The Effect of Hypoxia on Airway Smooth Muscle Function

by

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To my mother and father

and Alison, Sam and David

SUMMARY

Resistance to airflow in the respiratory tract is largely determined by the degree of tone in the smooth muscle layer surrounding the airways.

The tone of the airway smooth muscle *in vivo* is regulated by neural control mechanisms, locally released mediators as well as humoral factors. Environmental factors, such as hypoxia, may also influence the tone of the airway smooth muscle and in addition, alter its responsiveness to various pharmacological agonists. Since hypoxia can be a feature of respiratory disorders, such as asthma and chronic obstructive pulmonary disease, it may be of importance to determine if airway smooth muscle function is altered under hypoxic conditions.

Using various techniques, I assessed:

(i) The effect of acute changes in oxygen tension on responses to contractile agents and relaxatory agents in bovine isolated bronchi.

(ii) The effect of chronic hypoxia on contractile responses in rat isolated airways.

(iii) The effect of chronic hypoxia on endothelin receptor-mediated responses in rat isolated airways.

(iv) The effect of hypoxia on the proliferation of cultured human airway smooth muscle cells.

(v) The effect of changes in inspired oxygen tension on salbutamol-mediated bronchodilation and methacholine- and histamine-mediated bronchoconstriction in asthmatic patients *in vivo*.

In rings of bovine bronchi (3rd-5th order, 3-5mm internal diameter), isometric contractions were significantly potentiated when the oxygen tension in the Krebs-Henseleit solution was lowered from 524mm Hg (hyperoxia) to either 147 mm Hg (normoxia) or 26mm Hg (hypoxia). The ability of the dilator agents salbutamol, atrial natriuretic peptide (ANP), sodium nitroprusside (SNP) and isosorbide dinitrate (ISDN) to reverse methacholine-induced tone was also altered by changing the oxygen tension, although the pattern of response differed between the various agents: The ability of salbutamol to reverse the induced tone was attenuated in hypoxia, whereas ANP was more effective in hypoxia than either hyperoxia or normoxia. ISDN and SNP were similar in that they were both more effective in either hypoxia or normoxia than in hyperoxia.

In addition, the ability of these four dilators to confer protection against subsequent challenge with methacholine was compared under hyperoxic, normoxic and hypoxic conditions. Salbutamol attenuated responses to methacholine, but only under hyperoxic conditions, in normoxia and hypoxia it was ineffective. In hyperoxia, ANP protected against methacholine challenge, but in hypoxia, ANP actually potentiated the

methacholine-induced contractions. The ability of both SNP and ISDN to protect against methacholine challenge was enhanced when the oxygen tension was reduced from hyperoxia to either normoxia or hypoxia.

Tracheal rings (internal diameter ~2mm) isolated from rats exposed to 14 days of chronic hypobaric hypoxia (500-550mBar) produced contractions to methacholine, endothelin-1 (ET-1) and potassium chloride which were significantly less than responses in control rats. In both control and hypoxic rats, responses to methacholine and ET-1 were not altered by indomethacin (a cyclooxygenase inhibitor) but were potentiated by either L-NAME (a nitric oxide synthase inhibitor) or by removal of the epithelium. Responses to the nitric oxide donor, SNP, but not the β adrenoceptor agonist salbutamol were enhanced in chronically hypoxic rats. Taken together, these results indicate that nitric oxide or a nitric oxide-like substance is released from the epithelium of both control and chronically hypoxic rats and that this subsequently attenuates the contractile responses to methacholine and ET-1. In chronically hypoxic rats, however, the airway smooth muscle appears to be more sensitive to nitric oxide than control rats, which may explain why contractile responses are significantly smaller in the chronically hypoxic rats.

