine (10^{-5} M) followed by washout and

return to baseline. Subsequently, a full methacholine cumulative concentration-response curve (CCRC) was made, and the effective concentration that produced 50% of the maximal response (EC_{50}) was determined. After washout, this EC_{50} was applied to induce a baseline contraction plateau from which relaxation experiments could be carried out. Relaxation CCRC to either l-isoproterenol or forskolin were made; only one of the two agonists was administered to a given segment. After maximal relaxation with l-isoproterenol ($5x10^{-6}$M) or forskolin ($5x10^{-6}$M), full relaxation was obtained in calcium-free buffer containing EDTA ($4x10^{-3}$M). The relaxation responses to l-isoproterenol and forskolin were expressed as a percentage of the difference between methacholine EC_{50}-induced contraction and full relaxation in Ca free buffer. The spontaneous baseline contractile state of the ASM was defined as the difference (in μm) between the initial stable baseline and full relaxation in Ca-free buffer. From each CCRC, the EC_{50} was calculated using a computerised iterative curve fitting method, and the maximal shortening (S_{max}) or relaxation (R_{max}) were determined.

Cartilaginous bronchi were used to measure the neurally mediated NAI relaxation to EFS, because the NAI system is mainly present in central airways[9]. Bronchial strips were mounted in the organ baths and equilibrated at 37°C in aerated buffer under an isotonic load of 1 g. The Krebs-Henseleit buffer that was used in EFS experiments contained atropine ($1.2x10^{-6}$M) to inhibit cholinergic nerve-mediated contractions to EFS, indomethacin ($6x10^{-6}$M) and the leukotriene antagonist FPL-55712 ($11.5x10^{-6}$M), because we have previously found that human bronchi can produce bronchoconstricting prostaglandin- and leukotriene-like substances after EFS[21]. In separate strips from 18 subjects, we examined the effect of the nervous conductance blocker tetrodotoxin (TTX, $3x10^{-6}$ g/l), to assess the role of nervous conduction in the response to EFS. After an equilibration of 2 h, with frequent changing of the buffer, histamine ($5x10^{-6}$M) was added to induce a contraction plateau. EFS was applied via platinum plate electrodes parallel to the bronchial strips, and rectangular pulses of alternating polarity were delivered by a voltage-constant custom made tissue stimulator. Frequency-response curves to EFS were made by applying graded EFS as described previously[13], using 0.3 msec, 50 V pulses at frequencies of 1 to 50 Hz. After maximal relaxation to EFS, full relaxation of the strips was obtained in Ca-free buffer with EDTA and l-isoproterenol 10^{-5}M. Responses to EFS were expressed as a percentage of full relaxation. The frequency that produced 50% of the maximal effect of EFS (EF_{50}) was determined by linear interpolation.

Data analysis

Mean values of the negative logarithms of EC_{50}, the EF_{50}, and the maximal responses to methacholine, l-isoproterenol, forskolin and EFS, obtained in airways from patients with and without chronic obstructive bronchitis, were compared, and the significance of the differences between means was determined with Students t-test for unpaired samples (two tailed, $\alpha = 0.05$). Duplicate or triplicate measurements were avaraged before inclusion in the statistical analysis. All values are given as means ± 1 S.E.M.

Drug sources
Methacholine hydrobromide, l-isoproterenol and histamine dihydrochloride were purchased from Janssen pharmaceuticals, Belgium; indomethacin from Duchefa, The Netherlands; and TTX from Sigma, U.S.A. Forskolin was a gift from Hoechst Pharma, Holland, and FPL-55712 was provided by Fisons, U.K.. Buffer chemicals were analytical grade from Merck, F.R.G.

Results

The clinical, anthropometric and lung function data of the 20 subjects are given in Table 1. Ten subjects had chronic bronchitis and significant airflow limitation, and 10 were without symptoms and had a normal lung function. In both groups, 7 subjects currently smoked cigarettes; heavy smoking (more thans 25 cigarettes per day for more than 1 year) was more common in bronchitics. Five patients with chronic obstructive bronchitis used bronchodilator drugs: theophylline (no.14) and β-agonists (no.16,17 and 19), and three were treated preoperatively with oral dexamethasone (no.11,12 and 17). None of the control subjects received bronchoactive drugs. None of the subjects had central airway obstruction by tumor, as judged by preoperative bronchoscopy, and none had a clinical history suggestive of asthma.

In vitro measurements
All bronchiolar segments contracted dose-dependently to methacholine and relaxed to l-isoproterenol and forskolin. The mean values of -logEC_{50}, S_{max} and R_{max} in each subject are given in Table 2. The contractions induced by methacholine in normal bronchiolar ASM were significantly greater than in ASM from bronchitics (S_{max}: 1121 ± 146 μm and 718 ± 112 μm, respectively; $p < 0.05$). The mean -logEC_{50} was, however, similar (Figure 1). No differences were found between controls and bronchitics with respect to the mean -logEC_{50} and R_{max} to l-isoproterenol and forskolin (Table 2, Figures 2 and 3). Three bronchitics (cases no.15, 16 and 20) had a low R_{max} to l-isoproterenol that was outside the 95% confidence limits of the control group. The response to l-isoproterenol was not systematically reduced in the subjects that regularly used β-agonists, and no differences in *in vitro* responses were found in subjects that received dexamethasone. Forskolin was slightly less potent than l-isoproterenol (overall mean -logEC_{50}: 6.88 and 7.43 respectively), but always caused more relaxation (R_{max} 97% and 87% respectively).

Central airways were obtained from 9 controls and 10 bronchitics. No differences between the groups were found in the relaxation responses of bronchial strips to EFS, with or without TTX (Table 2, Figure 4). Relaxation responses to EFS were only 10-20% reduced in the presence of TTX, especially at lower stimulus frequencies (Figure 4). EFS was relatively ineffective, producing a mean maximal relaxation of 61,5%. All preparations had an intrinsic spontaneous baseline

Table 1. Patient characteristics

case no	sex	age (yr)	current smoking[a]	medication[b]	tumor location[c]	Lung function[d] IVC (ml)	IVC % pred.	FEV_1 (ml)	FEV_1/IVC % before bronchodilator	FEV_1/IVC % after bronchodilator	TLC (ml)	TLC % pred.
Normals												
1	F	23	–	–	RL	3470	92	2860	82	ND	4940	99
2	M	45	+	–	RU	3580	112	2890	75	77	5080	102
3	M	60	++	–	RL	4500	102	3200	72	ND	6900	99
4	M	62	+	–	RU	3440	114	2435	71	ND	5230	100
5	M	63	+	–	RU	3400	102	2700	79	ND	5540	97
6	F	54	+	–	RU	4320	119	3400	79	ND	6920	119
7	M	71	+	–	LL	3760	88	2680	71	ND	6430	89
8	M	65	ex	–	LU	3750	100	2780	74	ND	6050	93
9	M	77	pipe	–	RL	4000	116	2870	72	ND	ND	ND
10	M	58	ex	–	RU	4900	123	3600	73	ND	8700	139
Chronic obstructive bronchitis												
11	M	55	++	ster	RL	4700	91	2600	54	54	ND	ND
12	M	64	+	ster	RM	4500	92	2900	64	64	ND	ND
13	M	62	++	–	RL	4340	91	2860	66	70	7070	94
14	M	72	ex	theo	LL	4040	81	1820	45	47	ND	ND
15	M	70	++	–	LU	4700	107	2500	52	ND	8000	108
16	F	36	+	sal	LL	4100	111	2800	70	72	ND	ND
17	M	63	ex	sal,ster	RU	4060	93	2100	52	52	6950	98
18	M	73	+	theo	RL/RM	3030	69	1380	46	46	5820	83
19	M	63	ex	sal	LL	3500	90	2200	63	66	6700	102
20	M	72	+	–	RL	3550	91	1715	48	ND	7420	110

[a]Smoking: +, less than 25 cigarettes per day; ++, more than 25 cigarettes per day for one year or more prior to surgery; ex, ceased smoking at least 2 years previously.

[b]Medication: ster, corticosteroids; theo, theophyllin; sal, salbutamol.

[c]Abbreviations: RU, RM, RL: right upper, middle or lower lobe; LU, LL: left upper, left lower lobe.

[d]Abbreviations: IVC, inspiratory vital capacity; FEV_1, forced expiratory volume in 1 sec.; TLC, total lung capacity. Predicted values are from reference (18).

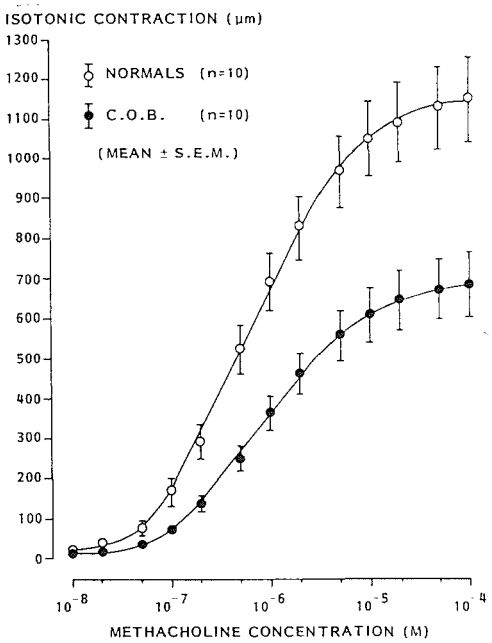

Figure 1. Mean cumulative concentration-response curves to methacholine of ASM preparations from control subjects (47 segments from 10 subjects) and patients with chronic obstructive bronchitis (38 segments from 10 subjects), represented by open and filled circles, respectively. The vertical axis depicts isotonic shortening of ASM, expressed in μm, and the horizontal axis shows the molar methacholine concentrations on a log scale. The difference in maximal responses is significant (Students t-test, $0.02 < p < 0.05$).

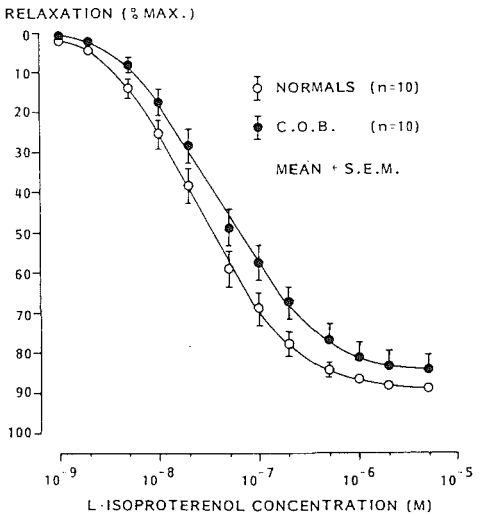

Figure 2. Mean cumulative concentration-response curves to l-isoproterenol of ASM preparations from control subjects (open circles, $n = 25$ segments from 10 subjects) and patients with chronic obstructive bronchitis (filled circles, $n = 19$ segments from 10 patients). The vertical axis depicts relaxation of methacholine EC_{50}-induced baseline contraction as a percentage of full relaxation obtained in Ca-free buffer containing EDTA (4×10^{-3}M). No difference was found between the curves.

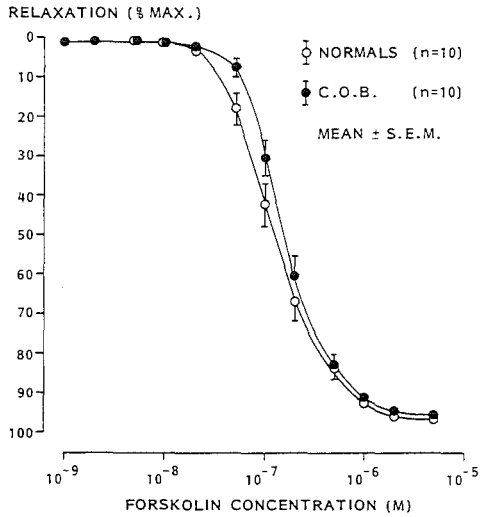

Figure 3. Mean cumulative concentration-response curves to forskolin of ASM preparations from control subjects (open circles, n = 22 segments from 10 subjects) and patients with chronic bronchitis (filled circles, n = 19 segments from 10 patients). The vertical axis depicts relaxation of methacholine EC_{50}-induced baseline contraction as a percentage of full relaxation obtained in Ca-free buffer containing EDTA ($4x10^{-3}$M). No difference was found between the curves.

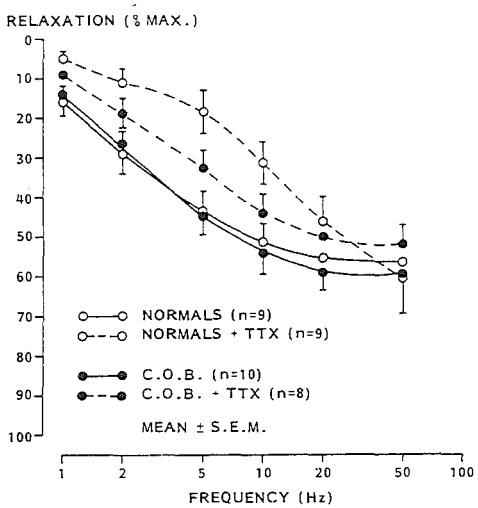

Figure 4. Mean frequency-response curves to graded electric field stimulation of bronchial strips from 9 control subjects (15 strips, open circles) and 10 patients with chronic obstructive bronchitis (20 strips, filled circles). Relaxation responses were obtained in the presence of atropine, indomethacin, FPL-55712 and histamine (see: methods) and are the consequence of stimulation of non-adrenergic inhibitory nerves in the bronchial wall. Broken lines indicate relaxation responses obtained in the presence of the nervous conductance blocker TTX (n = 11 strips from 9 controls and n = 8 strips from 8 bronchitics). The vertical axis depicts relaxation of histamine ($5x10^{-6}$M)-induced precontraction, as a percentage of full relaxation obtained in Ca-free buffer containing EDTA ($4x10^{-3}$M). The horizontal axis shows the frequency of EFS in Hz on a log scale. No significant differences were found between relaxation responses of bronchial strips from controls and from patients with chronic obstructive bronchitis.

Table 2. Results of *in vitro* studies

Case no	Methacholine		L-isoproterenol		Forskolin		Field stimulation	
	$-\log EC_{50}$	S_{max} (μm)	$-\log EC_{50}$	R_{max} (%)	$-\log EC_{50}$	R_{max} (%)	EF_{50} (Hz)	R_{max} (%)
Normals								
1	5.47	962	7.89	96	7.45	98	3.2	39
2	6.49	546	7.46	82	7.12	97	1.6	62
3	6.33	1061	6.80	91	6.71	97	10.0	67
4	6.10	1099	6.80	91	6.75	98	6.8	61
5	5.95	605	7.86	84	7.03	99	3.4	55
6	5.97	1290	7.77	82	6.66	97	2.9	47
7	5.85	2198	7.91	92	6.80	96	1.0	74
8	6.36	1205	7.33	87	6.75	94	2.2	83
9	6.40	901	7.56	93	7.18	99	1.5	83
10	6.65	1344	7.68	94	6.92	94	ND	ND
Mean	6.16	1121*	7.51	89	6.94	97	3.6	63
SEM	0.11	146	0.13	2	0.08	1	1.0	5
Chronic obstructive bronchitis								
11	6.25	924	7.07	100	7.05	100	3.5	39
12	6.04	358	7.52	96	6.89	98	1.9	49
13	6.06	690	7.56	90	6.73	95	5.0	36
14	5.91	316	8.05	96	6.87	94	5.4	38
15	5.91	564	7.14	75	6.75	95	1.7	91
16	6.74	216	7.31	68	6.59	96	2.6	61
17	6.07	736	7.51	87	6.98	92	2.0	78
18	6.20	1182	7.00	88	6.78	99	4.1	79
19	5.96	1154	7.09	92	6.57	95	2.4	78
20	5.96	1038	7.18	64	6.91	96	3.1	50
mean	6.11	718*	7.34	86	6.81	96	3.2	60
SEM	0.08	112	0.10	4	0.05	1	0.4	6

Individual values are the means of 2-6 measurements for pharmalogical experiments, and of 1-3 measurements for EFS experiments.
*indicates a significant difference between the 2 groups ($p < 0.05$).

contraction that was stable in time. There was no difference in the spontaneous contraction of ASM from bronchitics and controls (688.8 ± 93.9 μm and 709.6 ± 71.4 μm respectively). In addition, no relation was found between S_{max} to methacholine and intrinsic baseline contraction of the ASM.

Discussion

The present results indicate that ASM from subjects with chronic obstructive bronchitis relaxes normally in response to l-isoproterenol, forskolin and to EFS of NAI nerves. Contractions to methacholine were significantly higher in the

control group. Previous functional experiments on human airways have demonstrated no significant differences in the relaxation responses of human ASM from subjects with and without chronic bronchitis and/or bronchial hyperresponsiveness, which is in agreement with the present results[5,6,13]. Furthermore we confirmed the observation by Taylor et al[5] that ASM in chronic obstructive bronchitis can generate sufficient cAMP in response to forskolin to produce maximal relaxation. Receptor binding studies on human lung homogenates have shown no differences in the numbers of β-adrenoceptors in normal lungs and lungs from patients with chronic obstructive bronchitis[9]. In the lung, β-adrenoceptors are, however, not exclusively present on ASM cells, but also on many other cell types such as epithelial cells, mast cells and mucus secreting cells[9]. Therefore the studies in tissue homogenates did not allow conclusions on β-adrenoceptor density on ASM cells.

Airway inflammation is a uniform histopathologic feature of chronic bronchitis that is associated with *in vivo* bronchial hyperresponsiveness[22]. In the guinea pig, activated macrophages induce β-adrenoceptor desensitisation[23], probably via the production of hydroxyl radicals[24]. It is likely that oxygen radicals also play a role in the chronic airway inflammation in chronic bronchitis[25]. A decreased β-adrenoceptor function might therefore occur in airways from subjects with chronic bronchitis. Although, in the present functional studies, no differences have been found in the mean responses to β-receptor stimulation of airways from patients with and without chronic obstructive bronchitis, three bronchitics had a low R_{max} to l-isoproterenol of 75, 68 and 64%, with a normal EC_{50} (cases 15, 16 and 20 respectively). These R_{max} values fell outside the narrow 95% confidence limits of the control group. One of these 3 patients (no.16) used salbutamol, the other 2 had not received bronchodilators; none received corticosteroids. These 3 subjects had no common clinical features to which their abnormally low relaxation response to β-adrenoceptor stimulation could be attributed. Their ASM R_{max} to forskolin was 95, 96 and 96% respectively, so the impaired relaxability was probably due to β-receptor dysfunction, and not to deficient cAMP production. It seems therefore, that impaired β-receptor function may occur in a subgroup of patients with chronic obstructive bronchitis.

It has been speculated by Barnes[9] that airway inflammation may produce NAI hyporesponsiveness, because proteolytic enzymes that are released by inflammatory cells could inactivate the NAI neurotransmitter, which is probably a peptide. Such an inactivation might favour bronchoconstriction. We found normal relaxation responses to EFS in central airways from patients with chronic bronchitis, which argues against this hypothesis. The present results are, however, not conclusive because it is not known if inflammatory cells in isolated bronchus behave as they do *in vivo*.

There are few data in the literature on the function of the NAI system in human obstructive airway diseases. In one report on a relatively small number of non-asthmatic subjects, NAI responses were not related to bronchial hyperresponsiveness *in vivo*[5]. We have previously found that NAI responses in human asthmatic airways were in the low-normal range[13]. Thus, at present, there are

no convincing data to support a defect of the NAI system in subjects with chronic obstructive bronchitis and airway hyperresponsiveness. Since airflow limitation and hyperresponsiveness in asthma and chronic bronchitis have different characteristics[1,26], the present results do not exclude a defect of the NAI system in asthma. Clearly, more work needs to be done on this subject.

Surprisingly, ASM in the control group had a higher mean maximal contractile response to methacholine than ASM from bronchitics. In a previous study, we also found a trendwise, but not significant, difference in maximal methacholine responses (measured as isometric force) of ASM from similar populations of normals and bronchitics[7]. The significance of the difference ($0.02 < p < 0.05$) found in the present study may be related to the fourfold larger number of observations in the present study, and possibly to methodological factors such as the use of bronchiolar segments in stead of spiral strips. It is not likely that isometric and isotonic methods produce different results, because force development and ASM shortening are linearly related in experiments like the present[27]. *In vivo*, subjects with and without chronic obstructive bronchitis are often hyperresponsive to inhaled methacholine[1,26]. The apparent discrepancy between the *in vitro* and *in vivo* responses to methacholine may be related to the fact that we have studied methacholine responses only in small airways. Cholinergic receptor density decreases from central to peripheral airways[28]. In previous *in vitro* studies on central human airways, no differences in ASM responses to cholinergic stimulation were found between subjects with and without chronic bronchitis, airflow limitation or hyperresponsiveness[4,5]. In peripheral lung tissue homogenates, receptor binding studies have suggested a decreased number of muscarinic receptors in chronic bronchitis[29]. Decreased cholinergic responses in chronic bronchitis may therefore be limited to small airways.

We have considered the alternative possibility that a higher intrinsic muscle contraction limited the maximal response to methacholine in bronchitics. This appeared not to be the case, however, because a similar baseline contractile state was found in airways from normals and bronchitics. Furthermore there was no relation between S_{max} and baseline ASM contractile state within the two groups. Finally it could be that the 0.5 g isotonic load that was imposed on all ASM segments was not optimal for ASM from bronchitics. In preliminary studies, maximal contractions were invariably obtained with a 0.5 g preload, irrespective of the clinical diagnosis. However we did not perform length-tension experiments routinely before and after each CCRC, so this possibility cannot be excluded.

In summary, we found normal relaxation responses to l-isoproterenol and forskolin, and normal responses after stimulation of NAI nerves in ASM from patients with chronic obstructive bronchitis. There are indications, however, that β-receptor function may be defective in a subgroup of patients with chronic obstructive bronchitis, independent of the use of β-adrenergic drugs. In addition we found a reduced ASM contractility to methacholine in bronchitics. These results suggest that airflow limitation and bronchial hyperresponsiveness in

chronic obstructive bronchitis are neither due to an increased cholinergic responsiveness of small airway ASM, nor to a general impairment of ASM relaxability, or to a defective NAI system. Other possible mechanisms, including differences in baseline airway caliber, mucosal inflammation, abnormal autonomic control or changes in airway mechanics, may therefore be more important.

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Chapter 12

Comparison of human bronchiolar smooth muscle responsiveness *in vitro* with histological signs of inflammation

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Summary

A study was carried out to test the hypothesis that chronic inflammation is associated with an increased sensitivity or contractility of human airway smooth muscle. Bronchiolar strips from 30 patients, 12 of whom had chronic bronchitis, were examined in the organ bath for their responses to histamine, methacholine and leukotriene (LT) C_4. The same airways were also studied histologically and small airway disease (S.A.D.) was quantified by subjective grading of the degree of inflammatory cell infiltration, smooth muscle hypertrophy, fibrosis and goblet cell hyperplasia. We found a wide variation of S.A.D. in patients with and without chronic bronchitis. Multiple regression analysis failed to demonstrate increased sensitivity ($-\log EC_{50}$) to histamine, methacholine or LTC_4 in relation to S.A.D.. In contrast the only significant correlations found were between a decreased $-\log EC_{50}$ to histamine and methacholine and an increased S.A.D. score. Contractile responses (T_{max}) to histamine and methacholine in peripheral airways from patients with chronic bronchitis tended to be higher than in airways from patients without chronic bronchitis. This increased T_{max} was not related to the degree of S.A.D. These results suggest that chronic airway inflammation does not cause *in vitro* hyperresponsiveness of human small airway smooth muscle.

Key words: airway inflammation, chronic bronchitis, small airway disease, bronchial smooth muscle, airway responsiveness, methacholine, histamine, leukotriene C_4

De Jongste JC, Mons H, Van Strik R, Bonta IL, Kerrebijn KF. Comparison of human bronchiolar smooth muscle responsiveness *in vitro* with histological signs of inflammation. Thorax 1987, in press. Reprinted with permission of the British Medical Association.