ET-1 acts via at least two G protein-coupled receptor subtypes, termed ET_A and ET_B. Contractile responses to ET-1 were attenuated in chronically hypoxic rats, whereas responses to sarafotoxin S6c (an ET_B receptor agonist) were not altered. The ET_A receptor antagonist, FR 139317 at a concentration of 10^{-8}M potentiated contractile responses to ET-1 in trachea from control but not chronically hypoxic rats. The ET_B receptor antagonist, BQ 788, potentiated responses to ET-1 in both control and chronically hypoxic rat trachea. It was found that ET-1 responses were only blocked by simultaneous blockade of both ET_A and ET_B receptors, either by using the non-selective ET receptor antagonist, SB 209670, or by combining BQ 788 and FR 139317.

The proliferative response of cultured human airway smooth muscle cells was examined under different environmental oxygen tensions. Cell proliferation was assessed by cell counting and by measuring uptake of tritiated [^{3}H] thymidine. Cells were quiesced for 24 hours in normoxia and then stimulated with either ET-1 or platelet-derived growth factor (PDGF) for 24 hours, either in a normal CO_2 incubator under an oxygen tension of 147mm Hg or in a hypoxic incubator under an oxygen tension of 30mm Hg. Hypoxia stimulated proliferation of the cultured airway smooth muscle cells *per se*, and potentiated the mitogenic effects of both ET-1 and PDGF. ET-1-induced mitogenesis in hypoxia was blocked by the ET_A receptor antagonist BQ 123, but not by the ET_B receptor antagonist BQ 788, indicating that this is an ET_A-mediated response. In the presence of the protein kinase C inhibitor, staurosporine, the hypoxic enhancement of ET-1- and PDGF-induced mitogenesis was abolished. This suggests

that the stimulatory effect of hypoxia on mitogenesis in these cell is mediated via activation of protein kinase C.

Bronchodilator responses to salbutamol and bronchoconstrictor responses to methacholine and histamine were measured in asthmatic patients inspiring gas mixtures containing different oxygen contents. In parallel, responses to each of these agonists were measured in human isolated bronchial rings under hyperoxic, normoxic and hypoxic conditions.
The bronchodilator response to salbutamol in asthmatic patients was not altered by changing the inspired oxygen tension, whereas responses *in vitro* were significantly attenuated in hypoxia. In human isolated bronchial rings, contractile responses to both methacholine and histamine were significantly attenuated when the oxygen tension was reduced from hyperoxic levels. In asthmatic patients, methacholine-induced bronchoconstriction was enhanced when inspiring the hypoxic gas mixture, whereas responses to histamine were unaltered by changing the gas mixture.

In conclusion, changes in oxygen tension can have a significant effect on airway smooth muscle function.

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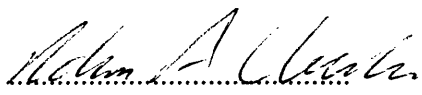
Last but not least, I would like to thank my mother and father for the sacrifices they had to make to put me through University and for always believing in me.

DECLARATION

This thesis is entirely my own composition and the experimental work detailed within was undertaken wholly by myself, with the exception of figures 7.2, 7.3, 8.3, 8.4, 8.5 and 8.6 which were produced in collaboration with Dr Ken Dagg. In the case of the aforementioned figures, I acted as the second-operator (see section 2.3.2) while Dr Dagg conducted the drug administration and performed the lung-function tests.

Signed 

Some of the results within this thesis have been published, details of which are given below.

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LIST OF ABBREVIATIONS

ANP	atrial natriuretic peptide
Ca^{2+}	calcium
cAMP	cyclic adenosine 3', 5'-monophosphate
cGMP	cyclic guanoosine 3', 5'-monophosphate
CO_2	carbon dioxide
DAG	diacylglycerol
dpm	disintegrations per minute
EC_{400mg}	the concentration of an agonist which evokes a contraction of 400mg wt
ERK	extracellular regulated kinase
$ETCO_2$	end-tidal carbon dioxide
ET-1	endothelin-1
$ET_{A, -B}$	endothelin receptor subtypes, A and B.
FEV_1	forced expiratory volume in one second
i.d.	internal diameter
IP_3	inositol 1,4,5-trisphosphate
ISDN	isosorbide dinitrate
MAPK	mitogen activated protein kinase
mbar	millibar
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
PC20	the concentration of agonist evoking a 20% fall in FEV1
pD2	the negative log of the concentration of agonist which evokes 50% of the maximum response
PGI_2	prostacyclin
PIP_2	phosphatidylinositol, 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
RTK	receptor tyrosine kinase
SaO_2	partial pressure of O_2 in arterial blood
SAPK	stress activated protein kinase
SxS6c	sarafotoxin S6c
SEM	standard error of the mean
SNP	sodium nitroprusside
~	approximately
%	percentage