Introduction

Clinical observations have led to the view that airway inflammation may be an important modulator of nonspecific bronchial responsiveness in patients with asthma or chronic bronchitis and even in normal individuals[1–5]. Moreover, we recently found an increased histamine responsiveness in isolated peripheral airways from patients with chronic bronchitis and airflow limitation[6]. The mechanisms by which inflammation could enhance bronchial responsiveness are not clear. Few studies have examined the responsiveness of isolated human airways in relation to the histological appearance of the bronchial tissue, and these studies have shown no relation between the degree of airway disease and function[7,8]. In the present study, we measured the functional responses to pharmacological stimulation of isolated peripheral airways from patients with and without chronic bronchitis and/or airflow limitation and quantified various histological aspects of small airway disease (S.A.D.) in the same preparations by means of a subjective grading method. Functional data were related to histological findings to determine whether inflamed peripheral airways are more responsive *in vitro* than non-inflamed airways.

Methods

Patients

Thirty patients who underwent lobectomy or pneumonectomy, 29 for bronchial carcinoma and one for carcinoid, were included in the study. All patients had lung function tests before surgery, including inspiratory vital capacity (VC) and forced expiratory volume in 1 sec (FEV_1) before and after 10 mg of nebulised isoproterenol. Chronic bronchitis was diagnosed according to the Medical Research Council criteria of cough and phlegm production for more than two years and more than three months in a year[9]. Subjects were classified as normal when they reported no respiratory symptoms, apart from recent symptoms caused by their tumor, and had a normal lung function with an FEV_1/VC of at least 70%. In 11 patients bronchial responsiveness was measured *in vivo* before surgery. Nebulised histamine was administered via a Wiesbadener Doppel Inhalator in doubling concentrations, and the provocative concentration that caused a 20% fall in FEV_1 from baseline ($PC_{20}FEV_1$) was calculated[10]. The study protocol was approved by the ethical committee of the University Hospital.

In vitro studies

Immediately after surgical resection a macroscopically normal piece of lung tissue was collected 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) aerated with 5% CO_2 and 95% O_2, and rapidly transported to the laboratory. Bronchiolar strips (10th to 13th generation) were dissected, stored overnight and studied isometrically with Grass FTO3D transducers, in 10 ml organ baths containing aerated Krebs-Henseleit buffer at 37°C as described previously[11]. A

baseline tension of 500 mg was applied. Histamine hydrochloride, methacholine bromide (Janssen pharmaceuticals, Belgium) and leukotriene (LT) C_4 (a gift from Dr. J. Rokach, Merck Frosst, Canada) were used as contracting agents, and three cumulative concentration-response curves (CCRC's) were made in succession on a single bronchiolar strip from each patient. The sequence of the agonists was determined by a random digits table. From each CCRC, the EC_{50} value (the molar agonist concentration that produced 50% of the maximal effect on a given strip) was derived as a measure of smooth muscle sensitivity. We have previously shown that for EC_{50} measurements this procedure does not lead to interactions between these three agonists[12]. The maximal force (T_{max}, contractility) developed by a strip was calculated only for the first CCRC on each strip because interactions occur regarding T_{max} when agonists are applied successively[12].

Histology

After dissection of the bronchiolar strips, a proximal segment was removed, fixated in formalin and processed for histological examination. We used a simplified version of Cosio's subjective grading method[13], adapted for bronchiolar strips, to assess the severity of S.A.D.. The items scored were inflammatory cell infiltration, smooth muscle hypertrophy, goblet cell hyperplasia and fibrosis. Each item was assigned a score (0-3) by comparison with pictorial reference standards, 0 representing the normal picture and 1-3 reflecting increasing degrees of abnormality (Figure 1). The total S.A.D. score was the sum of scores for the four items, so 12 was the maximally attainable score. The validity of the scoring system was confirmed in a preliminary study (Kappa-coefficients for within and between observer agreement were 0.77 ± 0.07 and 0.66 ± 0.12 respectively (mean $\pm$ S.E.M)).

Statistical analysis

Data for patients with and without chronic bronchitis were compared by using Student's t-tests (two tailed). For each agonist, the EC_{50} and T_{max} values of 30 strips from 30 patients were related to the total S.A.D. score by linear regression analysis. The relation between EC_{50} and T_{max} values and the four separate S.A.D. items was examined by multiple regression analysis. The significance of the regression was determined by analysis of variance and the F-test ($\alpha = 0.05$).

Results

Of the 30 patients, 12 had chronic bronchitis as defined, and 18 reported no cough or phlegm. None of the patients had a history of asthma. The clinical findings are summarised in Table 1. Eight of the 18 subjects without symptoms of chronic bronchitis had airflow limitation ($FEV_1/VC < 70\%$). Although this subgroup had a higher mean age and may have had airway obstruction or compression by tumor, we analysed the results for this subgroup separately.

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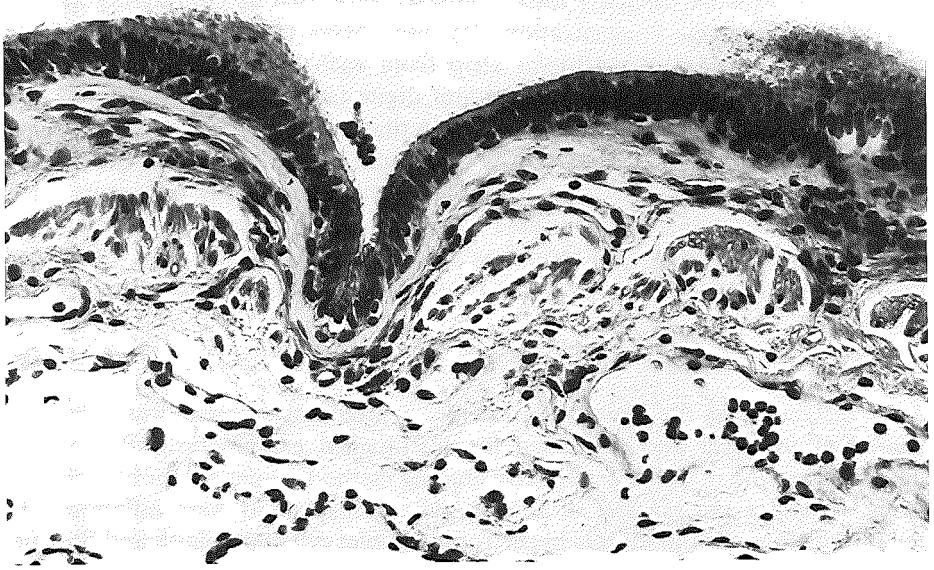

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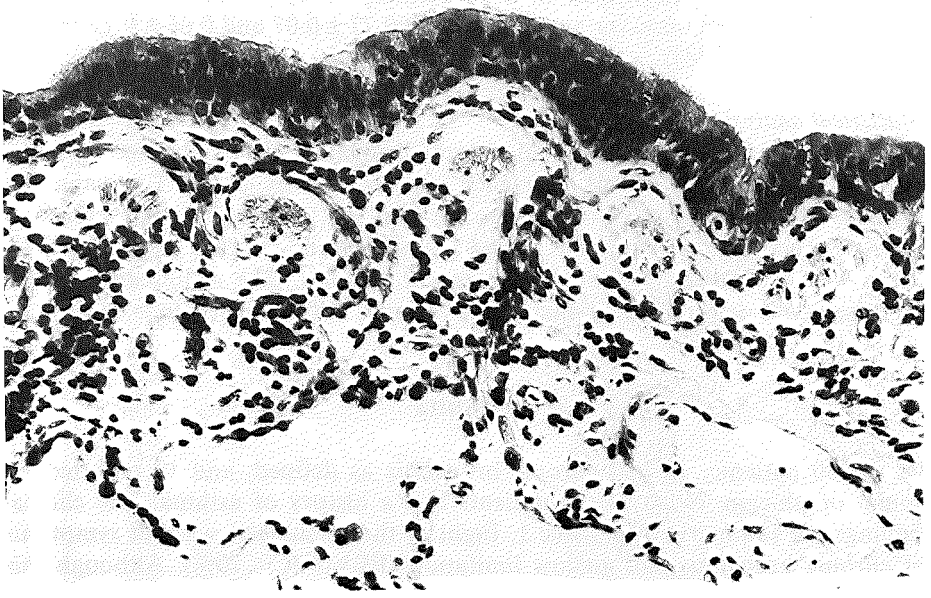

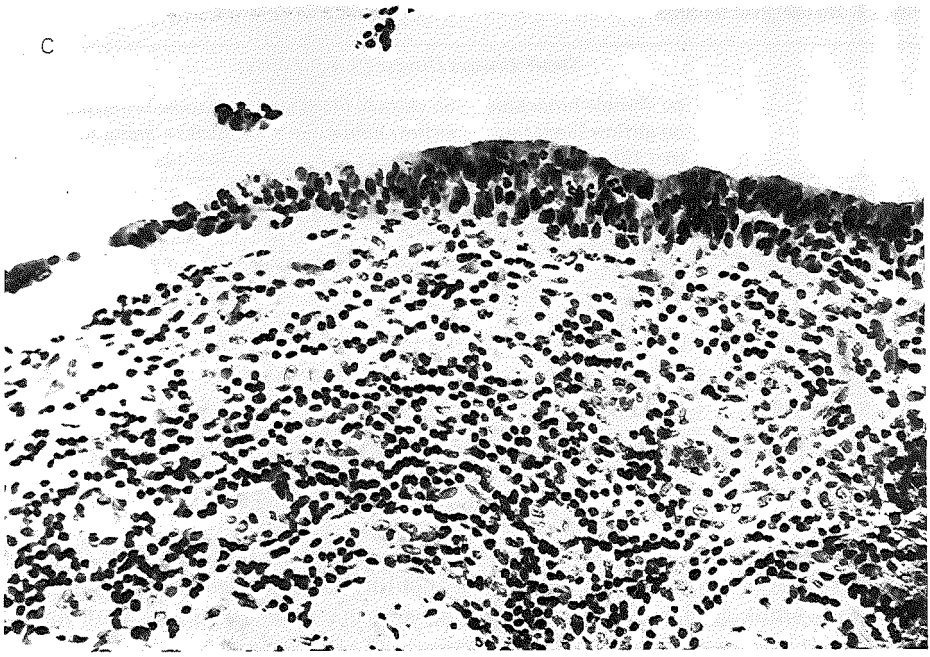

Figure 1. Photomicrographs showing various degrees of inflammatory cell infiltration in bronchiolar strips. A, B and C are increasingly abnormal pictures of inflammatory cell accumulation corresponding to cellular infiltration scores 1, 2 and 3 respectively. Magnification: 160x. Stain: May-Grünwald-Giemsa.

Table 1. Patient characteristics (mean values ± S.E.M.)

	no chronic bronchitis no airflow limitation (n = 10)	no chronic bronchitis airflow limitation (n = 8)	chronic bronchitis airflow limitation (n = 12)
Age (years)	49 ± 5	65 ± 3	55 ± 5
Inspiratory vital capacity (VC) (% predicted)	105 ± 4	108 ± 7	97 ± 5
Forced expiratory volume in 1 sec (FEV_1) as a % of VC	77 ± 2	59 ± 3	55 ± 4
% increase in baseline FEV_1 after isoproterenol	1 ± 1	4 ± 1	10 ± 3
Smoking habits (n)			
current	4	4	8
ex (> 2 years)	5	4	4
never	1	0	0
Response to inhaled histamine ($PC_{20}FEV_1$, mg/ml)	> 64 (n = 6)	> 64 (n = 2)	> 64 (n = 2) 32 (n = 1)

Table 2. Histological findings

	Small Airways Disease (S.A.D.) scores		
	no chronic bronchitis no airflow limitation (n = 10)	no chronic bronchitis airflow limitation (n = 8)	chronic bronchitis airflow limitation (n = 12)
S.A.D. item			
Cellular infiltration	0.9 ± 0.4	0.5 ± 0.3	0.7 ± 0.2
Muscle hypertrophy	0.9 ± 0.3	0.4 ± 0.2	0.7 ± 0.2
Fibrosis	1.2 ± 0.2	1.9 ± 0.2	1.8 ± 0.3
Goblet cell hyperplasia	0.7 ± 0.3	1.0 ± 0.3	1.3 ± 0.3
Total S.A.D. score	3.7 ± 0.9	3.8 ± 0.5	4.5 ± 0.7

Values are expressed as means ± S.E.M.; no significant differences existed between the groups.

Current cigarette smoking was reported by 8 of the 12 patients with, and by 5 of those without symptoms of chronic bronchitis. Three subjects without chronic bronchitis smoked pipe or cigars. Only one subject had never smoked, all others had ceased smoking at least 2 years previously (Table 1).

The response to histamine *in vivo* was measured in 8 patients without and 3 with chronic bronchitis. The $PC_{20}FEV_1$ was greater than 64 mg/ml in all patients except one of the 3 with chronic bronchitis (32 mg/ml) (Table 1).

A wide range of S.A.D. was found in patients both with and without chronic bronchitis. Severe S.A.D. was slightly more common in chronic bronchitis, but group mean S.A.D. scores did not differ significantly (Table 2). Current cigarette smoking was not associated with the S.A.D. score within the groups. The scoring system did not take account of the composition of the inflammatory cells in the airway wall, but most were neutrophilic granulocytes and mononuclear cells (Figure 1); eosinophils were rare.

The results of the *in vitro* experiments are summarised in Table 3. The $-\log EC_{50}$ values of methacholine, histamine and LTC_4 were similar in patients with and without chronic bronchitis or airflow limitation. As a consequence of the randomisation procedure, histamine was applied as the first drug to 15 of the 30 airway preparations, and methacholine and LTC_4 to 9 and 6 strips respectively. Because of the relatively large variation of T_{max} within-subjects[12], no conclusions about possible differences in T_{max} between groups of patients can be drawn from the small numbers of observations. There was, however, a tendency for histamine and methacholine T_{max} to be higher in airways from patients with chronic bronchitis and airflow limitation (Table 3). Because no differences were found in S.A.D. scores or EC_{50} values between groups of patients, the relation between S.A.D. and EC_{50} was analysed on the total population. The methacholine $-\log EC_{50}$ showed a weak but significant negative correlation with the total S.A.D. scores ($P < 0.05$), that is severely diseased airways tended to be less sensitive to

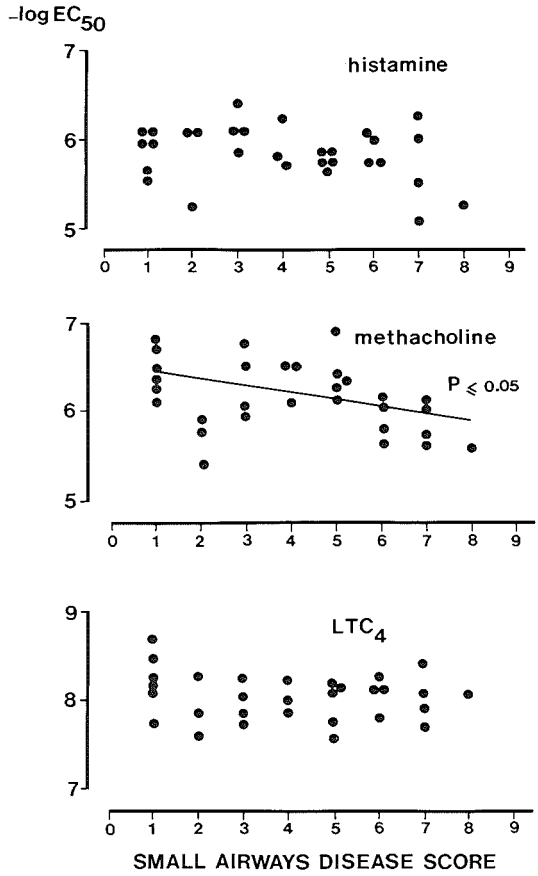

Figure 2. Relation between -log EC_{50} values for histamine, methacholine and LTC_4 and total S.A.D. scores for 30 bronchiolar strips from 30 patients with and without chronic bronchitis.

Table 3. Results of *in vitro* studies

		no chronic bronchitis no airflow limitation (n = 10)	no chronic bronchitis airflow limitation (n = 8)	chronic bronchitis airflow limitation (n = 12)
Histamine	-log EC_{50}	5.77 ± 0.09	5.96 ± 0.07	5.79 ± 0.11
	T_{max}	51 ± 8 (4)	73 ± 21 (5)	113 ± 21 (6)
Methacholine	-log EC_{50}	6.22 ± 0.13	6.22 ± 0.13	6.04 ± 0.10
	T_{max}	89 ± 13 (4)	78, 91 (2)	176 ± 68 (3)
LTC_4	-log EC_{50}	8.24 ± 0.08	8.00 ± 0.06	7.89 ± 0.08
	T_{max}	53, 79 (2)	18 (1)	40 ± 12 (3)

[a] T_{max} values are expressed as mg isometric force per mg dry tissue weight. The number of observations is indicated between brackets; only the first CCRC in each strip was included (see methods). Mean ± S.E.M. or, in case of less than 3 observations, individual values are given.
EC_{50} values are the mean ± S.E.M. from 30 measurements.

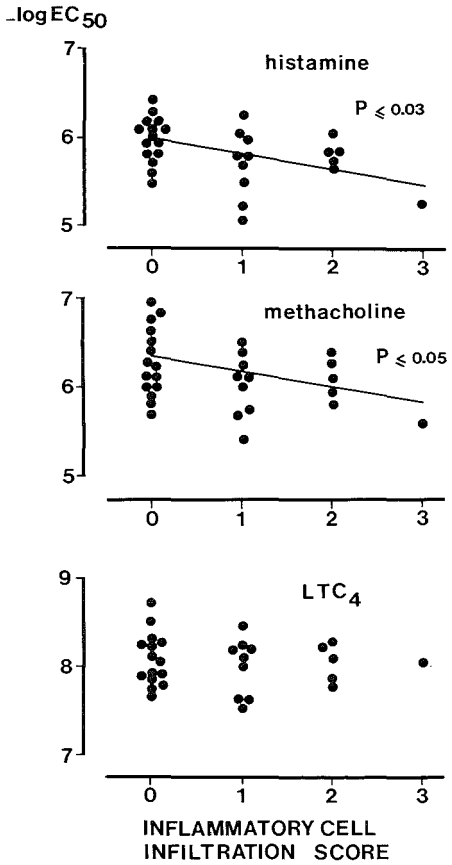

Figure 3. Relation between -log EC_{50} values for histamine, methacholine and LTC_4 and inflammatory cell infiltration scores of 30 bronchiolar strips from 30 patients with and without chronic bronchitis.

methacholine. No relation between histamine or LTC_4 EC_{50} and total S.A.D. scores was found (Figure 2). Multiple regression analysis showed that inflammatory cell infiltration was the only single variable that correlated negatively with both methacholine -logEC_{50} ($P<0.05$) and histamine -logEC_{50} ($P<0.05$)(Figure 3). T_{max} values for histamine, methacholine and LTC_4 were not related to the severity of S.A.D.

Discussion

Our results indicate that S.A.D. and, in particular, inflammatory cell infiltration of the airway wall in patients with and without chronic bronchitis is not associated with increased sensitivity or contractility of small airway smooth muscle to histamine, methacholine and LTC_4 *in vitro*. Infiltration of inflammatory cells

was negatively correlated with histamine and methacholine sensitivity, which was the opposite of what we expected. In accordance with our earlier findings[6], histamine T_{max} tended to be higher in airways from patients with chronic bronchitis and airflow limitation than in airways from normals. In this study mean T_{max} for methacholine was also higher in the patients with chronic bronchitis. Statistical analysis was not carried out because of the small numbers of observations and the large intrinsic variation in T_{max}[11]. There was no correlation between T_{max} and S.A.D. scores.

Airway inflammation scores were determined before tissues were stored and possibly the storage procedure and further processing of the bronchiolar strips altered smooth muscle function, conceivably by removing humoral factors or inflammatory cells. We did not repeat the histological examination after the experiments, but preliminary investigations did not show any deleterious effects of storage or organ bath studies on the histological appearance of the airways. Moreover, we and others have shown that prolonged storage in cold buffer does not affect *in vitro* human airway smooth muscle responsiveness to histamine or methacholine[11,14]. It therefore seems unlikely, though the possibility cannot be excluded, that we have missed an effect of inflammation on smooth muscle function as a result of factors relating to tissue processing.

In the dog, ozone-induced acute airway inflammation is associated with airway hyperresponsiveness[4,15] and this has been linked to granulocyte infiltration in the airway wall. In the guinea pig, ozone and cigarette smoke also produce acute airway inflammation and hyperresponsiveness[16,17], though in this model hyperresponsiveness preceded cellular infiltration and the granulocytes persisted longer than the increased responsiveness. Possibly in chronic inflammation airway hyperresponsiveness does not persist if inflammatory cells continue to be present after the acute phase, and this would explain the contradictory results in human and animal airways regarding inflammation and responsiveness. Our findings are in agreement with the clinical observation that patients with severe chronic non-asthmatic airway inflammation, such as occurs in cystic fibrosis or bronchiectasis, usually have only mild bronchial hyperresponsiveness or none at all, despite large numbers of leukocytes in their airways.

Our results confirm and extend findings by Armour et al, who did not find an association between inflammation and *in vitro* responsiveness of human airways to histamine and carbachol in a smaller group of patients[7,8]. In the present study and in other reports on *in vivo* and *in vitro* responsiveness of human airways, there has been only a weak correlation or none between the amount of airway smooth muscle and airway responsiveness[3,7,18,19]. This suggests that not only the amount, but also the intrinsic properties of airway smooth muscle may determine airway responsiveness. In addition, the arrangement of smooth muscle, rather than its amount, is likely to be responsible for a large part of the high intrinsic variability of T_{max} in spirally cut strips.

Mullen et al found that in non-asthmatic smokers inflammation of membranous bronchioles correlated with *in vivo* airway responsiveness[3]. These authors also found a relation between bronchiolar inflammation and *in vivo* airway caliber,

but the effects of these factors on airway responsiveness seemed to be independent[3]. Our present findings do not support a direct effect of inflammation on bronchiolar smooth muscle function. It remains possible, however, that peripheral airway inflammation modulates airway responsiveness *in vivo* by other mechanisms, for example, by altering autonomic regulation processes. Furthermore, our results do not exclude an effect of inflammation on smooth muscle function in central airways. Such an effect seems unlikely, however, as in the study by Mullen et al no relationship was found between inflammatory changes in central airways and *in vivo* bronchial responsiveness[3]. In the tissues we studied, we found no S.A.D. scores above 8. Although our results do not point in this direction, we can not exclude the possibility that smooth muscle sensitivity or contractility would have been abnormal in bronchioles with a higher score. This seems very unlikely, however, because severe S.A.D. is not a characteristic of subjects with a greatly increased *in vivo* airway responsiveness[3].

We, like others[20], found S.A.D. to a variable degree in lungs from both patients with and patients without chronic bronchitis and airflow limitation, with a large overlap. This indicates that histological signs of small airways disease are not specific for chronic bronchitis, and may occur in otherwise healthy, non-smoking symptomless subjects with normal lung function. This, together with the finding that small airway inflammation had no relation to *in vitro* small airway smooth muscle function, suggests that airway hyperresponsiveness in chronic bronchitis is not due to a direct effect of chronic inflammation on airway smooth muscle function. Our present findings do not exclude the possibility that inflammation may potentiate the mechanisms that underly airway hyperresponsiveness in asthma. We have recently reported an increased contractility of isolated airways from an asthmatic patient showing eosinophilic infiltration of the bronchial mucosa and smooth muscle hypertrophy[21]. Airway hyperresponsiveness in asthma and chronic bronchitis has different characteristics, however, and may have a different pathogenesis[22].

More studies are needed to establish to what extent airway inflammation or alternative mechanisms, such as mucosal edema, changes in central airways, potentiation of local or central nervous reflexes or other abnormalities in neural control contribute to the pathogenesis of airway hyperresponsiveness in chronic bronchitis and asthma.

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Chapter 13

Comparison of maximal bronchoconstriction *in vivo* and airway smooth muscle responses *in vitro* in non-asthmatic humans

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Summary

We tested the hypothesis that maximal bronchoconstriction in humans *in vivo* is limited by the maximal contractility of airway smooth muscle by comparison of complete *in vivo* and *in vitro* dose-response curves to methacholine in 10 non-asthmatic subjects who were scheduled for thoracotomy because of malignancies. The provocative dose of methacholine that produced a 10 and 20% decrease of baseline FEV_1 ($PD_{10,20}$ FEV_1) and the maximal fall in FEV_1 (M_{FEV1}) at the response plateau to inhaled methacholine were determined prior to surgery. Small airway smooth muscle preparations, obtained from the 10 resected lung tissue specimens, were examined *in vitro* to determine the sensivity (-log EC_{50}) and maximal isotonic shortening (S_{max}) to methacholine. In addition the relaxation responses to the β-agonist l-isoproterenol were measured. The degree of small airways disease (S.A.D.) was examined histologically.

Nine subjects showed a maximal response plateau to inhaled methacholine *in vivo*. The maximal fall in FEV_1 at the plateau was $26 \pm 3\%$. All airway smooth muscle preparations ($n = 30$) contracted to methacholine (-log EC_{50} 5.94 ± 0.09; S_{max} 1320 ± 219 μm) and relaxed to l-isoproterenol (-log EC_{50} $7.60 \pm 0,11$; maximal relaxation (R_{max}) $87 \pm 3\%$). No significant correlations were found between S_{max} or R_{max} of the airway smooth muscle *in vitro* and the M_{FEV1} *in vivo*, and between -log EC_{50} for methacholine or l-isoproterenol *in vitro* and PD_{10} or PD_{20} FEV_1 for methacholine *in vivo*. The severity of S.A.D. was significantly correlated with the degree of baseline airflow limitation ($p < 0.05$), but not with *in vivo* or *in vitro* responses to methacholine. These results suggest that, in non-asthmatic subjects, the maximal bronchoconstriction and sensitivity to inhaled methacholine *in vivo* are not determined by the maximal contractility and sensitivity, respec-

tively, of airway smooth muscle to methacholine, are not due to an impaired relaxation response to β-receptor stimulation and are not related to the severity of S.A.D.

Key words: human airway smooth muscle, bronchial responsiveness, methacholine, isoproterenol, small airways disease, bronchoconstriction, chronic airflow limitation.

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Introduction

Patients with asthma or chronic airflow limitation often have an increased sensitivity of airway narrowing to inhaled methacholine or histamine[1]. The aetiology of this non-specific bronchial hyperresponsiveness is not clear. To determine whether abnormalities of airway smooth muscle (ASM) play a role in bronchial hyperresponsiveness, a number of studies has been done in which contractile responses of isolated airways from subjects with bronchial hyperresponsiveness were measured[2–8]. These studies have shown that ASM from subjects with varying degrees of bronchial hyperresponsiveness, as defined by the position of the dose-response curve *in vivo*, responded *in vitro* within a remarkably narrow sensitivity range, which could not explain the wide variation of *in vivo* responsiveness.

ASM could contribute to bronchial hyperresponsiveness not only by a higher sensitivity, which means that a given contractile state of the muscle is reached at a lower stimulus intensity, but also by a higher contractility to a given stimulus (i.e. a higher maximal response), or by a combination of these two mechanisms[9–10]. Furthermore, a decreased response of ASM to relaxing agents may underly an increased bronchial responsiveness *in vivo*[11,12]. Studies of human ASM function *in vitro* have mainly focussed on sensitivity, usually measured as the effective concentration of an agonist that produces 50% of the maximal ASM response (EC_{50}). The maximal responses *in vitro* were considered less valuable because of a high intrinsic variability[8]. Theoretical considerations and a number of recent papers, however, have led to the view that ASM may contribute to bronchial hyperresponsiveness, both in asthma and chronic airflow limitation, by an increased maximal contractility[9,13–16].

Bronchial responsiveness *in vivo* is commonly measured by determining the provocative dose (PD) or concentration (PC) of an inhaled bronchoconstrictor that produces a given worsening of a lung function parameter, e.g. a 10% fall in FEV_1 (PD_{10} FEV_1).

It has recently been shown that a reproducible maximal bronchoconstrictor response can be measured *in vivo* after administration of a relatively high dose of inhaled histamine[17] or methacholine[18] in non-asthmatic subjects and in patients with mild asthma or chronic airflow limitation. In these patients, the *in vivo* dose-response curves reach a plateau at relatively mild degrees of airway narrowing, the cause of which is still not clear[9]. We have shown that, by using a carefully standardised method and a well-defined ASM preparation, it is also possible to measure maximal contractile responses of ASM *in vitro* with sufficient accuracy to detect differences between subjects[19,20].

In the present study, we have tested the hypothesis that the maximal response to methacholine in humans *in vivo* is limited by the maximal isotonic contractility of ASM to methacholine, or is related to differences in ASM relaxability. This was done by comparison of complete *in vivo* dose-response curves to methacholine with *in vitro* dose-response curves to methacholine and l-isoproterenol in patients who underwent thoracotomy and lung resection. Because airway inflammation, muscle hypertrophy or epithelial changes may modulate airway responsiveness[1], we have also examined the airways by light microscopy and compared these morphological findings with the various functional data.

Methods

Subjects and design

Ten adults who were scheduled for thoracotomy because of bronchial malignancies underwent lung function measurements and a methacholine inhalation test within a 2 weeks period prior to surgery, and specimens of bronchial tissue were studied *in vitro* within 24 h following surgical resection. Anthropometric and pre-operative lung function data are given in Table 1. None of the subjects had a history of asthma and none had used bronchodilators or corticosteroids.

In vivo studies

Methacholine inhalation tests were performed as described previously[18,21]. Methacholine chloride, dissolved in normal saline, was administered via a DeVilbiss 646 nebuliser (output 0.13 $mlxmin^{-1}$) in doubling doses up to a mouth dose of $3.4x10^{-4}M$. The aerosol was inhaled by tidal breathing during 2 min at 5 min intervals. The forced expiratory volume in 1 sec (FEV_1) was measured with a dry rolling-seal spirometer (Mijnhardt Volugraph), and the mean of 3 reproducible values was taken to calculate the baseline values. The response to inhaled methacholine was expressed as a percentage fall in FEV_1 from baseline value. The provocative doses that caused a 10 or 20% fall in FEV_1 (PD_{10} and PD_{20} FEV_1 respectively) were obtained by linear interpolation and expressed in μM of nebulised methacholine. A response plateau was considered if the FEV_1 values after 2 or more highest methacholine doses decreased less than 5%. The maximal response to methacholine (M_{FEV1}) was calculated by averaging all data points on the plateau, and was expressed as a % fall from baseline[18].

Table 1. Patient characteristics

Case	Sex	Age (yr)	Height (m)	Atopy[a]	Current smoking	Lung function[b] I.V.C. L	I.V.C. % pred.	T.L.C. L	T.L.C. % pred.	$F.E.V._1$ L	$F.E.V._1$ % pred.	$F.E.V._1/V.C.$ %
1	M	23	1.63	0	never	3.47	92	4.94	99	2.86	86	82
2	M	68	1.75	0	+	4.05	98	6.57	95	2.68	87	66
3	M	62	1.54	0	+	3.44	114	5.23	100	2.44	102	71
4	M	63	1.60	0	+	3.40	102	5.54	97	2.70	104	79
5	M	52	1.76	5	+	4.90	106	7.57	108	3.39	94	69
6	F	54	1.76	1	+	4.32	119	6.92	119	3.40	112	79
7	M	72	1.73	0	+	3.56	91	7.42	110	1.72	59	48*
8	F	39	1.67	1	ex	3.00	84	5.07	97	1.85	61	62*
9	M	71	1.79	0	cigars	3.77	88	6.43	89	2.68	85	71
10	M	65	1.67	0	+	2.92	79	5.95	95	1.90	68	65*

[a] Atopy: number of positive skin prick tests to common allergens (A.L.K., Copenhagen)

[b] Abbreviations: I.V.C., inspiratory vital capacity; T.L.C. total lung capacity; $F.E.V._1$, forced expiratory volume in 1 sec. Predicted values are from reference 25.

* $>$ 1.64 SD below predicted mean values (25).

In vitro studies.

From all subjects a macroscopically normal piece of the middle lobe of the right lung was obtained within 30 min after surgery, and was quickly transported to the laboratory 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) that was previously gassed with 95% O_2 and 5% CO_2. From each tissue specimen, 2-4 bronchiolar strips (approx. 10th to 13th generation) were dissected and stored overnight as described previously[19]. This procedure has been shown not to affect the response of ASM to cholinergic stimulation[19]. After mounting in the muscle bath, strips were allowed to equilibrate for 2 h at 37°C in Krebs-Henseleit buffer that was continuously gassed with 95% O_2 and 5% CO_2. Throughout the experiments, an isotonic load of 500 mg was applied, because in preliminary experiments we found that this load stretched bronchiolar strips from non-asthmatic subjects to their optimal lengths. Contractile responses were measured isotonically with high precision angular position transducers (Penny & Giles)[22]. Cumulative concentration-response curves (CCRC) to methacholine were made by adding the drug to the organ bath in volumes smaller than 50 μl, to obtain final bath concentrations of 10^{-8} to 10^{-4} M. On each strip, 2 successive methacholine CCRC were made, separated by an interval of 2 h to restore a stable baseline. Only the second curve was used for calculations, because we have previously found that the first curve on a strip always had a considerably lower maximal response than all subsequent CCRC, which were then reproducible[19]. After the second CCRC, methacholine was applied at its EC_{50}, and, when the resulting contraction had reached a plateau, a CCRC to l-isoproterenol (10^{-10} to 10^{-5} M) was made. After maximal relaxation by l-isoproterenol (10^{-5} M), a high concentration of EDTA (3.10^{-4} M) was used to obtain full relaxation. From the measured values, CCRC were constructed by means of a computerised iterative fitting method. The following parameters were derived from each CCRC: -log EC_{50}, as a measure of sensitivity, and maximal shortening (S_{max}, in μm) or maximal relaxation (R_{max}) as a percentage of maximal relaxation in the presence of EDTA as measures of contractility and relaxability, respectively.

Pieces of lung tissue were processed for histological examination and the severity of small airways disease (S.A.D.) was assessed in membranous bronchioles using a modification of the subjective grading method described by Wright et al[23,24]. Scores (0 = normal, 1-3 = increasingly abnormal) were determined for each of the following items: inflammatory cell infiltration, fibrosis, smooth muscle hypertrophy and goblet cell hyperplasia, by comparing with pictorial reference standards. The sum of these four scores was taken as the total S.A.D. score.

Drugs

Methacholine and l-isoproterenol were purchased from Janssen Pharmaceuticals, Belgium; EDTA from Sigma, USA; buffer chemicals were analytical grade from E. Merck, FRG. Drugs were dissolved in 0,9% NaCl, prepared fresh daily and kept on ice for the duration of the experiments.

Data analysis

The results of 2 to 4 *in vitro* experiments were averaged within subjects before being used in statistical calculations. The significance of the between-subjects variation of S_{max} *in vitro* was estimated by means of analysis of variance and the F-test in the 8 subjects from whom 3 or more strips were examined ($\alpha = 0.05$). The relation between *in vivo* and *in vitro* experimental data was analysed by calculation of Spearman's rank correlation coefficient (two tailed, $\alpha = 0.05$). All values are presented as means $\pm$ S.E.M.

Results

In vivo studies

Baseline lung function measurements showed mild to moderate airflow limitation in 3 of the 10 subjects and normal values (within $\pm$ 1.64 SD from predicted[25]) in the remaining 7 (Table 1). Eight subjects currently smoked cigarettes. All subjects reached a PD_{10} and 7 a PD_{20} FEV_1 (Table 2, Figure 1). A plateau in the dose-response curve was reached in 9 subjects. In one patient, no plateau could be measured because of severe degree of airway obstruction during the test. This subject (no.7) had the worst baseline FEV_1/VC (48%). Case no 3 showed a response plateau between 21.3 and 170 μM of nebulised methacholine, but showed a 5.9% further decline after 340 μM. We considered this nevertheless as a plateau, because the response at 340 μM was only 3.6% lower than the response after 85.1 μM (Figure 1). Patients 2, 7 and 10 had a PD_{20} FEV_1 in the asthmatic range (i.e. < 10.6 μM), although there was no clinical history of wheezing attacks. Two of these 3 patients had significant baseline airflow limitation (Table 1). The mean fall from baseline FEV_1 after the highest methacholine doses was $26 \pm 3\%$. Individual results of the methacholine inhalation tests are given in Table 2.

In vitro studies

A total of 30 bronchiolar strips was examined. All preparations responded concentration-dependently to methacholine and l-isoproterenol. The -log EC_{50} to methacholine showed a range within 1 log unit (5.47 to 6.40; mean: 5.95 ± 0.09). The S_{max} to methacholine varied much more from 105 to 2198 μm (mean: 1320 ± 219 μm). Analysis of variance was performed on the results of experiments in which 3 or more strips within-subjects were studied and the outcomes indicated a significant between-subjects variation ($p < 0.05$, F-test). For relaxation responses to l-isoproterenol, the -log EC_{50} ranged between 6.80 and 7.91 (mean: 7.60 ± 0.11) and R_{max} varied between 64% and 96% (mean: $87 \pm 3\%$). Individual values are given in Table 2. The relation between the maximal plateau bronchoconstriction *in vivo* (M_{FEV1}) and maximal contraction *in vitro* (S_{max}) to methacholine is depicted in Figure 2. No correlation was found (Spearman ρ -0.06, $p > 0.10$). Also, the R_{max} to l-isoproterenol was not correlated with M_{FEV1} *in vivo* (Spearman ρ -0.33, $p > 0.10$). Measures of sensitivity *in vivo* (PD_{10} FEV_1, PD_{20} FEV_1) were

Table 2. Bronchial responses *in vivo* and *in vitro*

Responses to methacholine *in vivo*						Responses to methacholine and isoproterenol *in vitro*			
Case	PD_{10} FEV_1 (μM)	PD_{20} FEV_1 (μM)	D_{PL} (μM)	D_{max} (μM)	M_{FEV1} (%)	Methacholine −log EC_{50}	S_{max}(μm)	L-isoproterenol −log EC_{50}	R_{max} (%)
1	85.1	–	85.1	340.0	11.9	5.47	962	7.89	96
2	3.9	8.2	42.6	85.1	36.7	5.59	105	7.49	93
3	8.8	19.2	85.1	340.0	24.1	6.10	1099	6.80	91
4	3.1	13.8	85.1	340.0	33.7	5.95	605	7.86	84
5	15.6	67.2	42.6	340.0	19.9	6.40	1821	7.62	93
6	16.0	–	21.3	340.0	14.0	5.97	1290	7.77	82
7	0.5	2.9	–	10.6	38.3	5.96	1038	7.18	64
8	6.2	–	10.1	21.3	16.5	6.16	1925	7.71	89
9	9.1	49.2	42.6	340.0	21.5	5.85	2198	7.91	92
10	1.1	4.9	85.1	340.0	39.3	6.00	2152	7.72	90
Mean ± S.E.M.					25.6 ± 3.3	5.94 ± 0.09	1320 ± 219	7.60 ± 0.11	87 ± 3

Abbreviations: $PD_{10,20}$ FEV_1, provocative methacholine doses that caused a 10 or 20% decline of FEV_1 from baseline values; D_{PL}, dose of methacholine at which the response plateau is reached; D_{max}, maximal dose of methacholine that was given; M_{FEV1}, maximal *in vivo* response to methacholine, expressed as the mean of FEV_1 values on the plateau (% fall from baseline); EC_{50}, effective concentration that produces 50% of the maximal effect; S_{max}, maximal isotonic shortening; R_{max}, maximal relaxation, expressed as a % of relaxation in Ca-free buffer.

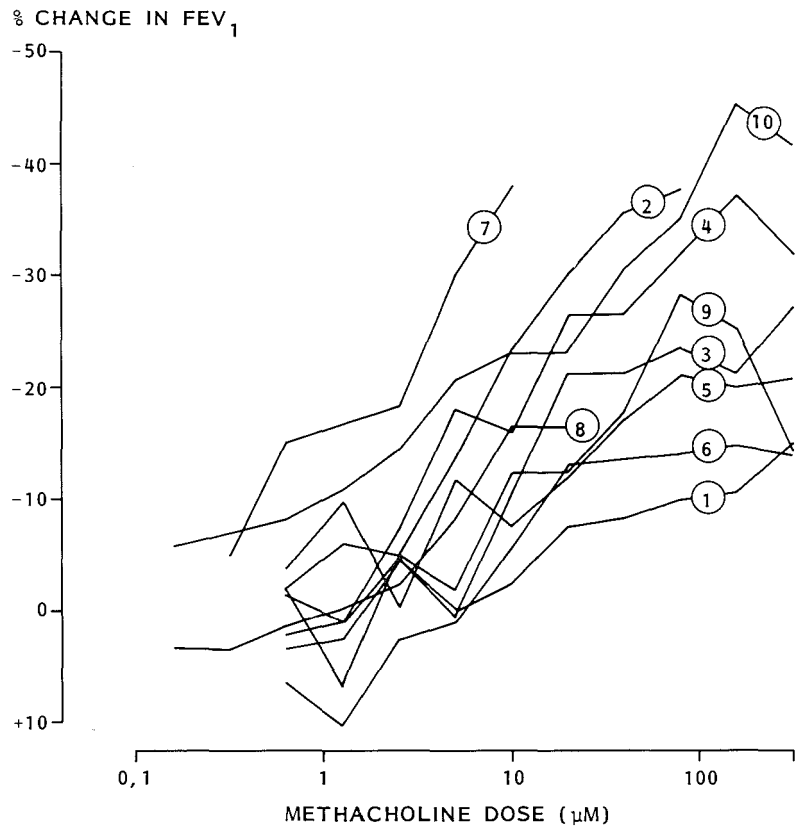

Figure 1. *In vivo* dose-response curves to methacholine. The vertical axis depicts the decline in FEV_1 expressed as a % fall from baseline, and the horizontal axis the dose of nebulised methacholine. Case numbers are indicated in the circles. All subjects except no. 7 exhibited a plateau in their response to methacholine.

not correlated with measures of sensitivity *in vitro* (-log EC_{50} to methacholine or isoproterenol) (Figure 2).

Histological examination of membranous bronchioles showed mild to moderate S.A.D. The most consistent findings were smooth muscle hypertrophy, inflammatory cell infiltration of the bronchiolar wall, predominantly with mononuclear cells, and thickening of the basal membrane. The bronchiolar epithelium was intact and relatively normal in all tissues studied, with only minimal degrees of goblet cell hyperplasia. Especially no obvious differences in mucosal thickness were found between subjects. The S.A.D. score was determined in 8 of the 10 patients, because histological sections contained insufficient numbers of suitable bronchioles in subjects 9 and 10. The mean scores for the separate items and the total S.A.D. scores for patients 1-8 are shown in Table 3. The total S.A.D. score showed a weak but significant ($0.02 < p < 0.05$) negative correlation with

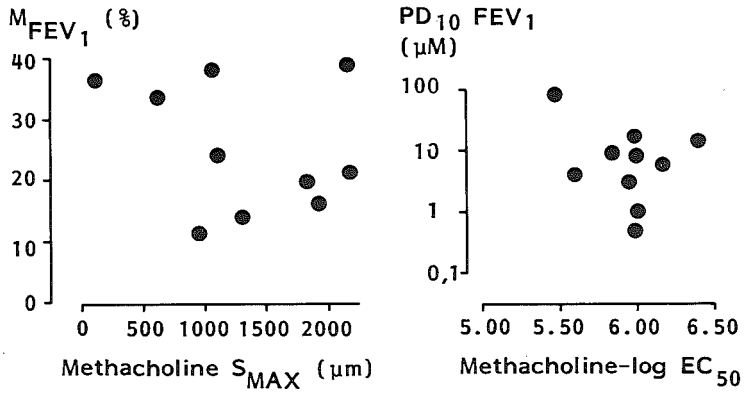

Figure 2. Comparison of *in vivo* and *in vitro* bronchial responses to methacholine Left panel: relationship between the maximal response to methacholine *in vivo* (mean % decline in FEV_1 at the response plateau, M_{FEV1}, vertical axis) and the maximal airway smooth muscle contraction *in vitro* (S_{max}, expressed as µm isotonic shortening, horizontal axis) in the 9 subjects that reached a response plateau. No correlation was found (Spearman ρ -0.06, $p > 0.10$). Right panel: relationship between the position of the dose-response curve to methacholine *in vivo*, as indicated by the PD_{10} FEV_1 (vertical axis) and the sensitivity to methacholine of airway smooth muscle *in vitro* (-log EC_{50}, horizontal axis). No significant correlation was found (Spearman ρ -0.02, $p > 0.10$; $n = 10$).

Table 3. Histological examination of small airways

Case no.	Inflammatory cell infiltration	Fibrosis	Smooth muscle hypertrophy	Goblet cell hyperplasia	Total S.A.D. score
1	1.0	0.0	0.3	1.0	2.3
2	1.3	0.3	0.7	1.2	3.5
3	1.0	0.0	1.3	0.7	3.0
4	1.2	0.1	0.4	0.5	2.2
5	1.3	0.8	1.4	1.3	4.8
6	0.7	0.3	1.3	0.3	2.6
7	1.5	0.5	1.5	1.5	5.0
8	1.5	0.5	1.5	1.0	4.5
Mean ±S.E.M.	1.2±0.1	0.3±0.1	1.1±0.2	0.9±0.2	3.5±0.4

Abbreviation: S.A.D., small airways disease.
All figures are mean scores for 2-24 bronchioles within a subject.

baseline FEV_1/VC (Figure 3). No other significant correlations were found between S.A.D. variables and *in vivo* or *in vitro* measures of bronchial responsiveness.

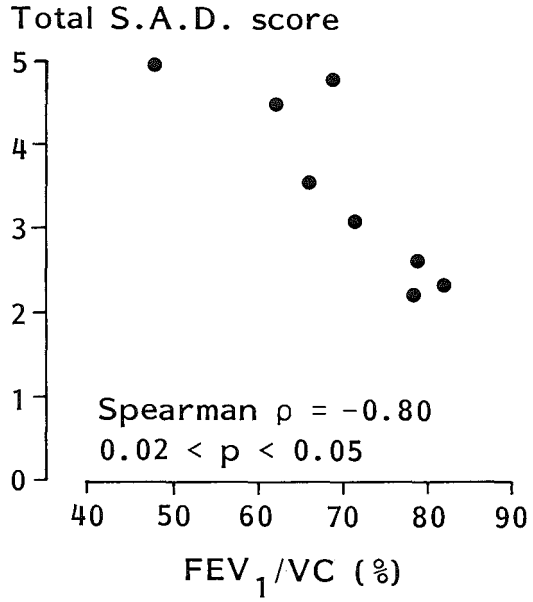

Figure 3. Relation between mean total S.A.D. scores and baseline airway caliber, expressed as FEV_1/VC%.

Discussion

The present results show that, in non-asthmatic subjects, the response of the airways to inhaled methacholine *in vivo* is not related to the responses of isolated ASM to either methacholine or isoproterenol *in vitro*. This was not only shown for the position of the dose-response curves, which confirms previous reports[4,5,7], but also for the maximal responses, which has not been reported before.

Several reports in the literature have indicated that ASM is not more sensitive to e.g. histamine or methacholine in subjects with nonspecific airway hyperresponsiveness than in normals[2–8]. In addition, no relationship between PD *in vivo* and maximal isometric responses *in vitro* has been found[2–8]. However, the *in vivo* dose-response curves that were obtained in these previous studies made it impossible to determine 'sensitivity' in the pharmacological sense because a maximal effect was not measured. Moreover, human ASM has a relatively high intrinsic within-subject variability of maximal responses *in vitro*, which made it often impossible to draw valid conclusions on this aspect of ASM function[2–8].