CHAPTER 1

INTRODUCTION TO RESEARCH

1.1 CONTROL OF RESPIRATION

1.1.1 INTRODUCTION

Oxygen is required for the survival of all higher life forms due to its central role in the synthesis of ATP by oxidative phosphorylation. Even transient, localised oxygen deficits can produce irreversible cellular damage, causing tremendous morbidity and mortality in humans. Breathing is therefore a tightly controlled event, so that even in the face of wide changes in metabolic demand, the arterial oxygen tension (Pa_{O2}) is normally kept within restricted limits (~85 to 95mmHg). The respiratory control system is comprised of two basic elements: the central controller in the brain and sensors which gather information.

1.1.2 CENTRAL CONTROL

Pioneering work by Lumsden (1923) indicated that the periodic nature of inspiration and expiration is generated by three major respiratory centres within the brainstem, termed the medullary respiratory centre, the pneumotaxic centre and the apneustic centre.

1.1.2.1 Medullary respiratory centre

Respiratory units in the medulla are primarily concentrated into two major aggregates: the dorsal respiratory nucleus (DRN) and the ventral respiratory nucleus (VRN). The DRN is believed to contain predominantly (Vibert *et al.*, 1976) or exclusively (von Baumgarten *et al.*, 1957; von Baumgarten & Kanzow, 1958; von Euler *et al.*, 1973) inspiratory units, whereas the VRN contains both inspiratory and expiratory units (Bianchi, 1971; Mitchell & Berger, 1975). Inspiratory units are nerve cells having a phasic discharge pattern linked to the inspiratory phase and provide excitatory drive to motoneurones innervating inspiratory muscles such as the diaphragm and the external intercostals. In expiratory units, the phasic discharge occurs during expiration and excitates motoneurones innervating expiratory muscles such as the internal intercostals and the abdominals.

Lumsden (1923) and others (Tenney & Ou, 1977) demonstrated that removing all but the medulla and the pons does not markedly alter the pattern of ventilation, indicating that some intrinsic pattern generator exists within these sites. A subregion of the VRN known as the preBotzinger complex is now thought to be the site of the respiratory rhythm generator (Smith *et al.*, 1991; Connelly *et al.*, 1992). Rhythmogenesis is still a poorly understood phenomenon, however, with models including pacemaker neurones

(Feldman & Cleland, 1982), interconnected networks of platonic cells (Llinas, 1988) and hybrids between the two (Ogilvie *et al.*, 1992).

1.1.2.2 Pneumotaxic centre

Stimulation of precise areas in the upper pons provokes a "phase-switch" in the respiratory cycle (Bertrand & Hugelin, 1971; Cohen, 1971), indicating that this is the site of the pneumotaxic centre. This area appears to terminate or inhibit inspiration and thus regulate ventilation. The role of this centre may be fine-tuning of the respiratory rhythm since normal respiration can exist in the absence of this centre.

1.1.2.3 Apneustic Centre

When a precise area in the lower pons of experimental animals is isolated by transection (Lumsden, 1923) or electrically stimulated (Ngai & Wang, 1957), the pattern of respiration is characterised by prolonged inspiratory gasps (apneuses) interrupted by transient expirations. Destroying this centre suppresses tidal volume but increases the frequency of ventilation (St. John & Wang, 1976), suggesting that the apneustic centre is primarily involved in controlling tidal volume. Tidal volume, however, is a complex process involving interaction between each of the brainstem respiratory centres, indeed, the apneustic centre's role in the control of normal human respiration is questionable.