Recent insight in airway mechanics has led to the view that an increased maximal contractility might be an important aspect of airway hyperresponsiveness[9]. Therefore it is relevant to compare maximal responses of the airways *in vivo* and *in vitro*. Maximal bronchoconstriction can be measured *in vivo* in normals, in patients with mild asthma and in subjects with chronic airflow

limitation by administering relatively high doses of methacholine or histamine by inhalation[17,18,21,26]. The maximal response (e.g. decline in FEV_1) thus obtained is, however, not merely a reflection of ASM contractility, but also depends on many other variables, such as aerosol deposition, penetration of the agonist through the mucosa, clearance of the agonist, baseline airway caliber, elastic load of the airway wall and lung parenchyma, inflammatory changes (e.g. mucosal swelling) in the airways or on humoral or neural regulation processes[9,12]. In contrast, contractile responses of isolated airways reflect mainly ASM function[19]. We can elucidate the role of ASM contractility on the maximal degree of airway narrowing *in vivo* by comparison of *in vivo* and *in vitro* responses in the present study.

The sensitivity (-log EC_{50}) of ASM can be measured accurately and reproducibly *in vitro*[19,20]. The relatively high intrinsic variation within-subjects of maximal responses, however, has been a problem. In the present study we have tried to limit the variability of absolute ASM responses *in vitro* by a careful dissection technique and by using only bronchioles obtained from the same site in the lung (the right middle lobe). We have previously shown that it is possible to detect differences between-subjects regarding maximal responses by using a similar method[13,14,19,20]. Because of the many confounding variables that influence the airway responses *in vivo*, the lack of correlation between *in vivo* and *in vitro* data is not surprising. From the factors that co-determine FEV_1, several may in particular be relevant to the comparison of *in vivo* and *in vitro* maximal responses to methacholine in this study. Baseline airway caliber is an important determinant of bronchial hyperresponsiveness in non-asthmatic subjects *in vivo*[27]. This seems also true for some subjects in the present study (Table 1). The contraction of a spiral strip *in vitro* will certainly not reflect this factor. Furthermore, part of the *in vivo* response may result from central airway constriction, whereas the *in vitro* experiments were only done using peripheral airways. It is not likely, however, that airway hyperresponsiveness *in vivo* is mainly a central airway phenomenon[28]. In addition, lung elastic recoil is likely to be a major determinant of the maximal degree of airway narrowing *in vivo*[29], but not *in vitro*. Finally, airways smooth muscle function is dependent on the initial length of the muscle. It is not known whether in humans ASM functions near its optimal length *in vivo*[9]. We did not perform length-tension experiments before each CCRC *in vitro*. Therefore, differences in length-tension relationships of airways from subjects with varying degrees of *in vivo* bronchial responsiveness may have biased our results and those of others. Further studies are needed to clarify this point. Airway smooth muscle, especially of non-cartilaginous airways, probably functions semi-isotonically *in vivo*[9]. Unlike most previous studies, the present results were obtained by measuring the isotonic shortening of the ASM preparations, which seemed more relevant than isometric tension recording. It is not likely, however, that in this type of study both methods will produce important differences[30].

We found no correlation between the -log EC_{50} and R_{max} to l-isoproterenol of isolated ASM and airway responsiveness *in vitro*. Such a relation was suggested by studies of Paterson et al[31] and Goldie et al[32], who used autopsy tissue from asthmatics, most of whom died during an asthma attack, and by a recent report by Cerrina et al[8] who examined airways from elderly asthmatics who also had considerable baseline airflow limitation. We have recently reported normal relaxation responses of asthmatic human airways to isoproterenol *in vitro*[14] and found a normal β-adrenergic relaxability in airways from patients with chronic obstructive pulmonary disease (unpublished observation). The present results are also in agreement with those of Taylor et al[7] who, in 10 subjects, found no relationship between non-asthmatic airway hyperresponsiveness *in vivo* and relaxation responses of isolated bronchi to the β_2-agonist salbutamol. Therefore, differences in responsiveness to β_2-adrenergic stimulation may not explain the degree of maximal airway narrowing that is possible *in vivo*.

We found a negative correlation between the degree of baseline airflow limitation and the severity of S.A.D., which confirms earlier reports[33,34]. Both the airflow limitation and the S.A.D. score were, however, not significantly correlated with the *in vivo* and *in vitro* responsiveness to methacholine. Similarly, we previously found no relation between the sensitivity or contractility of human bronchiolar smooth muscle to methacholine *in vitro* and the degree of bronchiolar inflammation[24]. Mullen and coworkers have found a weak significant correlation between both baseline airflow limitation and S.A.D. scores, and *in vivo* airway responsiveness to histamine or methacholine[34]. It seems that such relationships are more likely to reach significance if more patients are studied than we have done in the present report.

There is a recent interest in the role of mucosal thickening or edema in the pathogenesis of airway hyperresponsiveness[9]. However, no apparent differences in the structure of the epithelium or the submucosal connective tissue were found in bronchioles from the subjects in this study, who had widely varying degrees of airway hyperresponsiveness. This suggests that other mechanisms may be more important.

In conclusion, we found that, in non-asthmatic subjects, the maximal methacholine-induced bronchoconstriction *in vivo* is not related to the maximal isotonic contractility of ASM to methacholine *in vitro*. We also confirmed earlier reports that the position of the *in vivo* response curve to methacholine is not related to the sensitivity of the ASM to methacholine, and is not determined by the relaxation response of ASM to β_2-receptor stimulation. The lack of correlation may however also be due to the many variables that can influence the outcomes of *in vivo* measurements. Although the severity of histologically determined S.A.D. correlated significantly with baseline airflow limitation, no relation was found between S.A.D. variables and measures of bronchial responsiveness to methacholine *in vivo* and *in vitro*. The present results do not exclude a possible role of ASM abnormalities in asthma, because bronchial hyperresponsiveness in asthma and in chronic airflow limitation has different characteristics[27,35], and

may therefore have a different etiology. Moreover several reports have suggested increased ASM contractility in asthma[14–16]. Further studies are needed to investigate whether alternative mechanisms, such as decreased lung elasticity, increased airway compressibility[9] or abnormal autonomic control mechanisms are responsible for the variation in bronchial sensitivity and maximal response in humans.

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Chapter 14

Leukotriene generation and small airway smooth muscle responsiveness in human lung

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Summary

The hypothesis was tested that a high endogenous leukotriene (LT) production in the lung causes desensitisation of airway smooth muscle to LT. The synthesis of LTB_4, C_4, D_4 and E_4 by human lung tissue, obtained at thoracotomies, after stimulation with Ca-ionophore was assessed by HPLC. Functional studies of small airway smooth muscle from the same tissue specimens were carried out using LTC_4 and methacholine as the contracting agents. Generation of LTB_4, C_4, D_4 and E_4 was 453 ± 82, 84 ± 15, 71 ± 27 and 40 ± 16 pmol/g fresh tissue respectively (mean $\pm$ S.E.M., $n = 10$). All airway smooth muscle preparations responded to LTC_4 in a concentration dependent way with a -log EC_{20} of 8.56 ± 0.13, a -log EC_{50} of 7.95 ± 0.08 and a T_{max} of 82 ± 11 mg force/mg tissue weight, corresponding to $79 \pm 4\%$ of the maximal response to methacholine (mean $\pm$ S.E.M.; 27 preparations from 10 patients). No correlations were found between any of the functional parameters (-logEC_{20}, -logEC_{50}, T_{max} to LTC_4 and methacholine) and the amounts of LT's generated by the lung tissue. Furthermore airway smooth muscle contractility was not significantly reduced after repeated exposure of bronchiolar strips to LTC_4 *in vitro*. These findings suggest that the contractility of human peripheral airway smooth muscle is not decreased in lungs that can produce relatively large amounts of sulfidopeptide LT's.

Key words: human airway smooth muscle, bronchial responsiveness, leukotrienes, methacholine.

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Introduction

An increased bronchoconstriction in response to non-allergic stimuli is a characteristic of asthma and occurs frequently in chronic obstructive pulmonary disease (COPD)[1]. The mechanism of this bronchial hyperresponsiveness is not clear, although airway inflammation or increased bronchial smooth muscle contractility may play a role[2,3]. It is likely that leukotrienes (LT's) are involved in the pathogenesis of asthma and COPD[4,5]. Human lung parenchyma, bronchial epithelium, mast cells, alveolar macrophages and leukocytes can produce the LT's B_4, C_4, D_4 and E_4, which induce bronchial smooth muscle contraction, mucus secretion, capillary leakiness and recruitment of inflammatory cells. LT's may therefore contribute to airways obstruction and bronchial hyperresponsiveness both directly, by an effect on airway smooth muscle, and indirectly, by enhancement of airway inflammation[5,6]. It has recently been shown that the relative potencies of the sulfidopeptide LT's C_4, D_4 and E_4 as compared with methacholine or histamine are markedly lower in asthmatics than in normals[7,8], i.e. asthmatics are less hyperresponsive to LT's than to methacholine or histamine. This discrepancy could result from smooth muscle desensitisation to LT's, but not to methacholine or histamine. This could possibly be due to chronic exposure of smooth muscle to LT's in inflamed airways. The present study was done to test the hypothesis that a high endogenous LT synthesis by the lung causes specific desensitisation of airway smooth muscle to LT's. For this purpose, we measured the LT generation by human lung tissue *in vitro* and related these data to the maximal contractile responses and the sensitivity of isolated airway smooth muscle preparations to LTC_4 and methacholine. Furthermore, we examined whether repeated exposure to LTC_4 produced tachyphylaxis in isolated human airways.

Methods

Human lung tissue was obtained from 10 patients at thoracotomies. Preoperatively, the medical history was taken and patients underwent lung function studies, including the determination of the inspiratory vital capacity (VC) and the forced expiratory volume in 1 sec (FEV_1). Immediately after surgical resection, a piece of macroscopically normal lung tissue was collected in Krebs-Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, CaCl 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.55) gassed with 5% CO_2 and 95% O_2. For the measurements of LTB_4, C_4, D_4 and E_4 synthesis, lung parenchyma was kept at 4°C, prepared free of macroscopically visible blood vessels, airways and pleura and chopped in pieces of approx. 0.5 mm^3 using a mechanical tissue chopper. At least 10 g of chopped parenchyma was washed in 25 ml of fresh aerated buffer, followed by 2 min centrifugation at 400 g, three times. Finally, the pellet was resuspended in 25 ml of aerated cold buffer to which the Ca-ionophore A 23187 ($4x10^{-6}$ M), glutathion ($2x10^{-6}$ M) and arachidonic acid ($13x10^{-6}$ M),

as a substrate, were added. The tissue fragments were incubated at 37°C for 10 min while the buffer was stirred (900 rpm) and gassed. At the end of the 10 min incubation, known amounts of ^{3}H-LTC$_4$ and ^{3}H-LTD$_4$ were added and the suspension was centrifuged at 1400 g for 10 min, at 4°C. The clear supernatants were now processed for High Performance Liquid Chromatography (HPLC) as described previously[9]. Shortly, the supernatant was passed through SEP-PAK C_{18} and silica cartridges (Waters). In the redissolved extract, LT's were detected by reversed-phase HPLC on a Nucleosil 5 C_{18} column (Chrompack, The Netherlands) using a Hewlett-Packard 1082 HPL chromatograph. The absorption at 280 nm (LTC$_4$, D$_4$ and E$_4$) and 270 nm (LTB$_4$) was recorded and compared to a standard. Samples were collected and radioactivity measured in order to calculate the recovery of LT's. The amount of LT recovered was expressed in pmol LT per g fresh tissue. The reproducibility of LT measurements with this method has been determined in a previous study[9]. Small airway smooth muscle preparations were dissected and stored overnight as described previously[10]. Two or 3 airway smooth muscle strips from each patient were mounted in 10 ml organ baths and allowed to equilibrate for 2 hours under an isometric tension of 0.5 g, at 37°C in aerated buffer. Isometric responses to LTC$_4$ (10^{-10} to 10^{-7} M) were measured with Grass FTO3D force transducers. Cumulative concentration response curves (CCRC's) were made as described previously[11]. When the response to the highest concentration of LTC$_4$ (10^{-7} M) had reached its plateau, methacholine (10^{-4} M) was added to obtain a maximal contraction. From each CCRC the following parameters were derived: -logEC$_{20}$ and -logEC$_{50}$ (the negative logarithm of the concentrations of LTC$_4$ that produced 20% and 50% of the maximal response) as a measure of sensitivity and T_{max} (the maximal tension in the presence of 10^{-7}M LTC$_4$ or 10^{-4}M methacholine, expressed in mg isometric force per mg dry tissue weight) as a measure of contractility. In separate experiments we examined whether repeated exposure of isolated human airways to exogenous LTC$_4$ would cause tachyphylaxis. Under conditions described above, strips were stimulated 6 times with 10^{-8} M LTC$_4$ during 15 min, followed by washing and restoration of baseline tone. Exposures were separated by 30 min intervals. The responses were expressed as a percentage of the initial response of that strip.

Drug sources

Synthetic LT's B$_4$, C$_4$, D$_4$ and E$_4$ were a gift of Dr. J. Rokach (Merck Frosst, Canada). Methacholine hydrobromide was purchased from Janssen, Belgium; Ca-ionophore A 23187 from Hoechst, Calbiochem-Behring, USA; reduced glutathion from ICN, Cleveland, USA; arachidonic acid from Bio Data Corporation, Hatboro, USA and ^{3}H-LTC$_4$ and ^{3}H-LTD$_4$ from the Radiochemical Centre, Amersham, UK. HPLC liquids and buffer chemicals were analytical grade from E. Merck, Darmstadt, FRG.

Data analysis

From each of the 10 tissue specimens, the mean values of -logEC$_{20}$, -logEC$_{50}$

and T_{max} from 2 or 3 LTC_4 CCRC's were used for calculations.The relation between these functional data and the produced quantities of LTB_4, C_4, D_4, E_4 and total sulfidopeptide LT's ($C_4+D_4+E_4$) was examined by calculating Spearman's rank correlation coefficient (ρ)(two tailed $\alpha=0.05$).

Results

Patients

Lung tissue was obtained from 10 male patients. Their mean age was 63 ± 2.1 years (mean ± S.E.M., range: 54 to 72 years). The mean VC was 89 ± 6% of predicted values, FEV_1/VC was 59 ± 5%. All subjects had bronchial carcinoma. Seven had chronic bronchitis, and 7 reported current cigarette smoking. None of the patients had a history suggestive of asthma. Three used bronchodilators (β-agonists, theophylline, ipratropiumbromide) and 1 of these received a short course of dexamethasone prior to surgery.

LT generation by lung tissue fragments

All specimens produced LTB_4, C_4 and D_4. LTE_4 was recovered from 8 out of the 10 supernatants. The individual and mean data are provided in Table 1. Although the quantities of the various LT's varied markedly between individuals, LTB_4 was always the major metabolite recovered. The mean total sulfidopeptide ($C_4+D_4+E_4$) LT synthesis was less than 50% of that of LTB_4 (204 ± 16 and 453 ± 82 pmol/g fresh tissue respectively). Of the sulfidopeptide LT's, LTC_4 was produced in the largest amounts, followed by LTD_4 and LTE_4 (84 ± 15; 71 ± 27; 40 ± 16 pmol/g respectively).

Table 1. *In vitro* synthesis of LT's by lung fragments and functional responses of airway smooth muscle to LTC_4 and methacholine

	LT production[a]					*Functional responses*[b]			
Case	LTB_4	LTC_4	LTD_4	LTE_4	LTC_4 +D4+E_4	-log EC_{20} LTC_4	-log EC_{50} LTC_4	T_{max} LTC_4	T_{max} Methacholine
1	363	69	8	4	81	8.52	7.90	51	75
2	210	105	18	0	123	8.00	7.53	45	67
3	282	60	9	11	80	8.86	8.15	44	51
4	147	44	64	0	108	8.57	8.17	101	116
5	893	181	291	63	535	8.97	8.01	82	97
6	608	57	44	74	175	8.79	8.16	71	86
7	399	53	34	38	125	9.28	8.24	134	160
8	216	66	48	39	153	7.97	7.69	100	105
9	696	153	137	162	452	8.32	7.93	131	149
10	720	51	60	13	124	8.29	7.72	60	123
Mean	453	84	71	40	196	8.56	7.95	82	103
S.E.M.	82	15	27	16	51	0.13	0.08	11	11

[a] LT production is expressed as pmol LT per gram of fresh tissue.

[b] T_{max} is expressed as mg isometric force per mg dry tissue weight.

Responses of airway smooth muscle

Concentration-dependent contractions to LTC_4 were seen in all bronchiolar strips. Values of $-\log EC_{20}$, $-\log EC_{50}$ and T_{max} are given in Table 1. The $-\log EC_{50}$ of LTC_4 varied within 1 log concentration unit (range: 7.53 to 8.24) and was similar to our earlier findings[11]. Methacholine 10^{-4}M always produced an additional increase in tone when LTC_4 responses were at their plateau. The mean increase was $21 \pm 4\%$ (range 5 to 51%) of the maximal response after methacholine (10^{-4}M). The maximal responses to LTC_4 and methacholine were significantly linearly related ($R = 0.89$, $p < 0.001$)(Figure 1). The production of LTC_4 in relation to

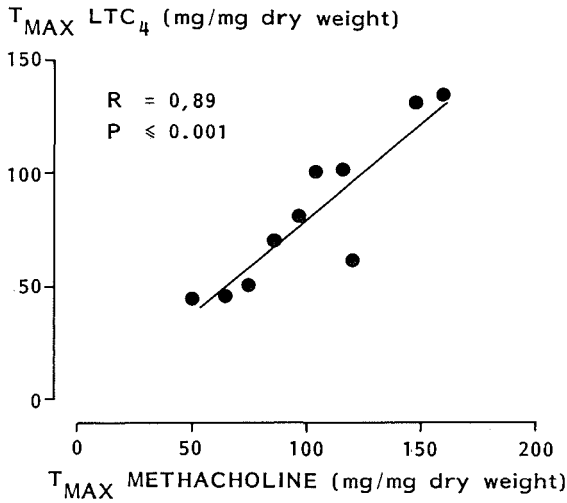

Figure 1. Relation between the T_{max} to LTC_4 (vertical axis) and to methacholine (horizontal axis) in human bronchiolar strips. Each dot represents the mean of 2-3 measurements. T_{max} is expressed in mg force per mg dry tissue weight. The linear correlation coefficient $R = 0.89$ ($p < 0.001$).

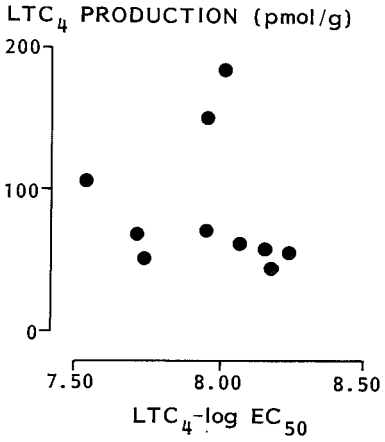

Figure 2. Relation between LTC_4 production, expressed in pmol LT per g of fresh tissue, by human lung fragments (vertical axis) and $-\log EC_{50}$ of LTC_4 in bronchiolar strips (horizontal axis). $-\log EC_{50}$ values are the mean of 2-3 measurements in each subject.

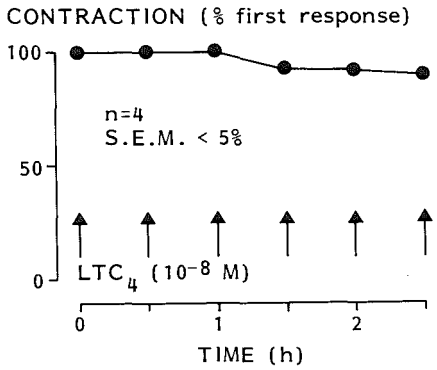

Figure 3. Effect of repeated LTC_4 administration 10^{-8}M to isolated human peripheral airway smooth muscle strips on isometric force development. Incubations lasted for 15 min until the contractile response had reached a plateau, and were separated by 30 min intervals. Responses to each of 6 stimulations are expressed as a percentage of the first response of a given strip. The mean values of 4 separate experiments are shown, S.E.M. values were smaller than 5%.

EC_{50} of LTC_4 is shown in Figure 2. No significant correlation was found (Spearman $\rho = 0.16$, $p > 0.10$). Also no significant correlations were found between the amounts of LTB_4, C_4, D_4 or E_4 or of the total sulfidopeptide LT's ($C_4 + D_4 + E_4$) synthetised and any of the other functional parameters ($-\log EC_{20}$, $-\log EC_{50}$ and T_{max}). Contractile responses after repeated exposure to LTC_4 are shown in Figure 3. No tachyphylaxis was found.

Discussion

The present results show that the sensitivity and contractility of airway smooth muscle to LTC_4 are not decreased in lungs that have a high capacity to produce LT. Because some conversion of LTC_4 in LTD_4 and E_4 might have occurred during the processing of the chopped tissue, we also examined the relation between the total amounts of sulfidopeptide LT synthetised and EC_{50} and T_{max} to LTC_4, but no significant correlations were found. Repeated LTC_4 exposure *in vitro* caused no tachyphylaxis. These findings do not support our initial hypothesis that a high endogenous LT synthesis leads to desensitisation of airway smooth muscle.

The results of the experiments with chopped lung tissue are in agreement with previous studies[12,13]. The predominant synthesis of LTB_4 suggests that alveolar macrophages and/or neutrophilic granulocytes were activated, because these cell types have been shown to produce mainly LTB_4[14]. This LT has chemotactic activity, and produces bronchial hyperresponsiveness in dogs[15]. Furthermore, LTB_4 has been demonstrated in bronchial secretions from patients with chronic bronchitis, that did not contain LTC_4 or D_4[16]. LTB_4 is, however,

only a weak and indirectly acting bronchoconstrictor[17]. The sulfidopeptide LTC_4 and D_4 are highly potent bronchoconstrictors both *in vivo* and *in vitro*[7,11,12]. Therefore we chose LTC_4, which probably acts via the same receptor as LTD_4[18], for the *in vitro* functional experiments. We found a close correlation of T_{max} to methacholine and LTC_4 (Figure 1), which is unlike the discrepancy between methacholine and LTC_4-induced airway obstruction in asthmatics *in vivo* found by Adelroth[7]. Furthermore, it was previously found in non-asthmatic subjects that, *in vivo*, LTC_4 is roughly 500-1000 times more potent than methacholine in causing airway narrowing[7], whereas the potencies *in vitro* differ only by a factor 100[11]. These differences in relative and absolute potencies of LTC_4 and methacholine *in vivo* and *in vitro* suggest that *in vivo* LTC_4 acts not only directly on airway smooth muscle, but has also indirect effects that cause an additional airway narrowing. Such effects may include the release of bronchoconstricting substances in the lungs, or induction of vagal reflex bronchoconstriction.

In the present study, we did not measure bronchial responsiveness *in vivo*, which could be relevant to airway smooth muscle function. It is possible that airway smooth muscle of asthmatics differs from that of normals or patients with chronic bronchitis. In a previous study, we have found that the response to LTC_4 of airway smooth muscle from subjects with and without chronic bronchitis and airflow limitation was not different[19]. We have also documented, however, that airway smooth muscle from an asthmatic patient had a highly increased maximal response to LTC_4 and methacholine, with normal EC_{50} values[20]. Fresh asthmatic lung tissue is rarely available for pharmacological studies. In the present study, we have therefore examined lung tissue from non-asthmatic subjects with and without chronic bronchitis and airflow limitation; it cannot be excluded that different results would have been obtained had asthmatic airways been studied.

A bias may have resulted from the various medications used by some of the patients prior to surgery. Several drugs may have suppressed LT generation (dexamethasone) or LT effects (β-agonists, theophylline). This seems unlikely, however, because our thorough washing procedure should have removed any residual drug activity before the start of the experiments[10], and airways from patients using bronchodilators were not obviously less responsive to LTC_4 and methacholine than those of patients who used no bronchoactive drugs.

In conclusion, we found that airway smooth muscle from human lung tissue that produced relatively large amounts of LT was not less sensitive to LT, and furthermore that no tachyphylaxis to exogenously added LTC_4 occurred. This suggests that the relatively low potency of LTC_4 in asthmatics with a highly increased bronchial responsiveness to methacholine may not be due to tachyphylaxis of airway smooth muscle to endogenous LTC_4.

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Chapter 15

Human asthmatic airway responses *in vitro*

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Summary

Responses of human isolated airways, obtained from an asthmatic patient at thoracotomy, were compared to responses of airways from 10 non-asthmatic subjects *in vitro*. Asthmatic bronchiolar strips exhibited increased contractile responses to histamine, methacholine and leukotriene (LT) C_4, and relaxed normally in response to l-isoproterenol and forskolin. The non-adrenergic inhibitory response to electric field stimulation was low, but was not significantly different from control values. These results suggest that increased airway smooth muscle contractility may contribute to the asthmatic airways hyperresponsiveness in this patient.

Key words: airway smooth muscle, asthma, bronchial hyperresponsiveness, non-adrenergic inhibitory system, methacholine, histamine, leukotriene C_4, isoproterenol, forskolin.

De Jongste JC, Mons H, Bonta IL, Kerrebijn KF. *In vitro* responses of airways from an asthmatic patient. Eur J Respir Dis 1987;71:23-29. Reprinted with permission of Munksgaard International Publishers.

Introduction

The mechanisms which underlie the nonspecific bronchial hyperresponsiveness in asthmatics are poorly understood. A number of studies have compared *in vitro* responses of isolated human airways to *in vivo* responsiveness. A narrow *in vitro* response range was found in airways from patients who exhibited a wide variation of *in vivo* responses to inhaled methacholine or histamine[1-7]. The

subjects included in these studies were, however, not asthmatic but had various degrees of COPD, and usually smoked. Bronchial hyperresponsiveness may have a different pathogenesis in asthma and COPD. In COPD, bronchial hyperresponsiveness is well correlated with baseline airway caliber, whereas in most asthmatics this relation is poor or absent[8–9]. Furthermore, bronchial responsiveness in COPD is usually moderate, whereas in asthma the response can progress to severe bronchoconstriction. There have been few reports on functional *in vitro* studies of human asthmatic airways[5,6,10–14]. We now report here our measurements of *in vitro* responses of isolated airways from an asthmatic patient. In addition to pharmacological experiments, we performed electrical field stimulation (EFS) in order to assess the function of the non-adrenergic inhibitory (NAI) system, which probably represents the only inhibitory innervation of human airways[15]. We have compared our findings to our own measurements of *in vitro* responses of airways from subjects without asthma or COPD.

Patients and methods

The patient was a male aged 41 years, who had had asthma since early childhood. House mite atopy was confirmed by skin tests and IgE-RAST. He had never smoked. Lung function was normal except for a largely reversible bronchoconstriction (inspiratory vital capacity (VC) 5810 ml (112% of predicted value); forced expiratory volume in 1 s (FEV_1) 3530 ml (61% of VC) before, and 70% of VC after inhalation of 0,4 mg salbutamol). The histamine responsiveness, determined by inhalation of doubling concentrations of histamine by tidal breathing for 30 s, was highly increased (provocative concentration that produced a 20% fall in FEV_1 1 mg/ml; normal value $>$ 16 mg/ml). Maintenance treatment consisted of inhaled beclomethasone. No bronchodilators had been used prior to surgery. A resection of the apical segment of the right upper lobe was performed for a solitary coin lesion, which turned out to be a tuberculoma.

In vitro studies

Lung tissue was collected immediately after surgical resection in ice-cold Krebs-Henseleit buffer (composition in mM : NaCl 118, KCl 4.7, $CaCl_2$ 2.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.55), aerated with 95% O_2 and 5% CO_2. Strips of segmental bronchus (internal diameter 3-4 mm) and of bronchioles (internal diameter 1 mm) were dissected carefully, as described previously[16]. Bronchiolar strips were stored overnight and studied the next day for their response to pharmacological stimulation. Bronchial strips were examined on the day of surgery for their response to EFS.

Pharmacological stimulations were carried out as described previously[17]. Cumulative concentration-response curves (CCRC) were made using histamine, methacholine and leukotriene (LT)C_4 as contracting agents. Tension changes were measured with Grass FTO3D isometric force transducers, employing a baseline tension of 500 mg. Maximal responses (T_{max}) were expressed as mg

isometric force per mg dry weight of the bronchiole. The EC_{50} (the molar concentration of an agonist that produces 50% of the maximal effect) was derived from each CCRC by means of a computerized iterative curve fitting method. Relaxation of methacholine EC_{50}-induced contraction by l-isoproterenol and forskolin was measured isotonically using Penny & Giles transducers, because reference values had been obtained previously with the respective techniques[18]. Relaxation responses to l-isoproterenol and forskolin were expressed as a percentage of the difference between methacholine EC_{50} induced contraction and maximal relaxation, determined after each experiment by adding EDTA ($4x10^{-3}$ M) and, after forskolin CCRC, also isoproterenol 10^{-5} M. EFS of bronchial strips was carried out via platinum plate electrodes. Rectangular 0,3 ms pulses of alternating polarity and supramaximal voltage (50 V) were delivered by a custom made tissue stimulator. An isotonic load of 1 g was applied to bronchial strips. In all field stimulation experiments, atropine ($1.2x10^{-6}$M), indomethacin ($6x10^{-6}$M) and the leukotriene (LT) receptor antagonist FPL 55712 ($11.5x10^{-6}$M), were added to the organ bath in order to block the effects of cholinergic nerve stimulation, the production of prostaglandins and the effects of LT formation. This was done because we had previously found that EFS, apart from stimulating cholinergic and NAI nerves, induces production of contracting prostaglandins and leukotrienes by fresh bronchial tissues, which may obscure NAI responses[19]. EFS frequency-response curves were preceded by a histamine CCRC followed by washing; histamine was then applied at its EC_{50} to precontract the airway before EFS. Frequency-response curves were made by applying continuous EFS with stepwise increasing frequency (1 to 50 Hz), and relaxation responses were expressed as described for pharmacological relaxation curves.

A paired experiment was performed simultaneously on a second bronchial strip from the same patient which was pretreated with the nervous conductance blocker tetrodotoxin (TTX, $3x10^{-6}$ g/ml) in order to estimate the role of nerve conduction. Proximal segments were removed from the bronchiolar strips before the *in vitro* experiments and were processed for histological examination. The amount of airway smooth muscle was assessed semi-quantitatively by subjective grading, using a modification of the method described by Wright et al[20]. A score 0-3 was determined for normal (score 0) or increasingly abnormal (score 1-3) airways, using pictorial reference standards.

Data analysis

Responses of asthmatic tissue were compared to reference values, obtained in previous experiments with airways from 10 non-asthmatic subjects (mean age: 59 ± 3 years; VC 104 ± 4%; FEV_1/VC 75 ± 2%, mean $\pm$ S.E.M.), who had no respiratory symptoms prior to surgery[18]. Geometric mean values (for contractile responses) or individual values (for relaxation responses) of asthmatic tissues were compared to the mean values and 95% confidence limits of non-asthmatic airways.

Table 1. Responses of human asthmatic and non-asthmatic airways *in vitro*

	$-\log EC_{50}$			T_{max} (mg force/mg dry weight)		
	histamine	methacholine	LTC_4	histamine	methacholine	LTC_4
asthmatic[a]	5.93 ± 0.28	6.29 ± 0.26	8.06 ± 0.13	321 ± 99	301 ± 109	222 ± 83
normals[b] (n = 10)	5.85 (5.34–6.36)	6.24 (5.35–7.13)	8.09 (7.65–8.53)	52 (26–76)	100 (0–231)	74 (0–163)
	$-\log EC_{50}$			R_{max}[d]		
	l-isoproterenol	forskolin	EFS (EF50)[c]	isoproterenol	forskoline	EFS
asthmatic	7.44	6.77	2.4	92	84	46
normals[b] (n = 10)	7.25 (5.48–9.02)	6.80 (6.29–7.31)	2.1 (0.4–3.8)	92 (67–100)	95 (82–100)	71 (33–100)

[a] Geometric mean values ± S.E.M. of triplicate measurements for each agonist.
[b] Geometric mean values and 95% confidence limits.
[c] EF_{50} = the frequency (Hz) that produces 50% of maximal relaxation in a given strip.
[d] R_{max} = maximal relaxation, expressed as a percentage of the difference between histamine or methacholine-induced contraction and maximal relaxation in the presence of isoproterenol 10^{-5}M and EDTA 4×10^{-3}M.

Results

The results of the pharmacological experiments are shown in Figures 1 and 2. Contractile responses to histamine, methacholine and LTC_4 were significantly higher in asthmatic ($n=3$ for each agonist) compared to non-asthmatic airways ($n=10$). The absolute contractile responses of asthmatic airways showed a large scatter, which was not unexpected, because of the relatively high intrinsic variability of T_{max} in human bronchiolar strips[17]. The EC_{50} values of asthmatic and non-asthmatic airways were similar (Table 1). No differences were found in the relaxation responses to both isoproterenol and forskolin between one

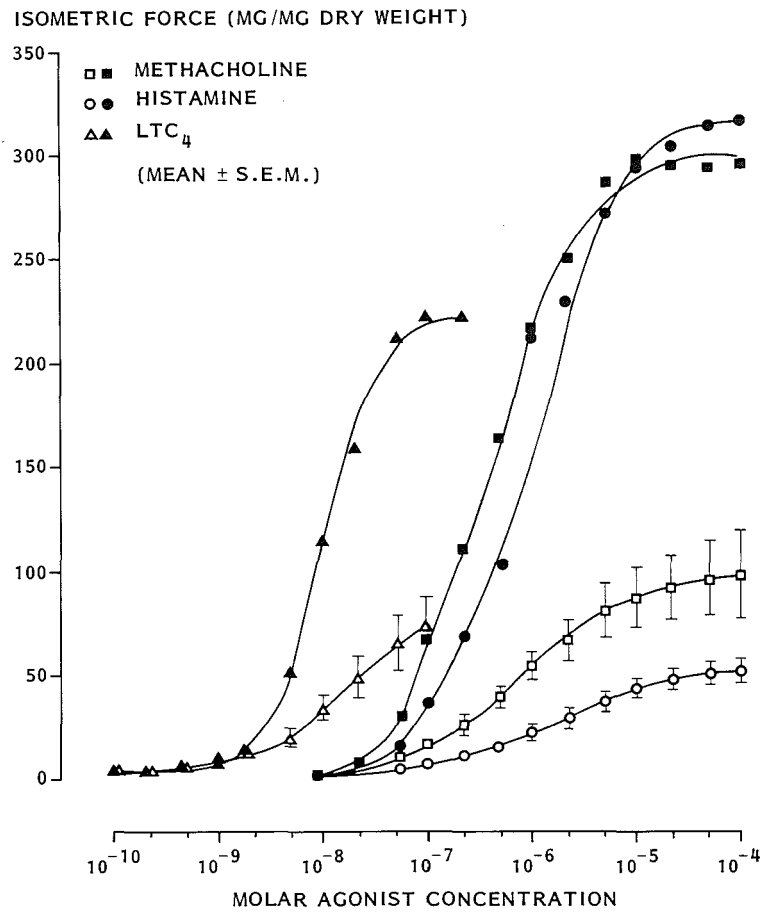

Figure 1. Cumulative concentration-response curves of human asthmatic (closed symbols) and non-asthmatic (open symbols) bronchiolar strips to histamine, methacholine and leukotriene C_4. Asthmatic and non-asthmatic curves are from 1 and 10 subjects respectively; asthmatic curves are the mean of triplicate measurements for each agonist.

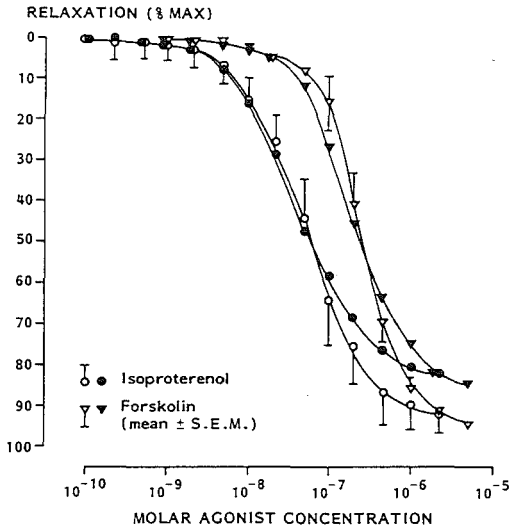

Figure 2. Cumulative concentration-response curves of human asthmatic (closed symbols) and non-asthmatic (open symbols) bronchiolar strips to l-isoproterenol and forskolin as relaxing agents. Asthmatic curves are from 1 bronchiolar strip each, nonasthmatic curves are the mean of 10 separate experiments. Relaxations are expressed as a percentage of the difference between initial methacholine EC_{50}-induced tone and maximal relaxation in calcium-free buffer containing EDTA ($4x10^{-3}$ M) and isoproterenol 10^{-5}M.

asthmatic and 10 non-asthmatic tissues (Table 1, Figure 2). EFS produced frequency-dependent relaxations in the two asthmatic and 10 non-asthmatic bronchi. Individual responses of the asthmatic and mean frequency- response curves of 10 non-asthmatic airways with and without TTX are shown in Figure 3 and in Table 1. The maximal NAI-response of one asthmatic bronchus (46% of histamine EC_{50}-induced contraction) was considerably less then the mean maximal response of 10 non-asthmatic tissues ($71 \pm 6\%$) but fell within the 95% confidence limits of the normal values. In the presence of TTX, only a minor relaxation (14%) remained in the other asthmatic bronchus, whereas in 10 non-asthmatic airways the mean relaxation was reduced to $55 \pm 8\%$ (NS).

Histological examination of three asthmatic airways showed characteristic abnormalities such as derangement of the epithelium, thickening of the basal membrane, goblet cell hyperplasia and eosinophil infiltration in the submucosal layer; smooth muscle hypertrophy (score 3) was always a prominent feature (Figure 4). The mean muscle hypertrophy score of 10 non-asthmatic airways was 0.6 ± 0.3 (mean $\pm$ S.E.M., range 0-2).

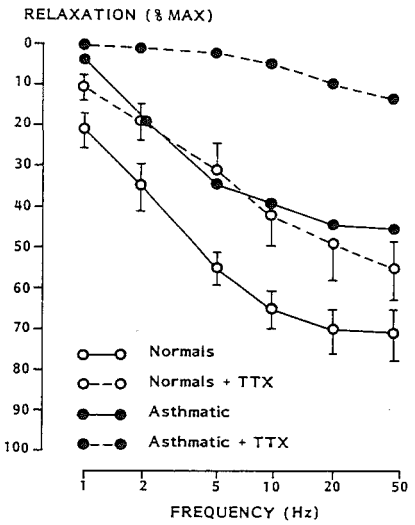

Figure 3. Non-adrenergic inhibitory responses of human asthmatic (closed symbols) and non-asthmatic (open symbols) segmental bronchus to electric field stimulation in the absence (continuous lines) and presence (broken lines) of the nervous conductance blocker tetrodotoxin ($3x10^{-6}$ g/ml). Asthmatic curves are from one bronchial strip each, non-asthmatic curves are the mean of 10 separate experiments. The vertical axis depicts relaxation responses as a percentage of the difference between initial histamine EC_{50}-induced contraction and maximal relaxation in calcium-free buffer containing EDTA ($4x10^{-3}$M) and isoproterenol 10^{-5}M. All experiments were performed in the presence of atropine ($1.2x10^{-6}$M), indomethacin ($6x10^{-6}$M) and FPL 55712 ($11.5x10^{-6}$M).

Discussion

In bronchial tissues from an asthmatic patient we found significantly increased contractile responses to histamine, methacholine and LTC_4, and normal relaxations to isoproterenol and forskolin. NAI relaxations to EFS were low but not significantly different from control values. The patient was well characterized by clinical history, the demonstration of atopy, a reversible bronchoconstriction and a highly increased bronchial responsiveness to histamine. This asthmatic represents a rare case because he had no features of chronic bronchitis, never smoked and underwent lung tissue resection without having bronchial malignancy.

There are only few reports in the literature on *in vitro* functional responses of isolated asthmatic human airways[5,6,10–14]. Increased contractile responses to histamine, carbachol and LTC_4 have been observed by Schellenberg et al[13] and selective hyperresponsiveness to histamine was found in a single asthmatic bronchus by Schellenberg and Foster[12]. Histamine and carbachol or methacholine responses of asthmatic cases reported by Paterson et al[10], Roberts et al[5,6], and Cerrina et al[14], however, were similar to those of non-asthmatic airways. There are important differences in patient characteristics and *in vitro* methodology

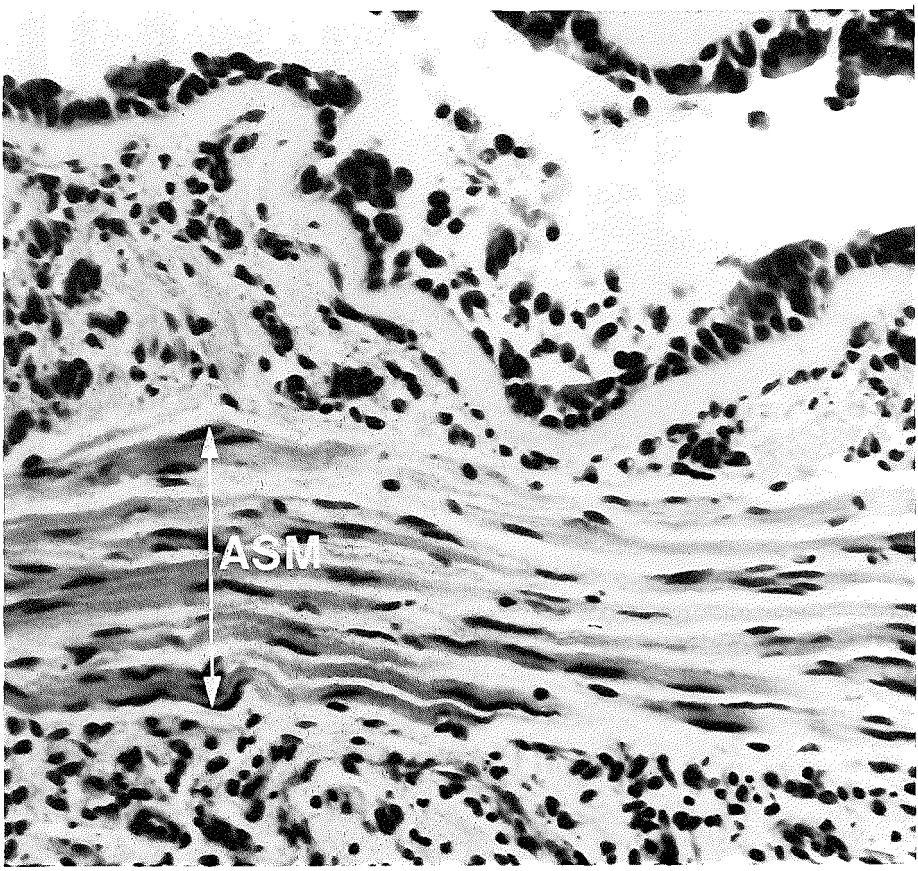

Figure 4. Photomicrograph of a section through an asthmatic bronchus, showing epithelium derangement, thickening of the basal membrane, infiltration of the submucosa with inflammatory cells, including many eosinophils, and severe hypertrophy of airway smooth muscle (ASM). Stain: May-Grünwald-Giemsa; magnification: 200x.

between these studies that make it difficult to compare the results. In contrast to the present case, most asthmatics in two of the previous studies were elderly subjects with chronic bronchitis and more or less severe airflow obstruction, who smoked cigarettes and used bronchodilators[5,14]. These factors may influence *in vivo* and, perhaps, *in vitro* airway responsiveness[8,9,18]. The asthmatic lung tissue studied by Paterson et al[10] was obtained 4-16 h post mortem. Prolonged hypoxia and acidosis may have attenuated the absolute responses to contractile agonists, in spite of unchanged EC_{50} values.

In the present case, a remarkable degree of smooth muscle hypertrophy suggested that the exaggerated isometric force development may have resulted from an increased amount of muscle, although muscle volume and isometric force do not seem to be closely correlated in non-asthmatic human airways[2,3].

In animal experiments, hyperresponsiveness may result from atopic sensitization[21–23]. However, human airways from non-asthmatic individuals with positive skin prick tests and elevated serum IgE levels have not been found to be hyperresponsive *in vitro*[1,2,5,6]. Since atopy often occurs without asthma, the relevance of the animal experiments seems questionable.

Finally, asthmatic airways may be more responsive because they are more inflamed. Animal studies have shown that granulocyte infiltration of airways produces hyperresponsiveness[24] and we found histamine hyperresponsiveness in small airways from patients with chronic bronchitis[18]. However, small airway inflammation is not specific for chronic bronchitis, and is not related to *in vitro* responsiveness[3,4,25]. It cannot be excluded, however, that asthmatic airway inflammation, which may be different from airway inflammation in chronic bronchitis, has an effect on human airway smooth muscle function.

In two previous reports, a defective isometric response of human asthmatic airways to β-adrenergic stimulation has been described[10,14]. Experiments on sensitized 'asthmatic' airways from experimental animals have produced conflicting results in this respect[23,26]. We observed normal isotonic relaxations to isoproterenol and forskolin in two asthmatic bronchiolar strips, indicating normal β-receptor function and sufficient cAMP generation to produce near-maximal relaxations in these asthmatic airways. Again, methodological factors may be responsible for the discrepancy of our results and those of others; it is unlikely, however, that isotonic and isometric measurements produce different results[27].

NAI relaxations of asthmatic bronchus were unusually low, but not significantly different from control values. NAI responses of human asthmatic airways have not been reported before; Taylor et al. found normal NAI responses of non-asthmatic airways from hyperresponsive COPD patients[7]. Clearly, these limited data allow no conclusions on possible NAI dysfunction in asthmatic airways[15].

In conclusion, we found significantly increased absolute force development and normal sensitivities as measured by EC_{50}, to histamine, methacholine and LTC_4, normal relaxation responses to isoproterenol and forskolin, and low NAI relaxations in isolated airways from an asthmatic patient. It is suggested that the increased contractile responses may be the result of smooth muscle hypertrophy, although alternative mechanisms have not been excluded. The characteristic *in vivo* airway hyperresponsiveness in this case seems at least partly due to exaggerated responses of the airway smooth muscle.

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Chapter 16

Cholinergic and non-adrenergic inhibitory nerve-mediated responses of isolated human airways from patients with and without COPD or asthma

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Summary

Abnormalities in the balance between the excitatory and inhibitory components of the autonomic innervation of the airways may contribute to the airways obstruction and hyperresponsiveness in chronic obstructive pulmonary diseases (COPD) and asthma. This balance is reflected by the nerve-mediated biphasic contraction-relaxation response of isolated human airways to electric field stimulation (EFS). We measured the responses to EFS of human airway smooth muscle preparations from 10 normal subjects, 5 patients with COPD and 2 asthmatics. All airways from normals and COPD patients showed biphasic responses to EFS consisting of a rapid cholinergic contraction followed by a slower non-adrenergic relaxation. Precontraction of airway smooth muscle with methacholine led to a decrease of the cholinergic component; the non-adrenergic inhibitory (NAI) component increased when the airway was precontracted from 0 to 60% of the maximal response to methacholine, and decreased when the strip was further contracted to 100%. For a given contractile state of the muscle, the response to EFS was relatively constant. No differences in nerve-mediated cholinergic and NAI responses were found in airways from normals and COPD patients. The 2 asthmatic airways, however, showed exaggerated contractions to methacholine and EFS. The relaxation response of asthmatic bronchi was reduced. In airways from normal subjects the inflammatory mediators histamine and $PGF_{2\alpha}$ had no effect on the response to EFS but LTC_4 reduced the NAI relaxation response significantly ($P < 0.05$).

These results suggest that the cholinergic/NAI balance in human airways is normal in COPD, but may be abnormal in asthma due to an increased cholinergic response and a reduced activity of the NAI system. Reduced NAI responses might result from exposure to LTC_4.

Key words: airway smooth muscle, human bronchus, electric field stimulation, non-adrenergic inhibitory nerves, cholinergic nerves, asthma, COPD, methacholine, histamine, prostaglandin $F_{2\alpha}$, leukotriene C_4.