1.1.2.4 Cortical Control

Breathing in humans is, to a considerable extent, under voluntary control, allowing the respiratory centres in the brainstem to be temporarily overridden. The cortical mechanisms involved in this are largely unknown (for review, see Davenport & Reep, 1995).

1.1.3 SENSORS

The respiratory centres mentioned above receive sensory input from various receptors, the most important of which are chemoreceptors and mechanoreceptors.

1.1.3.1 Chemoreceptors

Since the purpose of respiration is to supply O_2 to and remove CO_2 from the blood, it is unsurprising to find that the pattern of ventilation is exquisitely sensitive to feedback from receptors which respond to variations in blood O_2 and CO_2 levels.

1.1.3.1.1 Peripheral Chemoreceptors

The principal O_2 sensors are the so-called peripheral chemoreceptors, localised in the carotid and aortic bodies (Duffin, 1971; Sorensen, 1971), which signal the brain via afferent pathways in the glossopharyngeal and vagus nerves, respectively. These

chemoreceptors increase their discharge as O_2 levels are decreased, with steep increases below ~50mmHg and this increased discharge stimulates breathing. Both carotid and aortic bodies are stimulated by a fall in Pa_{O2}, although the response of the aortic body is much smaller (Lahiri *et al.*, 1981). Unlike the carotid body, the aortic body is stimulated by reductions in arterial O_2 content which occur in carboxyhemoglobinemia, anemia and hypotension (Lahiri, 1980; Lahiri *et al.*, 1980), suggesting that the aortic body is more sensitive to total O_2 delivery whereas the carotid body is primarily responsive to changes in Pa_{O2} (Lahiri, 1991). Under normal conditions, O_2 sensors account for only a small part of the chemoreceptor drive to breathe, since removal of their sensory input by breathing pure O_2 reduces minute ventilation by only about 15% in awake mammals (for review, see Fidone & Gonzales, 1986).

1.1.3.1.2 Central Chemoreceptors

Although the peripheral chemoreceptors are also responsive to alterations in Pa_{CO2}, the principle detectors for Pa_{CO2} changes are the central chemoreceptors, which have been localised to the ventrolateral surface of the medulla (Mitchell *et al.*, 1963; Loeschke *et al.*, 1963; Schlaefke *et al.*, 1970). A very small increase in Pa_{CO2} increases the activity of these chemoreceptors which in turn stimulate ventilation, therefore Pa_{CO2} acting on the central chemoreceptors is the principle respiratory stimulus.

It has been debated for many years whether CO_2 activates peripheral and central chemoreceptors exclusively via its acidifying action or via some pH-independent effect. Several studies indicate that differences exist in the ventilatory response to CO_2 versus H^+ (Fukuda, 1983; Eldridge *et al.*, 1985; Harada *et al.*, 1985; Neubauer *et al.*, 1991), however, it is generally accepted that CO_2 stimulates chemoreceptors via intracellular acidification (Fidone & Gonzalez, 1986; Hanson *et al.*, 1981; Rocher *et al.*, 1991).

Thus, variations in Pa_{CO2} are detected by both the peripheral and central chemoreceptors, whereas changes in Pa_{O2} are detected by the peripheral chemoreceptors only. Indeed, hypoxia exerts a depressive effect on central respiratory centres (Grunstein *et al.*, 1981; Easton *et al.*, 1986), presumably via a direct attenuation of neuronal excitability (Fowler, 1989; Doll *et al.*, 1991; Cummins *et al.*, 1993).

1.1.3.2 Mechanoreceptors

Feedback from mechanoreceptors in the lungs is vital for the precise control of respiratory muscle activity. At least three types of mechanoreceptors exist in the lungs and all have afferent pathways travelling via the vagus nerve (Richardson & Ferguson, 1979).