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Introduction

In humans, the autonomic nervous system directly controls airway caliber by both a cholinergic excitatory and a peptidergic nerve supply to airway smooth muscle[1]. The peptidergic system in the airways probably contains both excitatory and inhibitory components[1]. Functional studies on human airways, however, have only provided evidence for a non-adrenergic inhibitory (NAI) innervation[1-8]. Obstructive disorders of the gastrointestinal tract have been demonstrated to be associated with a defect of the NAI system. Since the gastrointestinal tract and the bronchi have a similar ontogenesis, it seems likely that NAI defects may also occur in the airways, and give rise to airways obstruction[1,2,6,7]. The role of the NAI system in obstructive airway diseases, however, is difficult to assess because the neurotransmitter is not known, and specific blockers are not available. At present, most evidence suggests that the NAI transmitter might be vasoactive intestinal polypeptide (VIP) or a related peptide[1,5]. However, in guinea pig trachea electric field stimulation (EFS) still produces NAI relaxations in the presence of maximally effective concentrations of VIP[9]. Because the neurotransmitter is not known, the only valid way to study the NAI response of human airways is to measure the response of airway smooth muscle to selective stimulation of NAI nerves. Electric field stimulation (EFS) of human airways *in vitro* produces a biphasic contraction-relaxation (C-R) response, which is due to stimulation of cholinergic and NAI nerves in the bronchial wall[3,4,7,8]. The configuration of the C-R response to EFS is, however, not only determined by the relative contribution of the cholinergic and NAI innervation, but also by the baseline contractile state of the muscle, as has been shown for guinea pig central airways[10], where precontracted airways exhibit small C and large R responses, whereas relaxed airways have large C and absent R responses.

The present study was undertaken to determine the effects of airway smooth muscle contraction on the C-R response to EFS in human airways from patients with and without chronic obstructive pulmonary disease (COPD) or asthma, in order to document a possible cholinergic/NAI dysbalance in obstructive airway disease. In addition, we examined whether the presence of inflammatory mediators changed the C-R response of normal human airways, because inflammation is considered to be a determinant of airways obstruction and hyperresponsiveness[11].

Methods

Patients

Patients, scheduled for pneumonectomy or lobectomy, preoperatively underwent lung function measurements (inspiratory vital capacity, VC, and forced expiratory volume in 1 sec, FEV_1) using a water-locked spirometer. The reversibility of airways obstruction was determined by repeated spirometry after inhalation of 10 mg nebulised isoproterenol. The medical history was taken and a physical examination was done. Subjects were classified as normal when they had no respiratory symptoms and a normal lung function. COPD was diagnosed according to the criteria of the American Thoracic Society, i.e. when patients reported cough and sputum production for two or more years during at least three months each year, and when they had airways obstruction ($FEV_1/VC\ \% < 70$) that did not normalise after inhalation of 10 mg nebulised isoproterenol. Asthma was diagnosed when patients reported episodes of wheezing with acute onset that responded to bronchodilator treatment, had a highly increased responsiveness to inhaled histamine as measured by the fall in FEV_1 after inhalation of doubling concentrations of nebulised histamine, and showed a 10% or more improvement of baseline FEV_1 after 10 mg of nebulised isoproterenol.

In vitro studies

Human lung tissue was obtained at thoracotomies for bronchial malignancy. Immediately after resection, a macroscopically normal piece of lung was collected in 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) aerated with 95% O_2 and 5% CO_2. Bronchial strips (fourth to seventh generation) were carefully dissected free from parenchyma and blood vessels as we have described previously[12], and were mounted in 10 ml double jacketed organ baths at 37°C.

We previously demonstrated that, after this procedure, the function of smooth muscle preparations was stable for many hours after surgery, which allowed reproducible measurements of sensitivity to pharmacological agonists with a small within-subject variability[12,13]. Contractile responses were measured isotonically using high precision angular displacement transducers[14] and employing an isotonic load of 1 gram which stretched the preparations to their optimal length, as found in preliminary experiments. The airway preparations were allowed to equilibrate for 2 h and were washed extensively to remove drugs used during anaesthesia (halothane, O_2/N_2O, fentanyl, pentothal, atropine, pancuronium) and substances possibly liberated during the dissection. After stabilisation of the baseline, all preparations were contracted with methacholine (10^{-8} to 10^{-4} M in 10 fold concentration steps) followed by washing and return to baseline.

Electric field stimulation

Rectangular pulses of alternating polarity were delivered by a custom-made tissue

stimulator via platinum plate electrodes (40x5 mm) glued to both sides of the organ bath parallel to the bronchial strip (electrode distance: 15 mm).

A series of experiments was carried out to determine optimal EFS stimulus characteristics. Voltage-response curves were made employing a constant frequency (30 Hz) and pulse width (0.3 msec) and an increasing voltage (5 to 50 V). Similarly, frequency-response curves (5 to 50 Hz, 30 V, 0.3 msec) and pulsewidth-response curves (0.2 to 0.75 msec, 30 V, 30 Hz) were made employing 30 sec pulse trains. All experiments were done on 5 bronchial strips from 5 different subjects without COPD. Before EFS, a moderate contraction was induced with histamine (10^{-6} M) to allow measurement of the NAI relaxation responses. Both C and R responses were expressed as a percentage of the maximum response in a given strip, and means ± SEM for a given stimulus were calculated.

To confirm the nerve-mediated cholinergic and NAI nature of the biphasic response to EFS, the effects of atropine (10^{-6} M), the nervous conductance blocker tetrodotoxin (TTX, $3x10^{-6}$ g/ml) and the β-receptor blocker timolol (10^{-6} M) were examined in separate experiments.

EFS responses in COPD, asthmatic and normal airways

EFS was applied to airways from patients with and without COPD and asthma in the absence and presence of increasing concentrations of methacholine (10^{-8} to 10^{-4} M) to precontract the airway smooth muscle, as examplified in Figure 1. The inherent high variability of the absolute responses of isolated human airways[12] made normalisation of responses necessary. We therefore followed the procedure proposed by Mansour and Daniel[15] and calculated all responses to pharmacological and field stimulation as a percentage of the difference between 'zero contraction', determined at the end of each experiment by adding L-

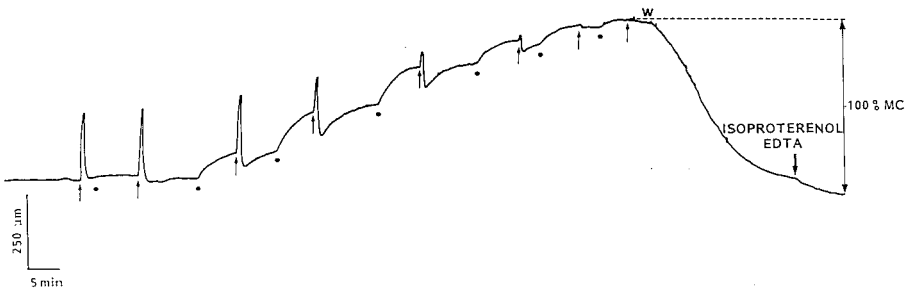

Figure 1. Representative example of a field stimulation experiment in the presence of increasing concentrations of methacholine. Arrows indicate onset of a 30 sec. train of 30 V, 30 Hz, 0.3 msec pulses. Dots indicate when methacholine was added in order to produce baseline elevation (final bath concentrations: 0, 10^{-8}, 10^{-7}, $3x10^{7}$, 10^{-6}, $3x10^{-6}$, 10^{-5} and 10^{4} M). At the end of the experiment, the strip was exposed to Ca-free buffer containing EDTA ($4x10^{-3}$ M) and isoproterenol (10^{-4} M) in order to produce maximal relaxation. The difference between maximal methacholine-induced contraction and maximal relaxation is indicated (100 % MC).

isoproterenol (10^{-4} M) and EDTA ($4x10^{-3}$ M), which relaxed the strips completely, and 'maximal contraction' in the presence of 10^{-4} M methacholine. Responses were expressed as a percentage of maximal contraction (% MC)(Figure 1).

Effects of inflammatory mediators on responses to EFS

Bronchial strips from normal subjects were precontracted by increasing concentrations of either histamine (10^{-8} to 10^{-4} M), prostaglandin (PG) $F_{2\alpha}$ (10^{-7} M to 10^{-5} M), or leukotriene (LT) C_4 (10^{-10} to 10^{-7}M), and EFS was applied when a contraction plateau was reached after a given mediator concentration.

Both the responses to the mediators and to EFS were expressed as a %MC. The maximal contraction (100 % MC) was determined by adding 10^{-4} M methacholine when the response to the highest concentration of a mediator had reached its plateau. From each pharmacological cumulative concentration-response curve (CCRC), the negative logarithm of the effective concentration of an agonist that produced 50% of its maximal effect (-logEC_{50}) was derived by linear interpolation.

Drug sources

Methacholine hydrobromide, histamine dihydrochloride and L-isoproterenol were obtained from Janssen, Belgium; EDTA, TTX and $PGF_{2\alpha}$ from Sigma, USA; Atropine sulphate from Brocacef; Timolol maleate from Merck, Sharp & Dhôme; synthetic LTC_4 was a gift from Merck Frosst, Canada.

Statistical analysis

The measured values of C and R responses to EFS at increasing levels of muscle contraction, induced by methacholine, were plotted against baseline contractile state. From these curves, C and R response amplitudes at 0,10,20 etc. up to 100% MC were obtained by linear interpolation. Mean $\pm$ SEM for C and R responses were calculated in different airway preparations at corresponding contraction levels. Mean C and R values at corresponding baseline contraction levels were compared in strips from normals and COPD patients. The amplitudes of C and R responses of normal airways in the presence of inflammatory mediators were compared to C and R responses at corresponding baseline contraction levels induced by methacholine. The statistical significance of differences in means was determined by Student's t-test for unpaired samples (two tailed, $\alpha = 0.05$). Whenever more strips from a patient were studied, the results of that patient were averaged before being used in statistical calculations.

Results

Patients

Lung tissue was obtained from 10 normals, 5 patients with COPD and 2 asthmatics. The clinical data of these patients are summarized in Table 1. Both asthmatics had a highly increased bronchial responsiveness to histamine (provocative concentration of inhaled histamine that produced a 20% fall in FEV_1:

Table 1. Patient characteristics

	N	Age (yr)	VC (% predicted)	FEV_1/VC (%)	Regularly used medication		Current smoking (n)[a]
Normals	10	57±3	108±6	71±5	—		4
COPD	5	62±3	89±5	53±4	Ipratropium	(1)	2
					Salbutamol	(1)	
					Theophylline	(2)	
Asthma	2	40, 41	107,112	62, 61	Salbutamol	(2)	1
					Theophylline	(1)	
					Beclomethasone	(1)	

Values reported as mean ± SEM

[a] Non-smokers were all ex-smokers who ceased smoking at least 3 years prior to surgery. One asthmatic had never smoked.

1 mg/ml in both subjects). Skin tests and IgE-RAST were positive for house dust mite in one asthmatic and negative in the other. All subjects were operated because of bronchial carcinoma except for one of the asthmatics, who had a coin lesion which turned out to be a tuberculoma.

Determination of optimal EFS stimulus characteristics

All bronchial strips from normals and COPD patients responded to EFS with biphasic responses. Voltage-response curves showed that the C-phase was maximal at 40 V and the R-phase at 30 V (Figure 2A). Frequency-response curves produced maximal C and R responses at 30 and 40 Hz respectively (Figure 2B), and pulsewidth appeared to have a relatively minor effect on both C and R responses, with a maximal C and R response at 0.4 msec (Figure 2C). In all further experiments, 30 sec trains of 30 V, 30 Hz and 0.3 msec pulses were used in order to obtain near-maximal responses. Atropine blocked the C-response completely; TTX, the nervous conductance blocker, blocked the C-phase completely and reduced the R-phase, and timolol, a β receptor-blocker, had no effect on both C and R phases (data not shown).

EFS responses in normal, COPD and asthmatic patients' airways

We examined 10 airway preparations from 10 normals, 10 preparations from 5 patients with COPD, and 2 strips from 2 asthmatics. All strips had some stable intrinsic contractile activity (spontaneous baseline contraction: 11 ± 3 %MC and 18 ± 3 %MC in normal and COPD tissues respectively, NS). EFS produced a C phase of 36 ± 5 %MC in normals and 42 ± 7 %MC in COPD (NS) and an R phase of 2 ± 1% MC in both groups. Inducing contraction with methacholine was followed by a gradual decrease of the C phase, which always disappeared at 100 %MC. The R response increased between 0-60 %MC. The maximum relaxation was 16 ± 3 %MC in normal airways and 17 ± 3 %MC in COPD airways,

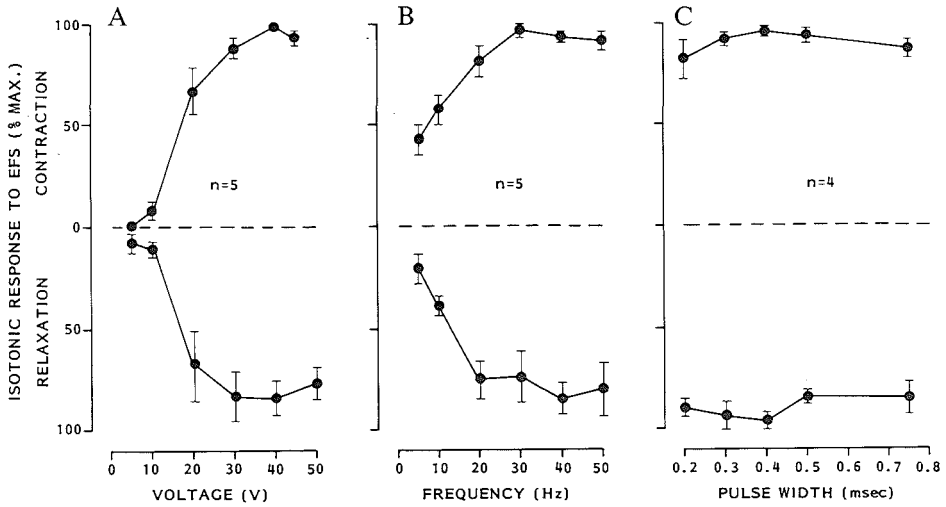

Figure 2. Effect of changes in pulse voltage, -frequency and -width on the contraction and relaxation responses of human bronchial strips. A: effect of voltage (0 to 50 V) with constant frequency (30 Hz) and pulse width (0.3 msec). B: effect of frequency (0 to 50 Hz) with constant voltage (30 V) and pulse width (0.3 msec). C: effect of pulse width (0.2 to 0.75 msec) with constant voltage (30 V) and frequency (30 Hz). Pulse trains of 30 sec. Contractions and relaxations are expressed as a percentage of the maximal response in a given strip. Mean values ± SEM of 4-5 experiments are shown.

at 60 %MC baseline. When the baseline was raised further up to 100% MC, R responses decreased and usually disappeared at 100 %MC (Figure 3). At corresponding levels of induced contraction, C and R responses showed relatively little variation and were not different in normal and COPD airways. The methacholine CCRC was also similar in normal and COPD airways (Figure 4), with a -logEC_{50} of 6.04 ± 0.13 and 6.13 ± 0.14 respectively (NS).

The 2 airways from asthmatic subjects had a spontaneous baseline contraction of 11 and 32 %MC, which was within the 95% confidence limits of the non-asthmatic tissues. EFS produced C phases of 22 and 33 %MC and R phases of 0 %MC. After precontraction with increasing methacholine concentrations, asthmatic airways also showed a decrease of the C response, but the R phase was grossly deficient, only being detectable as a 1 %MC relaxation to EFS at 40 %MC baseline in one of the two subjects' airways (Figure 5).

Methacholine CCRC's in asthmatic airways had a -logEC_{50} of 6.26 and 6.05. The maximal contraction to methacholine of asthmatic airways was, however, roughly 3-4 times higher than that of the most responsive non-asthmatic airways.

Effect of inflammatory mediators

The effect of histamine, $PGF_{2\alpha}$ and LTC_4 on the C-R response to EFS was

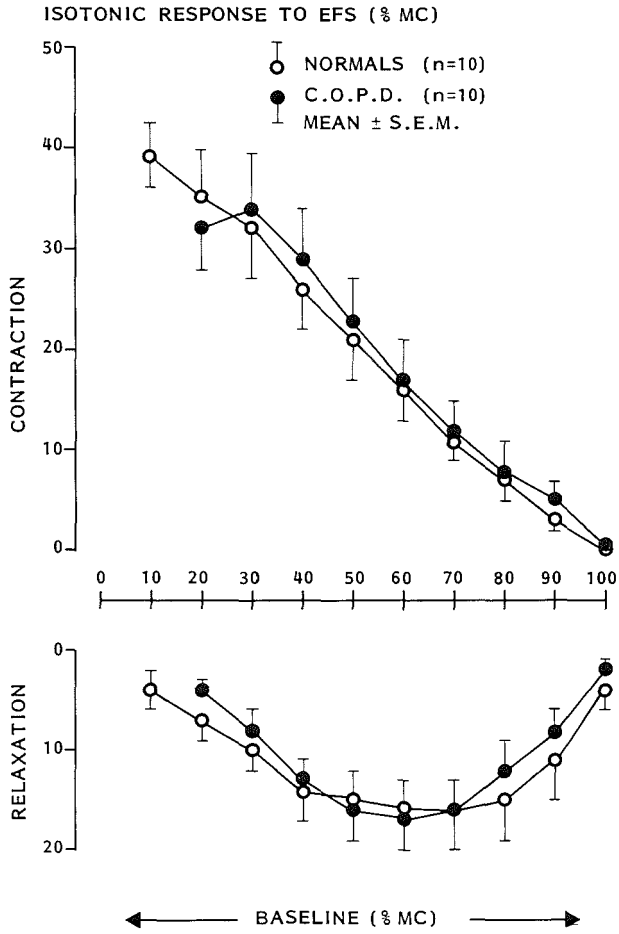

Figure 3. Response to EFS of 10 airway smooth muscle preparations from 10 normals (open circles) and 10 preparations from 5 patients with COPD (closed circles). Contraction and relaxation responses are expressed as % MC, baseline elevation was produced by adding increasing concentrations of methacholine (10^{-8} to 10^{-4} M) as shown in figure 1. At all levels of induced contraction, the contraction and relaxation responses in normals and COPD patients were not different ($P > 0.05$).

measured in 20 bronchial strips from normal subjects. Baseline conditions were not different regarding the patients ages, lung function and smoking habits; the spontaneous baseline contractile state of the bronchial strips was also not different in experiments with the various mediators and methacholine. Histamine, $PGF_{2\alpha}$ and LTC_4 produced CCRC's with a mean -logEC_{50} of 5.92 ± 0.14 ($n = 7$), 6.25 ± 0.31 ($n = 7$) and 7.87 ± 0.13 ($n = 6$) respectively. Maximal responses to histamine (10^{-4}M), $PGF_{2\alpha}$ (10^{-5}M) and LTC_4 (10^{-7}M) were 89 ± 4, 64 ± 7 and 61 ± 7 % MC respectively (Figure 4). The results of the induction of

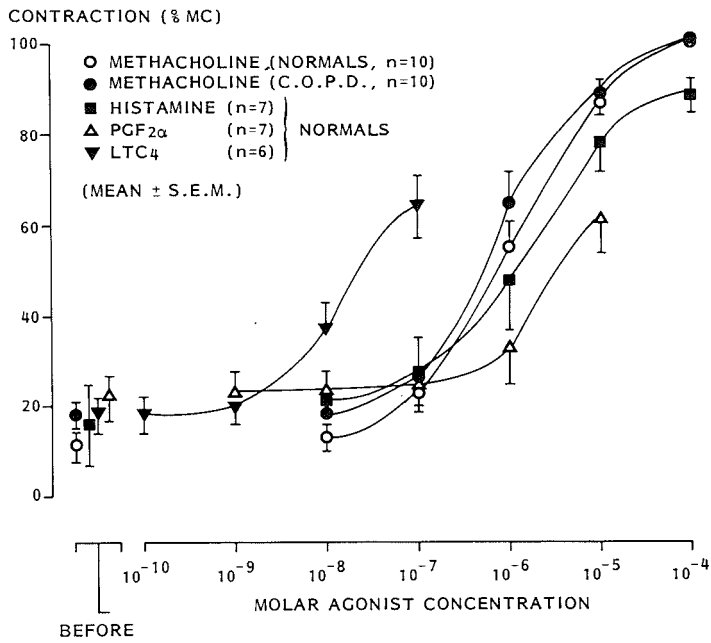

Figure 4. Cumulative concentration-response curves (CCRC) of human bronchi. Methacholine CCRC were made on 10 bronchial strips from 10 subjects without COPD and on 10 strips from 5 patients with COPD; CCRC to histamine, $PGF_{2\alpha}$ and LTC_4 were made on 6-7 strips from subjects without COPD. The vertical axis depicts contractions, expressed as a percentage of the difference between maximal contraction, induced by methacholine, and maximal relaxation by Ca-free buffer containing isoproterenol (see: methods). Spontaneous baseline contractile state of the preparations before each CCRC was similar, as shown left from the curves ('before').

contraction by these mediators on the C-R responses to EFS is shown in Figure 6. Although C responses in the presence of the inflammatory mediators (Figure 6) tended to be lower than C responses in the presence of methacholine (Figure 3), the mean values at corresponding baseline levels were not significantly different. R responses in the presence of LTC_4 were reduced compared to R responses in the presence of methacholine (shown in Figures 6 and 3 respectively). This reduction was present onwards from 30 %MC baseline, and reached significance at 60 %MC (the mean R phase was 16 ± 3 %MC in the presence of methacholine and 7 ± 2 %MC in the presence of LTC_4 at 60 %MC, $p < 0.05$). The mean concentration of LTC_4 necessary to produce 60 %MC was 3.47×10^{-8}M. Histamine and $PGF_{2\alpha}$ had no effect on the R phase.

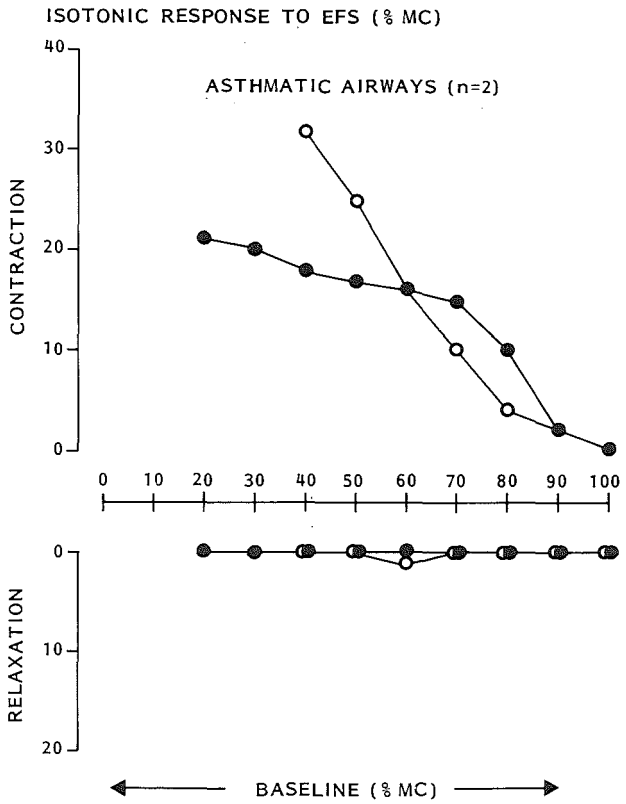

Figure 5. Responses to EFS of 2 bronchi from 2 asthmatic patients (individual values are shown). Baseline elevation was produced by methacholine (10^{-8} to 10^{-4} M) as shown in Figure 1. Note the absence of a relaxation response at all levels of induced contraction.

Discussion

In the present study, we used the contraction-relaxation response of human airways to EFS as a measure of the balance between the contractile and inhibitory components of the autonomic innervation of the airway smooth muscle. Our results suggest that this balance is similar in airways from patients with and without COPD. Airways from two asthmatics, however, showed increased contractile and reduced inhibitory responses. Furthermore, the inflammatory mediator LTC_4 appeared to reduce the relaxation phase in non-asthmatic airways. Our field stimulation characteristics chosen to produce near-maximal responses were similar to those reported by others[3,8]. The contractile phase was completely blocked by atropine and, thus, was due to muscarinic receptor stimulation. This cholinergic phase was neurally mediated because it could also be blocked by TTX. The relaxation phase was not affected by timolol, and was partially reduced

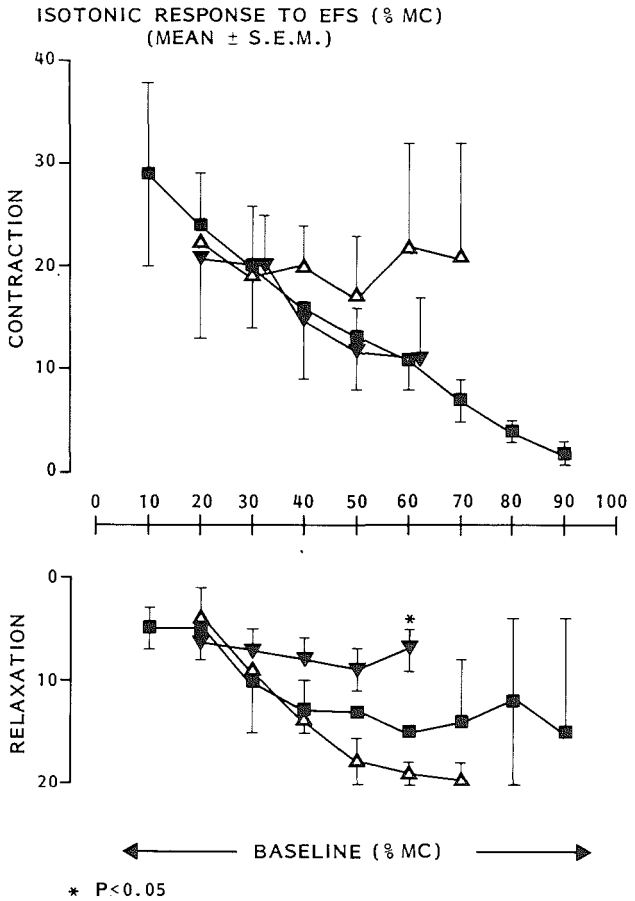

Figure 6. Responses to EFS of normal human bronchus in the presence of increasing concentrations of histamine (n=7, squares), $PGF_{2\alpha}$ (n=7, open triangles) or LTC_4 (n=6, closed triangles). Mean ± SEM of 3-7 separate experiments are shown; due to the submaximal contraction produced by these mediators and differences in spontaneous baseline contractile state of the preparations, no complete curves were obtained. Only when a given baseline level was reached in at least 3 experiments, the data were included in the figure. Relaxation responses at 60% MC were significantly reduced by LTC_4 ($P<0.05$), as compared to relaxations at the corresponding baseline level in the presence of methacholine (shown in figure 3).

by TTX. This phase was therefore non-adrenergic and partly nerve-mediated. Incomplete blockade of NAI relaxation responses to EFS by TTX has been documented previously and suggests a non-neural component in the NAI relaxation response[5,8,16,17]. The origin of this TTX-insensitive component is unclear, but may represent release of a mediator or transmitter from a non-neural site.

EFS-induced C-responses under basal conditions were roughly 20 to 40% of

maximal methacholine-induced contraction, which is in the same order as reported previously[17]. Figure 3 shows that only about one third of methacholine-induced contraction could be antagonised by stimulation of the NAI system. This is less than the 50 to 70% reported by others[8,17]. This difference may be explained by the fact that we did not block the cholinergic response, which may have reduced the NAI phase due to superposition of residual cholinergic contraction. In previous experiments, we have also found more effective NAI relaxations in the presence of atropine and histamine, which supports this explanation[16]. Our findings in normal and COPD airways confirm earlier reports by Roberts et al[18] and Taylor et al[17] and our own previous work[19] in that COPD airways responded normally to exogenous cholinergic stimulation by methacholine, and to intrinsic cholinergic nerve stimulation by EFS. At all levels of induced contraction, the C-responses of normal and COPD airways were similar, and decreased linearly when contraction was induced from 0 to 100 %MC. This was to be expected because both methacholine and EFS had their effect through stimulation of muscarinic receptors. Futhermore, NAI-responses of normal and COPD airways were similar, which confirms previous observations by others[17]. We have demonstrated that NAI responses of human airways are optimal between 40 and 70 %MC precontraction and decline when the baseline is elevated further. The degree of airway smooth muscle shortening that is possible *in vivo* is probably much less than the maximal contraction *in vitro* due to increasing loads that impede muscle shortening *in vivo*. Calculations have suggested that at most 30-45% of maximal smooth muscle shortening is possible *in vivo*[20]. Our findings suggest that in this contraction range, NAI relaxations are near-optimal and could therefore be important in counteracting bronchoconstriction.

It is difficult to obtain asthmatic lung tissue for pharmacological experiments. In the present study, we had the rare opportunity to examine airways from 2 patients with pertinent asthma. Our observations are highly suggestive of both an increased contractility to cholinergic stimulation and a decreased relaxation after stimulation of NAI nerves at all levels of induced contraction, although the number of observations is insufficient to draw firm conclusions.

We have reported the responses of one of the asthmatic patients' airways to pharmacological stimulation with a number of contractile and relaxant agonists and to graded EFS in more detail previously[16]. These experiments also indicated an extremely high contractility of asthmatic bronchi to cholinergic stimulation. NAI responses in the presence of atropine and after precontraction with histamine were, however, only slightly reduced in comparison with normal control tissues. It seems likely, therefore, that the virtual absence of NAI responses in asthmatic airways in the present study is not only due to a reduced activity of the NAI system, but is also the consequence of partial superposition of the pronounced cholinergic contractions. Exaggerated contractile responses of isolated human asthmatic airways to various agonists have also been reported by others[21,22]. No satisfactory explanation for this increased contractility has been found. Its non-specific nature suggests a postreceptor mechanism, such as an increased effectiveness of receptor activation-contraction coupling or an increased capacity

of the contractile elements to shorten. Also, smooth muscle hypertrophy might produce increased contractility[16,20]. We have not performed passive and active length-tension studies on asthmatic airways; the isotonic load of 1 g that we have used in all experiments was determined to be optimal for non-asthmatic airways in preliminary experiments. By definition however, sub-optimal loads could not result in a higher contractility of the asthmatic airways.

Reduced NAI responses of human asthmatic airways could also contribute to airways obstruction, as suggested in the introduction. Several mechanisms might be responsible for a NAI dysfunction. Acquired defects of the NAI system could result from chronic airway inflammation in case of inactivation of the peptide transmitter by proteolytic enzymes[1]. If this were true, a NAI defect would be expected in chronically inflamed COPD airways. We showed normal NAI responses in COPD airways, however. These airways were not examined histologically, but it has been shown that cartilaginous bronchi in COPD exhibit the various features of chronic inflammation[23]. Therefore, it seems unlikely that chronic inflammation such as occurs in COPD will cause NAI hyporesponsiveness.

Airway inflammation has also been reported in asthma. Inflammation in asthma is different from that in COPD in that eosinophils constitute an important part of the inflammatory cell population[24].

On histological examination, both asthmatic bronchi used in this study showed marked infiltration of the submucosa with inflammatory cells, including numerous eosinophils. Eosinophils seem to play an important role in the pathogenesis of asthma[24,25]. They have been shown to generate preferentially LTC_4[26], and LTC_4 is probably one of the main mediators of human asthmatic allergic bronchoconstriction[27]. We found reduced NAI relaxations after pretreatment of human non-asthmatic airways with LTC_4. Likewise, in a study by Taylor and coworkers[8], frequency- response curves to EFS on 6 human bronchi, that were precontracted with either histamine or LTC_4, showed a 10% lower mean maximal NAI response in the presence of a similar concentration of LTC_4 that inhibited NAI relaxations in the present study. This leads to the speculation that, in asthma, exposure to LTC_4 may induce or enhance NAI hyporesponsiveness. This may be of clinical relevance in the abovementioned airway smooth muscle contraction range that occurs during asthmatic attacks.

There is no good explanation for this putative action of LTC_4. Indirect evidence suggests that LT's may decrease the number of gap junctions, which are probably responsible for the electrical coupling of smooth muscle cells[28]. Reducing the number of gap junctions, therefore, would result in slower propagation of contractile or relaxation responses from cell to cell. It has been shown that cholinergic nerves are abundant in the airway generations we studied[29]. The NAI innervation may be less dense[30], especially in peripheral airways[5], although comparative quantitative data are not available. If NAI nerves are sparser than cholinergic nerves, NAI responses may be more dependent on smooth muscle cell-to-cell coupling than cholinergic responses, and may be reduced selectively

when the number of gap junctions is decreased, which would explain a possible effect of LTC_4 pretreatment on NAI responses to EFS. Further studies are needed to examine the effect of LT on EFS-induced responses in more detail.

A reduced NAI response in asthma may also result from an impaired relaxability that is not confined to the NAI system. Decreased relaxations to β-receptor stimulation have been found in human asthmatic airways[31,32], and both decreased β-adrenergic and NAI responses were found in an animal model of asthma by Souhrada et al[10]. However, we have recently reported normal relaxation responses to isoproterenol and forskolin of airways from one of the two asthmatic subjects, so impaired relaxability is not a general feature of asthmatic airways[16].

In conclusion, we found that near-maximal responses of human airways from normals and patients with COPD to EFS *in vitro* were similar both with respect to cholinergic contractions and NAI relaxations. Methacholine-induced contractions were also similar. Airways from 2 subjects with asthma who had a highly increased bronchial responsiveness *in vivo* showed exaggerated cholinergic contractions and reduced NAI relaxations. In normal airways, histamine and $PGF_{2\alpha}$ had no effect on the response to EFS, but LTC_4 reduced the NAI relaxation responses.

These results suggest an imbalance between the contractile and relaxant components of the autonomic regulation of airway caliber in asthma, but not in COPD, that may be related to exposure of the airways to LTC_4.

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Chapter 17
Summary and general discussion

Most patients with asthma and many patients with chronic bronchitis have an increased bronchial responsiveness, which means that their airways constrict after pharmacological, physical, chemical or allergenic stimuli that do not have such an effect in normals. In asthma, the degree of bronchial hyperresponsiveness correlates with the severity of respiratory symptoms, and hyperresponsive subjects probably have an increased risk of developing chronic airflow limitation. It seems important, therefore, to elucidate the mechanisms that underly bronchial hyperresponsiveness. That in hyperresponsive subjects so many unrelated stimuli can provoke bronchoconstriction suggests a dysfunction of the end organ. Airways contain bundles of smooth muscle in their walls, that run spirally around the bronchial circumference. Their contraction causes airway narrowing. The physical characteristics of airway smooth muscle suggest that it may well be involved in the pathogenesis of bronchial hyperresponsiveness. This thesis contains investigations on human airway smooth muscle function in patients with and without chronic bronchitis and asthma. Human lung tissue was obtained during surgery for bronchial malignancies, and methods were developed to measure the functional responses of isolated central and peripheral airways to pharmacological agents and to electric field stimulation. All patients preoperatively underwent lung function measurements and, when possible, a bronchial challenge with histamine or methacholine. We have compared airway smooth muscle function in patients with and without chronic bronchitis or asthma.

As an introduction to the studies, *Part I* contains a literature review on airway smooth muscle physiology and pathophysiology.

In *Chapter 1*, the structure of airway smooth muscle and the biochemistry, electrophysiology and mechanics of airway smooth muscle contraction and relaxation are reviewed.

Chapter 2 describes the organisation of the autonomic control of human airway smooth muscle, which consists of a direct cholinergic and non-adrenergic inhibitory innervation and, perhaps, the circulating adrenalin from the adrenal medulla.

The effects of inflammatory mediators on airway smooth muscle are summarized in *Chapter 3*.

Chapter 4 is a review of the present insights in the pathogenesis of nonspecific bronchial hyperresponsiveness. Although the many theories on this subject indicate the poor understanding of its etiology, it seems likely that indeed many

intrinsic and exogenous factors interact to produce bronchial hyperresponsiveness. Important factors seem to be acute airway inflammation and mucosal thickening.

The role of airway smooth muscle abnormalities in bronchial hyperresponsiveness is discussed in *Chapter 5*. From the literature data, the aims of this thesis are derived.

Part II contains four studies on the methodological aspects of the measurement of human airway smooth muscle function *in vitro*.

Chapter 6 gives a detailed description of the method to prepare human bronchiolar strips. With this preparation, it is possible to perform accurate and reproducible measurements of airway smooth muscle sensitivity (EC_{50}). Maximal effects could also be measured reproducibly, and with sufficient accuracy to detect differences between subjects. These responses were stable in time for a period of 55 h including 2 storage episodes in cold buffer.

In *Chapter 7*, we describe the effects of methacholine, histamine and LTC_4 on human bronchiolar strips. Methacholine and histamine were equipotent; LTC_4 was a factor 100 more potent than the latter 2 agonists. The mean maximal effects of these three agents were similar. We demonstrated that EC_{50} measurements for all three agonists could be performed successively on a single preparation without interactions. The maximal responses, however, were influenced: It was shown that histamine pretreatment induced an increase in smooth muscle maximal responsiveness to LTC_4.

Isometric and isotonic measurements of human airway smooth muscle function have been compared in *Chapter 8*. It was found that, with the isotonic method, the sensitivities to methacholine, histamine, LTC_4, $PGF_{2\alpha}$, isoproterenol and theophylline were slightly lower than with the isometric method. The maximal isotonic and isometric responses to methacholine were linearly related. We concluded that these differences were of little practical importance for conventional pharmacological studies.

Chapter 9 describes the response of fresh human isolated central airways to electric field stimulation. Maximal stimulation produced complex responses consisting of a rapid cholinergic nerve-mediated contraction, that, in 20% of all preparations, was followed by a non-neural smaller contractile peak that was due to synthesis of a PG-like substance. The next phase was a non-adrenergic nerve-mediated relaxation, followed by a fourth, sustained, non-neural contractile phase, caused by the synthesis of a LT-like substance. It was demonstrated that the sustained non-neural contractions were abolished by transient cooling to 4°C for 30 min, but returned in the course of several hours. The recognition of these non-neural components is important where the neural response patterns of human airways are studied.

Part III contains chapters on airway smooth muscle function in patients with chronic bronchitis or asthma.

Chapter 10 describes airway smooth muscle responsiveness in patients with

and without COPD. This study showed that the EC_{50} of human airway smooth muscle to contracting agonists showed no important between-patients variation, and was similar in patients with and without COPD. Bronchiolar strips from patients with COPD had a significantly higher maximal response to histamine, responded normally to LTC_4, and tended to contract less to methacholine than strips from control subjects. The difference in histamine contractility of airways from patients with and without COPD was probably not due to histamine tachyphylaxis or the production of relaxing prostaglandins in airways without COPD.

In *Chapter 11*, relaxation responses to isoproterenol, forskolin and electric field stimulation and responses to methacholine of bronchiolar smooth muscle from patients with and without chronic obstructive bronchitis have been compared. Nonspecific hyperresponsiveness and airflow limitation could result from an impaired relaxability of airway smooth muscle. The response of bronchitic airways to isoproterenol *in vitro* was normal, however, and complete relaxation occured in response to activation of adenylate cyclase by forskolin. This indicates that an impaired relaxability of airway smooth muscle is probably not involved in bronchial obstruction and hyperresponsiveness in chronic bronchitis. Although no significant differences were found between the group mean relaxations to L-isoproterenol, forskolin and electric field stimulation of NAI nerves, three patients with chronic obstructive bronchitis had a significantly decreased relaxability of their airways to L-isoproterenol but not to forskolin. In this chapter, the methacholine response of airways from patients with chronic bronchitis was significantly lower than of control airways. That this was not found in Chapter 10 may be due to the fourfold lower number of observations in that study. The reduced effect of methacholine in peripheral but not in central airways from patients with chronic bronchitis is unexpected, and could not be explained easily.

In *Chapter 12*, histological signs of small airway inflammation were quantified and compared to airway smooth muscle contractions *in vitro*. It was found that inflamed airways were not hyperresponsive, but, in contrast, tended to be less responsive to histamine, methacholine and LTC_4 than non-inflamed airways from subjects with varying degrees of chronic bronchitis. Acute airway inflammation is regarded as an important determinant of bronchial hyperresponsiveness *in vivo*. Our findings suggest that chronic inflammation does not enhance airway smooth muscle contractile function. We have not excluded, however, that the many substances that are released by inflammatory cells may have transient effects on muscle cells that facilitate bronchoconstriction during an acute inflammation.

Chapter 13 is a study on the relationship between the degree of bronchial hyperresponsiveness to methacholine in non-asthmatic subjects *in vivo* and the structure and function of bronchiolar strips *in vitro*. No correlations were found between the sensitivity and maximal response to methacholine *in vivo* and the responsiveness of bronchiolar smooth muscle to methacholine and L-isoproterenol *in vitro*. The severity of membranous bronchiole inflammation was

significantly correlated with the degree of baseline airflow limitation, but not with airway responsiveness *in vivo* or *in vitro*. These findings suggest that, in chronic bronchitis, the *in vivo* hyperresponsiveness to methacholine is not due to intrinsic abnormalities of the airway smooth muscle.

Chapter 14 compares the capacity of human lung parenchyma to synthetize leukotrienes to the *in vitro* responsiveness of bronchiolar smooth muscle to LTC_4 and methacholine. This was done to examine the possibility of endogenous desensitization of the muscle in response to high levels of LT's. The parenchyma produced LTB_4, C_4, D_4 and E_4 in decreasing amounts. Airway smooth muscle responses to LTC_4 were not decreased in tissue that had the highest capacity to generate LT's, and maximal responses after LTC_4 were linearly related to those after methacholine.

Chapter 15 is a case report of human asthmatic airway responses *in vitro*. Bronchiolar smooth muscle from an atopic asthmatic patient developed a four- to five-fold higher isometric tension in response to histamine, methacholine and LTC_4 than normal control tissues. The relaxation responses to L-isoproterenol and forskolin were normal. The NAI relaxation after field stimulation was low compared to the response of non-asthmatic tissues.

In *Chapter 16,* the balance between the cholinergic and the NAI components of the autonomic nervous system in human central airways has been studied in relation to baseline smooth muscle contractile state in patients with and without chronic obstructive bronchitis and asthma. The results suggested that the cholinergic/NAI balance in chronic bronchitis was normal. In 2 asthmatic airways, a reduction of NAI responses together with cholinergic hyperresponsiveness were found. Incubation of bronchiolar strips with the inflammatory mediators histamine, $PGF_{2\alpha}$ or LTC_4 indicated that LTC_4 reduced the effect of NAI stimulation.

Airway smooth muscle function in asthma

Since 1982, a few reports on asthmatic human airway smooth muscle function *in vitro* have been published. In some of these studies, an increased maximal tension development after histamine, cholinergic agonists and LTC_4 has been observed. Others found normal or low contractions, sometimes with a variably reduced sensitivity and maximal response to β agonists. We have reported a pronounced non-specific increase in maximal isometric tension of asthmatic human airway smooth muscle. We also found normal relaxation responses to forskolin, which stimulates cAMP production receptor-independently, and to L-isoproterenol. Non-adrenergic inhibitory responses to electric field stimulation were low in bronchi from two asthmatics who were highly hyperresponsive *in vivo*, and had a normal baseline lung function. Histologic examination showed marked airway smooth muscle hypertrophy. Discrepancies in the results of the various published *in vitro* studies on human asthmatic airways may be due to differences in methodology and patient selection. It is remarkable, for instance,

that increases in contractile force have been found in fresh tissue, whereas impaired relaxation responses were present in autopsy tissues from patients who died of asthma. This suggests that an impaired function of β-receptors is not an 'intrinsic' abnormality of airway smooth muscle in asthma, but may occur transiently during severe asthma attacks.

Airway smooth muscle function in chronic bronchitis

Several studies have been published on the function of airway smooth muscle in well-defined populations of patients with and without chronic bronchitis, airflow limitation and bronchial hyperresponsiveness. Previous studies have mainly concentrated on the sensitivity of human airway smooth muscle to constricting agonists, expressed as the EC_{50}. There is now general agreement that, in chronic bronchitis, the degree of non-specific bronchial hyperresponsiveness is not related to the sensitivity of airway smooth muscle to contracting agonists.

Absolute responses and maximal effects previously showed a troublesome within-subject variability. By using a carefully standardised method and a well-defined airway preparation, the bronchiolar strip, we accomplished a reduction of this variability so that we could detect differences in maximal responses between-subjects. We found no relation between the maximal response plateau after inhalation of methacholine *in vivo* and the maximal isotonic contraction of airway smooth muscle *in vitro*. This suggested that, in bronchial hyperresponsiveness without asthma, both the position and the maximal response plateau of the dose-response curve to inhaled methacholine are not determined by airway smooth muscle function. Bronchial hyperresponsiveness to methacholine in chronic bronchitis can therefore not be explained by an abnormal smooth muscle responsiveness to methacholine. However, things may be different with respect to histamine. Histamine is more active in peripheral airways, and produces more airway narrowing than methacholine in patients with chronic bronchitis and airflow limitation. Our finding that the maximal response of bronchiolar smooth muscle to histamine, but not to methacholine and LTC_4, is significantly higher in patients with chronic bronchitis than in controls, suggests that a histamine-specific hyperresponsiveness of airway smooth muscle in small airways may be partly responsible for the exaggerated bronchoconstriction after inhaled histamine in chronic bronchitis.

Not only an increased contractility, but also a decreased relaxability of airway smooth muscle could be important in causing bronchial hyperresponsiveness. We have found similar mean relaxation responses to L-isoproterenol and forskolin of bronchiolar segments from patients with chronic bronchitis and from control subjects without chronic bronchitis. Our findings suggest however that β-receptor dysfunction may occur in a subgroup of patients with chronic bronchitis.

Acute airway inflammation is probably a major factor in the pathogenesis

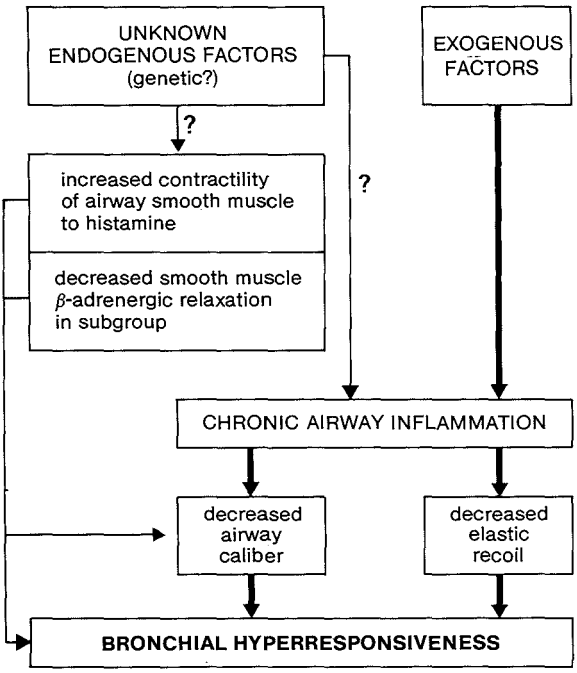

Figure 1: Mechanisms of hyperresponsiveness in chronic bronchitis.

of bronchial hyperresponsiveness. We have examined human airway smooth muscle function in relation to the degree of airway wall inflammation, and found a negative correlation between the severity of airway wall inflammation and the sensitivity of bronchiolar smooth muscle to histamine and methacholine, i.e. inflamed airways were less sensitive than non-inflamed airways. This suggests that, in non-asthmatic subjects, chronic airway inflammation does not cause bronchial hyperresponsiveness via a direct effect on airway smooth muscle. It is highly probable, however, that other effects of chronic inflammation, such as mucosal swelling, mucus hypersecretion, and, perhaps, a decreased elastic lung recoil, are important factors in the pathogenesis of hyperresponsiveness in chronic bronchitis. The good correlation between baseline airway caliber and the degree of bronchial hyperresponsiveness in chronic bronchitis strongly suggests that, *in vivo*, geometric factors are of major importance. These will not influence the function of the isolated bronchi in the organ bath.