1.1.3.2.1 Slowly Adapting Receptors

Also known as "stretch receptors" these receptors are myelinated nerve terminals localised mainly to the smooth muscle of conducting airways (Guz & Trenchard, 1971) and are stimulated by changes in tension across the airway wall. They are responsible for the Hering-Breuer reflex which inhibits sustained inspiratory activity to avoid over inflation of the lung (Bartoli *et al.*, 1973). Slowly adapting receptors probably have little influence on the breathing pattern of normal people at rest (Clark & von Euler, 1972), but they may play a role in the control of breathing in patients with chronic obstructive pulmonary disease who breath at high lung volumes (Bartoli *et al.*, 1973).

1.1.3.2.2 Rapidly Adapting Receptors

These receptors are also myelinated nerve terminals, but differ from slowly adapting receptors in that they adapt more quickly and fire irregularly. In addition to mechanical stimuli, they are activated by chemical stimuli such as ammonia, ozone, histamine and prostaglandins (Sampson & Vidruk, 1975) and are therefore known as "irritant receptors." These receptors are situated below the epithelium and between epithelial cells (Das *et al.*, 1978; Laitinen, 1985) and when stimulated, evoke bronchoconstriction by a reflex increase in vagal efferent activity (see section 1.4.1.1). The role of these receptors is unclear but they may act to limit penetration of potentially harmful substances into the lung and thereby prevent these substances from reacting with the gas exchanging surfaces of the alveoli.

1.1.3.2.3 C-fibre Receptors

Nonmyelinated nerve endings, termed C-fibres, are also found in the airways, usually within the airway epithelium (Rhodin, 1966; Laitinen, 1985). Unmyelinated nerves are also occasionally seen in alveolar walls (Fox *et al.*, 1980) and these are thought to correspond to J-receptors, which are closely associated with pulmonary capillaries. Activation of these receptors, by lung deflation and certain chemical stimuli such as capsaicin (Coleridge *et al.*, 1965) and bradykinin (Kaufman *et al.*, 1980), leads to the development of rapid, shallow breathing.

1.1.3.2.4 Other Receptors

While the chemoreceptors and mechanoreceptors mentioned above are the principal receptors involved in controlling respiration, the central respiratory controller receives feedback from a number of other sources. Receptors in the upper airways of the nose, pharynx and larynx are involved in airway reflexes such as sneezing, while input from joint and muscle receptors are believed to be an important part of the stimulus to ventilation during exercise.

1.2 STRUCTURE OF THE AIRWAYS

1.2.1 INTRODUCTION

The airways consist of a series of branching tubes which become narrower and more numerous as they penetrate deeper. The *upper respiratory tract*, comprising the nose, paranasal sinuses and the nasopharynx, is principally involved in filtering, humidifying and adjusting the temperature of inspired air, whereas the function of the *lower respiratory tract,* which begins at the trachea and continues down through the bronchi and bronchioles to the alveolar sacs, is to conduct inspired air to the gas exchanging regions of the lungs. The lower respiratory tract can be conceptually divided into three zones:

1. The conducting zone, comprising the trachea, bronchi and bronchioles which have no gas exchanging surfaces (alveoli).
2. The transitional zone, which both conducts air and exchanges gas. These consist of respiratory bronchioles which have both gas exchanging and non-gas exchanging epithelium in their walls.
3. The gas exchanging zone which consists of the alveolated airways (alveolar ducts and sacs).

1.2.2 AIRWAY STRUCTURE

Although the conducting airways share a common plan - muscular tubes lined by a ciliated epithelium - they differ in detail depending on size (Hayward & Reid, 1952; Horsefield, 1974). The walls of airways approximately 1mm or more in diameter are reinforced by cartilage and are called bronchi, whereas conducting airways without cartilage are termed bronchioles.