It has also been speculated that inflammation might produce hyperresponsiveness by impairing the action of relaxing peptidergic neurotransmitters. We examined the function of the cholinergic and the non-adrenergic inhibitory components of the autonomic innervation of airway smooth muscle, and showed that both were normally functioning in airways from patients with chronic bronchitis. It seems unlikely, therefore, that chronic inflammation causes hyporesponsiveness of the non-adrenergic inhibitory system.

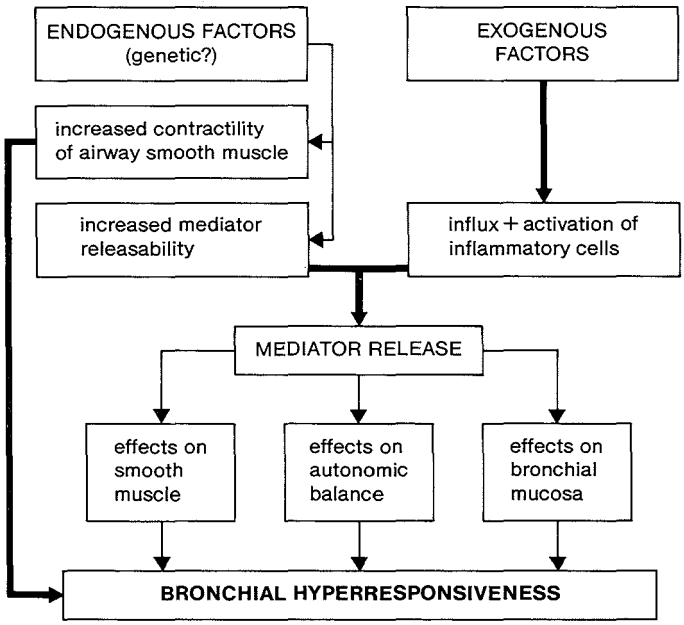

Figure 2: Mechanisms of hyperresponsiveness in asthma.

Conclusions

Comparative *in vivo-in vitro* studies have shown that in non-asthmatic humans with bronchial hyperresponsiveness, increased responsiveness of the airways *in vivo* is not caused by an abnormal sensitivity (EC_{50}) of airway smooth muscle to contracting stimuli, and is not due to an impaired relaxability to e.g. β-receptor stimulation. The strong correlation between baseline airway caliber and the degree of hyperresponsiveness indicates that mechanical factors such as mucosal thickening are of major importance as a mechanism underlying non-specific hyperresponsiveness in chronic bronchitis. An increased histamine efficacy in small airways may, however, contribute to the *in vivo* bronchoconstriction after histamine inhalation. The concept of the mechanisms that lead to bronchial hyperresponsiveness in chronic bronchitis is illustrated in Figure 1.

Because of the different nature of bronchial hyperresponsiveness in asthma and chronic bronchitis, the results obtained with airway smooth muscle from patients with chronic bronchitis are probably not relevant to hyperresponsiveness in asthma. In asthma, an intrinsic nonspecific increase of airway smooth muscle strength may exist which is dependent on the degree of smooth muscle hypertrophy. This is suggested by *in vitro* studies on fresh airways from stable asthmatics. An increased strength may lead to bronchial hyperresponsiveness

because when the stronger muscle contracts, it will more easily overcome the elastic recoil forces that may normally prevent excessive airway narrowing. During acute phases, inflammatory mediators may further enhance smooth muscle responsiveness via various putative mechanisms, such as presynaptic facilitation of cholinergic nervous transmission, modulation of the number of gap junctions, or stimulation of axon reflexes and local release of tachykinins. Furthermore, mucosal swelling and mucus plugging may occur that worsen airway obstruction and responsiveness.

The relaxability of airway smooth muscle in asthma is essentially normal. However, studies on autopsy tissue from asthmatics who died during attacks have suggested, that an impaired β-receptor function may occur during severe asthma attacks. This corresponds with the clinical observation that β agonists are excellent bronchodilators in asthmatic patients, but are usually only partly effective during severe attacks. Finally, the non-adrenergic inhibitory system may be impaired in asthma, secondary to asthmatic airway inflammation. Figure 2 depicts the mechanisms that may be relevant for the pathogenesis of bronchial hyperresponsiveness in asthma.

In summary, it seems that not only *in vivo*, but also *in vitro* there are fundamental differences in the nature of bronchial hyperresponsiveness in patients with asthma or chronic bronchitis (Table 1). Several important questions remain, however, that need to be answered. It is not known, for instance, whether these differences

Table 1. Putative abnormalities of airway smooth muscle in asthma and chronic bronchitis

	Airway smooth muscle responsiveness	
	Asthma	Chronic bronchitis
Contractility		
– histamine	↑↑	↑
– methacholine	↑↑	↓
– LTC_4	↑↑	n
Relaxability		
– β receptor	n, (↓↓ during attacks)	n, (↓ in subgroup)
– cAMP	n	n
Autonomic control		
– cholinergic	↑↑	n
– non-cholinergic excitatory	↑ (?)	?
– non-adrenergic inhibitory	↓	n
Inflammation		
– acute	↑↑	?
– chronic	(decreases airway caliber)	(decreases airway caliber) ↓ (decreases sensitivity to contracting agonists)

Abbreviation: n = normal

are genetically determined or acquired. It seems also important to know whether the asthmatic type of hyperresponsiveness can lead to chronic airflow limitation and a 'bronchitic-type' of hyperresponsiveness in later life. It is clear that much more work needs to be done both *in vivo* and *in vitro* to substantiate the concepts presented, and to further elucidate the mechanisms that underly bronchial hyperresponsiveness in chronic bronchitis and asthma.

Directions for future research

Thusfar, the study of human asthmatic airway smooth muscle has progressed slowly, due to a limited supply. The study of lung tissue from experimental animals or from patients with non-asthmatic hyperresponsiveness may not give relevant results.

It is further clear that the preparations used for *in vitro* studies by most investigators have serious disadvantages. Bronchial strips contain various components such as epithelium, connective tissue and inflammatory cells, that may influence muscle function, and can only be obtained at thoracotomies or after death. It is important, therefore, to develop techniques that will make it possible to study pure human airway muscle, either in small cell bundles or as single cells. It seems now technically feasible to do mechanical, pharmacological, biochemical and electrophysiological studies on small amounts of muscle or even on single cells. This makes it possible to examine airway tissue from endobronchial biopsies obtained via bronchoscopy. Bronchoscopy and biopsies can be safely performed in mild asthmatics and normal subjects, and smooth muscle cells thus obtained may be used directly or can be cultured with preservation of contractile function. It seems possible, that studies of this kind may eventually answer the question whether human airway smooth muscle is intrinsically abnormal in asthma.

It seems also important to study the interactions between muscle tissue and inflammatory cells. This requires a more or less pure muscle preparation, which makes it possible to examine the effect of activation of various types of inflammatory cells or epithelial cells on muscle tone, muscle responsiveness or its control by the autonomic nervous system. Studies of this kind will provide insight in the many possible ways in which the various cell types in the airways may interact to produce hyperresponsiveness, and allow the study of mechanisms of action of putative anti-asthmatic drugs.

Samenvatting

Bronchiale hyperreactiviteit is een basiskenmerk van astma en komt vaak voor bij patienten met chronische bronchitis. Bij deze patienten ontstaat bronchoconstrictie na farmacologische, fysische, chemische of allergene stimuli die dit effect niet hebben bij normale personen. De mate van bronchiale prikkelbaarheid correleert met de ernst van de symptomen bij astma, en patienten met bronchiale hyperreactiviteit lopen waarschijnlijk een verhoogd risico op chronische obstructieve longziekten. Het lijkt daarom belangrijk het mechanisme van bronchiale hyperreactiviteit te onderzoeken.

Dat zoveel verschillende soorten stimuli bronchoconstrictie kunnen veroorzaken bij patienten met bronchiale hyperreactiviteit duidt erop dat er een functiestoornis zou kunnen bestaan van het eindorgaan. In de wand van de luchtwegen bevinden zich bundels glad spierweefsel, die spiraalsgewijs verlopen zodat zij de luchtwegdiameter verkleinen wanneer zij samentrekken. De fysische eigenschappen van het bronchiaal glad spierweefsel wijzen erop dat het een rol zou kunnen spelen bij bronchiale hyperreactiviteit.

Dit proefschrift bevat de resultaten van een aantal studies naar de rol van het bronchiale spierweefsel bij bronchiale hyperreactiviteit en luchtwegvernauwing, zoals die voorkomen bij patiënten met chronische bronchitis en astma. Hiertoe werden methoden ontwikkeld om de functionele responsen van geïsoleerde perifere en centrale menselijke luchtwegen te meten na stimulatie met farmacologische stoffen en electrische veldstimulatie.

Longweefsel werd verkregen van patiënten die een thoracotomie ondergingen wegens maligniteit. Bij deze patiënten werd preoperatief longfunctieonderzoek en zo mogelijk een inhalatie-provocatietest met histamine of methacholine verricht.

Nadat de nauwkeurigheid en de reproduceerbaarheid van de *in vitro* methodieken was nagegaan, werd vergelijkend onderzoek verricht met luchtwegpreparaten van patiënten met en zonder chronische bronchitis of astma.

In *Deel I* wordt de fysiologie en pathofysiologie van bronchiaal glad spierweefsel beschreven aan de hand van literatuurgegevens.

Hoofdstuk 1 bevat een beschrijving van de structuur van bronchiaal glad spierweefsel en van de biochemische, electrofysiologische en mechanische aspecten van contractie en relaxatie.

Hoofdstuk 2 beschrijft de autonome regulatie van menselijk bronchiaal spierweefsel. Het autonome zenuwstelsel innerveert het bronchiale spierweefsel met cholinerge en met niet-adrenerge inhiberende vezels. Een niet-adrenerg exciterend systeem is mogelijk van belang bij locale axonreflexen. Circulerende catecholamines zijn waarschijnlijk vooral belangrijk tijdens stress.

De effecten van een aantal mediatoren van ontsteking op bronchiaal glad spierweefsel worden samengevat in *Hoofdstuk 3*.

Hoofdstuk 4 bevat een overzicht van de theorieën over de pathogenese van aspecifieke bronchiale hyperreaktiviteit bij patiënten met astma of chronische

bronchitis. Het grote aantal theorieën duidt erop, dat de mechanismen van bronchiale hyperreactiviteit nog onvoldoende worden begrepen. Het is echter waarschijnlijk, dat ontsteking en zwelling van het bronchiale slijmvlies een belangrijke rol spelen.

De mogelijke rol van bronchiaal glad spierweefsel bij bronchiale hyperreactiviteit wordt besproken in *Hoofdstuk 5*. Uit de literatuurgegevens worden conclusies getrokken die hebben geleid tot de vraagstellingen van het onderzoek, beschreven in dit proefschrift.

Deel II bestaat uit vier hoofdstukken, waarin de methodologische aspecten zijn beschreven van de meting van de functie van menselijk bronchiaal spierweefsel *in vitro*.

Hoofdstuk 6 bevat een gedetailleerde beschrijving van de techniek waarmee menselijke bronchiolus-strips worden geprepareerd. Met de bronchiolus-strip is het mogelijk om nauwkeurige en reproduceerbare metingen te verrichten van de gevoeligheid van bronchiaal glad spierweefsel. Maximale effecten konden ook reproduceerbaar worden bepaald, met voldoende nauwkeurigheid om verschillen tussen personen te kunnen aantonen. De responsen waren stabiel gedurende 55 uur na chirurgische resectie van het longweefsel, en werden niet beïnvloed door bewaren van de preparaten in koude buffer.

In *Hoofdstuk 7* beschrijven wij de effecten van methacholine, histamine en LTC_4 op menselijke bronchiolus-strips. Methacholine en histamine waren equipotent, LTC_4 was 100 maal potenter dan deze twee stoffen. De maximale effecten van de drie agonisten waren vergelijkbaar. Het bleek dat methacholine, histamine en LTC_4 geen interacties vertoonden met betrekking tot de gevoeligheid (EC_{50}), maar wel wat betreft de maximale respons. Histamine voorbehandeling leidde tot een toegenomen maximaal effect van LTC_4, hetgeen duidt op een interactie voorbij het niveau van receptoractivatie.

In *Hoofdstuk 8* worden isotonische en isometrische metingen met elkaar vergeleken. De EC_{50}, bepaald met de isotonische methode, voor methacholine, histamine, LTC_4, $PFG_{2\alpha}$, isoprenaline en theophylline was iets groter dan met de isometrische methode. De maximale isotonische en isometrische responsen na methacholine waren lineair gerelateerd. Wij concludeerden dat de gevonden verschillen tussen deze methoden van weinig belang waren voor conventionele farmacologische proeven.

In *Hoofdstuk 9* wordt het reactiepatroon van verse menselijke centrale luchtwegen na electrische veldstimulatie *in vitro* beschreven. Maximale veldstimulatie leidde tot complexe responsen die begonnen met een snelle, cholinerge contractie ten gevolge van stimulatie van cholinerge zenuwen in het preparaat. Bij 20% van alle bronchi werd vervolgens een tweede contractiepiek waargenomen, die berustte op het vrijkomen van een prostaglandine-achtige stof. De volgende component was een door niet-adrenerge inhiberende zenuwen veroorzaakte relaxatie, en deze fase werd in vrijwel alle bronchi gevolgd door een trage contractie, die veroorzaakt werd door de productie van een leukotriene-achtige stof. Deze langzame contractiefase verdween vrijwel na tijdelijke afkoeling tot

4°C gedurende 30 min, maar ontstond hierna geleidelijk opnieuw in de loop van enkele uren. Deze niet door zenuwstimulatie veroorzaakte contracties kunnen de cholinerge en vooral de niet-adrenerge respons sterk vervormen, hetgeen van belang is bij neurofysiologisch onderzoek.

Deel III bevat een zevental hoofdstukken waarin onderzoek wordt beschreven naar de functie van geisoleerd bronchiaal glad spierweefsel *in vitro* en kenmerken van patiënten met chronische bronchitis of astma, zoals luchtwegobstructie, bronchiale hyperreactiviteit en ontsteking van de bronchi.

Hoofdstuk 10 beschrijft de functie van bronchiaal glad spierweefsel bij patiënten met en zonder chronische bronchitis en luchtwegobstructie. Bronchiolus-strips van patiënten met bronchitis vertoonden een significant grotere maximale isometrische respons na histamine dan strips van controlepersonen. Er was een normale respons na LTC_4, en mogelijk een iets lagere contractiekracht ten aanzien van methacholine. Verschillen in EC_{50} werden niet gevonden.

In *Hoofdstuk 11* zijn de relaxatieresponsen van bronchiolus strips van patiënten met en zonder chronische bronchitis en luchtwegobstructie vergeleken, en werd bovendien het effect van methacholine *in vitro* nagegaan. Hoewel de relaxatie responsen na isoprenaline, forskoline en veldstimulatie van het niet-adrenerge inhiberende systeem niet significant van elkaar verschilden in de twee groepen, waren er drie patiënten met bronchitis, van wie het bronchiaal spierweefsel een significant verlaagde respons vertoonde na isoprenaline, maar niet na forskoline. In deze studie was de maximale respons na methacholine *in vitro* bij patiënten met chronische bronchitis significant lager dan bij controlepersonen. Dit verschil met de studie beschreven in het vorige hoofdstuk, wordt waarschijnlijk veroorzaakt doordat in hoofdstuk 11 een viermaal groter aantal preparaten *in vitro* werd onderzocht dan in hoofdstuk 10.

In *Hoofdstuk 12* zijn histologische kenmerken van ontsteking in kleine luchtwegen van patienten met en zonder chronische bronchitis gekwantificeerd en gerelateerd aan de functie van het bronchiale spierweefsel *in vitro*. Het bleek dat ernstig ontstoken luchtwegen niet hyperreactief, maar daarentegen minder gevoelig zijn voor histamine, methacholine en leukotriene C_4 dan minder ontstoken bronchioli.

Hoofdstuk 13 bevat de resultaten van een onderzoek naar de mate van bronchiale hyperreactiviteit bij patiënten zonder astma *in vivo* en de structuur en functie van bronchiolusstrips *in vitro*. Er werden geen correlaties gevonden tussen de gevoeligheid en maximale respons ten aanzien van methacholine *in vivo* enerzijds, en de gevoeligheid en maximale isotonische contracties en relaxaties van bronchiolus-strips na methacholine en isoprenaline *in vitro*, of de ernst van de histologische afwijkingen in membraneuze bronchioli anderzijds. Wel was de 'basale' luchtwegobstructie significant groter naarmate ernstiger histologische afwijkingen in de kleine luchtwegen bestonden.

In *Hoofdstuk 14* wordt het vermogen van het longweefsel om leukotrienen te produceren gerelateerd aan de *in vitro* responsen van bronchiaal spierweefsel na leukotriene C_4 of methacholine. Dit is gedaan om na te gaan of een hoge

endogene leukotrieneproduktie zou kunnen leiden tot desensitisatie van de spiercellen. Het bleek, dat longparenchym leukotriene B_4, C_4, D_4 en E_4 produceerde in afnemende hoeveelheden. De respons van bronchiolusstrips na leukotriene C_4 was niet lager in weefsel dat een hoge capaciteit had om leukotriene te produceren. De maximale responsen na leukotriene C_4 waren lineair gerelateerd aan de respons na methacholine.

Hoofdstuk 15 bevat een casuistische mededeling over de functie van bronchiaal spierweefsel van een astmapatiënt. Bronchiolusstrips van deze patiënt ontwikkelden een vier tot vijf maal grotere isometrische kracht na histamine, methacholine, leukotriene C_4 en veldstimulatie dan controlepreparaten van patienten zonder astma of chronische bronchitis, die een normale longfunctie hadden. De relaxatie na isoprenaline en forskoline was normaal. De niet-adrenerge inhiberende respons na veldstimulatie was lager dan controlewaarden.

In *Hoofdstuk 16* wordt een onderzoek beschreven naar het evenwicht tussen de cholinerge en niet-adrenerge inhiberende componenten van het autonome zenuwstelsel in centrale bronchi. Dit evenwicht is bestudeerd in relatie tot de basale contractietoestand van het bronchiale spierweefsel bij patiënten met en zonder chronische bronchitis of astma. De resultaten lieten zien dat het evenwicht tussen het cholinerge en het niet-adrenerge inhiberende systeem normaal was bij patiënten met chronische bronchitis. Twee bronchi van astmapatiënten vertoonden echter een sterk toegenomen cholinerge respons en een zeer lage niet-adrenerge inhiberende respons. Incubatie van bronchuspreparaten met onstekingsmediatoren (histamine, $PGF_{2\alpha}$, LTC_4) leidde alleen na voorbehandeling met LTC_4 tot verminderde niet-adrenerge relaxatie.

In de *algemene discussie* wordt gesteld dat bij patiënten met chronische bronchitis een afwijkende functie van bronchiaal glad spierweefsel werd aangetoond die een rol zou kunnen spelen bij de bronchoconstrictie na inhalatie van histamine. De gevonden afwijking vormt echter geen verklaring voor aspecifieke bronchiale hyperreactiviteit. Dit suggereert dat andere factoren dan het spierweefsel bij deze patienten van belang zijn, zoals bijvoorbeeld zwelling van de bronchiale mucosa tengevolge van chronische ontsteking. Dit zou geen invloed hebben op de contracties van bronchuspreparaten *in vitro*. Een verminderde relaxatie van bronchiaal spierweefsel na stimulatie van β-receptoren zou bij sommige patiënten met chronische bronchitis een rol kunnen spelen, maar is geen typisch kenmerk. Ook werd een normale respons na stimulatie van het cholinerge, en van het niet-adrenerge inhiberende zenuwstelsel gevonden.

De bevindingen met longweefsel van een astmapatiënt duidden echter wel op een aspecifiek toegenomen contractiliteit van het bronchiale spierweefsel. Histologisch onderzoek van astmatische luchtwegen toonde een sterke mate van hypertrofie van het bronchiale spierweefsel, die een verklaring zou kunnen vormen voor de toegenomen contractiliteit. Bovendien werden bij twee astmapatienten aanwijzingen gevonden voor een verminderde functie van het niet-adrenerge inhiberende systeem. Stimulatie van β-receptoren leidde tot een normale relaxatie van astmatische bronchi. Literatuurgegevens suggereren echter dat een vermin-

derde relaxatie na β-receptor stimulatie kan optreden na ernstige astma-aanvallen.

Tenslotte wordt uit de resultaten van eigen onderzoek en uit literatuurgegevens een concept afgeleid dat een verklaring poogt te geven voor bronchiale hyperreactiviteit. Hierbij lijkt dat, zowel *in vivo* als *in vitro*, de aard van de bronchiale hyperreactiviteit bij patienten met astma belangrijk verschilt van die bij patienten met chronische bronchitis.

Post scriptum

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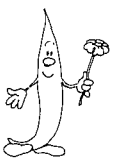

Curriculum vitae

Johannes Cornelis de Jongste was born on April the 7th, 1955, in Rotterdam, The Netherlands. He passed his secondary school exam (HBS-B) in 1972 at the 'Groen van Prinsterer Lyceum' in Vlaardingen. He started his medical training in 1972 at the Medical Faculty of the Erasmus University Rotterdam. In 1978 he obtained his medical degree. From November 1978 to March 1979 he was a clinical fellow in Internal and Tropical Medicine at the Harbour Hospital in Rotterdam (head: prof. dr. P.C. Stuiver). From March 1979 to April 1980, he completed a professional training at the Department of General Practise, Erasmus University Rotterdam (head: prof. dr. H.J. Dokter). During this year he worked as assistant General Practitioner in Zierikzee (practise: drs. A.W. van Geer) and followed clinical fellowships at the Department of Revalidation (head: drs. J.M. Oostlander), St. Franciscus Gasthuis, Rotterdam, and at the Department of Paediatrics, Sophia Children's Hospital, Rotterdam (head: prof. dr. H.K.A. Visser). In 1980 he was registered as General Practitioner. In this same year, he started his specialist training in Paediatrics in the Sophia Children's Hospital, which is part of the University Hospital Rotterdam. He was recorded as a Paediatrician in the register of the Royal Netherlands Medical Association in April 1984. From April 1984 to April 1987, he followed a clinical- and research fellowship at the Department of Paediatric Respiratory Diseases (head: prof. dr. K.F. Kerrebijn) of the Sophia Children's Hospital, under auspices of The Netherlands Asthma Foundation. The research bundled in this thesis was performed during this period at the Department of Pharmacology (head: prof. dr. I.L. Bonta) of the Erasmus University Rotterdam. Since April 1987, he is a staff paediatric pulmonologist at the Sophia Children's Hospital.

He is married to Klasine Karoline Breeman, M.D., and has two children: Evert-Jan and Arjen.

List of abbreviations

AA	arachidonic acid
ASM	airway smooth muscle
ATP	adenosine triphosphate
C	contraction
cAMP	cyclic adenosine monophosphate
CCRC	cumulative concentration-response curve
CE	contractile element
COB	chronic obstructive bronchitis
COPD	chronic obstructive pulmonary disease
D_{max}	maximal dose
D_{pl}	dose of agonist at which a response plateau is reached
DSCG	disodium cromoglycate
DW	dry weight
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
EJP	excitatory junctional potential
FEV_1	forced expiratory volume in 1 sec
HETE	hydroxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
IVC	inspiratory vital capacity
L_o	optimal length
LT	leukotriene
MC	maximal contraction
M_{FEV1}	maximal fall in FEV_1
NAI	non-adrenergic inhibitory
NANC	non-adrenergic, non-cholinergic
NCCRC	non-cumulative concentration-response curve
NS	not significant
PAF	platelet-activating factor
PC	provocative concentration
PD	provocative dose
PEC	parallel elastic component
PG	prostaglandin
PHI	peptide histidine isoleucine
PL	phospholipase
R	relaxation
R_{max}	maximal relaxation
SAD	small airways disease
SEC	series elastic component
SEM	standard error of the mean
sGAW	specific airway conductance

S_{max}	maximal isotonic shortening
TLC	total lung capacity
T_{max}	maximal isometric tension
TTX	tetrodotoxin
Tx	thromboxane
VC	vital capacity
VIP	vasoactive intestinal peptide

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