1.2.2.1 Bronchial level

The trachea divides into left and right primary bronchi which supply each lung. Each primary bronchus gives rise to secondary bronchi supplying the lobes of the lung before dividing again to form tertiary bronchi which supply the segments of each lobe. Within the lung, the bronchi branch asymmetrically, giving rise to progressively smaller vessels. The bronchi are lined by a pseudostratified ciliated columnar epithelium resting on a basement membrane (McCarter & Vazquez, 1966), beneath which is the lamina propria, composed predominantly of longitudinal elastic fibres (Miller, 1947). The smooth muscle of the bronchial wall lies just beneath the elastic fibres and is arranged in discrete bundles which wind down the airway wall in a spiral pattern (Von Hayek, 1960; Nagaishi *et al.*, 1972). Loose connective tissue and bronchial glands occupy the space between the muscle and the outermost layer of the bronchi, which consists of

heavy, circumferential bundles of collagen fibres and cartilage. The form taken by the cartilage varies between regions in the respiratory tract; in the trachea and extrapulmonary bronchi, the cartilage appears as U-shaped rings which are open dorsally, whereas the intrapulmonary bronchi have irregularly shaped islands of cartilage (Hayward & Reid, 1952).

The epithelium lining the bronchi fulfils a number of roles, including synthesis and release of various chemical factors (see section 1.4.3) as well as their degradation, but its principle function is the production and propulsion of mucus (Breeze & Wheeldon, 1977). Glands in the submucosa also contribute significantly to the production of mucus (Meyrick *et al.*, 1969). These glands decrease in number distally, ultimately disappearing at the same level as the cartilage.

1.2.2.2 Bronchiolar level

The walls of airways less than 1mm in diameter lack cartilage and consist mainly of smooth muscle enclosed in a thin connective tissue space. The proportion of the airway wall occupied by smooth muscle increases as the airway diameter decreases, reaching maximal prominence in the terminal bronchioles (Von Hayek, 1960; Nagaishi *et al.*, 1972). The epithelium is classified as simple, columnar, containing only two cell types; ciliated cells and non-ciliated secretory cells, known as Clara cells (Clara, 1937). Mucus producing cells are not normally found, but may be present in a number of disease states or after chronic exposure to tobacco smoke (Ebert & Terracio, 1975). In respiratory bronchioles, the proportion of ciliated cells decreases and in distal respiratory bronchioles often disappear completely, while the non-ciliated cells become cuboidal in shape (Basset *et al.*, 1971).

1.2.2.3 Respiratory Zone level

The respiratory zone of the lung comprises functional units termed acini which are supplied by a single terminal bronchiole. All the structures comprising an acinus (respiratory bronchioles, alveolar ducts and alveolar sacs) partake to some extent in gas exchange, since they all have alveoli. Respiratory bronchioles are vessels composed in part of muscular bronchial wall covered with cuboidal epithelium and in part by alveoli. Alveolar ducts are conducting structures lined entirely by alveoli and lead to a final generation of blind-ending, alveolus-lined spaces known as alveolar sacs. Airway smooth muscle and elastic fibres penetrate down to the level of the alveolar ducts (Whimster, 1975; Young *et al.*, 1980), whereas the alveolar walls are composed of alveolar epithelium, pulmonary endothelium and interstitial cells (Crapo *et al.*, 1982). The capillaries of the pulmonary vasculature are arranged as a tight mesh (Miller, 1947; Sobin *et al.*, 1970) around the alveoli to maximise diffusion of gases.

1.2.2.4 Overview

Each section of the respiratory tract has its own characteristic structural feature, but there is a gradual rather than abrupt, transition from one type of airway to the next. The principal variations in architecture which occur along the respiratory tree are as follows:

(a) The respiratory epithelium undergoes progressive transition from a tall, pseudostratified, columnar, ciliated form in the larynx and trachea to a simple, cuboidal, non-ciliated form in the smallest airways. Mucus producing cells are numerous in the trachea but decrease in number and are absent in the terminal bronchioles.

(b) A layer of smooth muscle lies deep to the mucosa (except in the trachea) and becomes increasingly prominent as the airway diameter decreases, reaching its greatest prominence in the terminal bronchioles.

(c) Submucosal connective tissue underlies the smooth muscle layer and contains mucus glands which become progressively less numerous in the narrower airways and are not present beyond the tertiary bronchi.

(d) Cartilage lies outside the submucosa and diminishes in prominence as the calibre of the airway decreases.

Figure 1.1

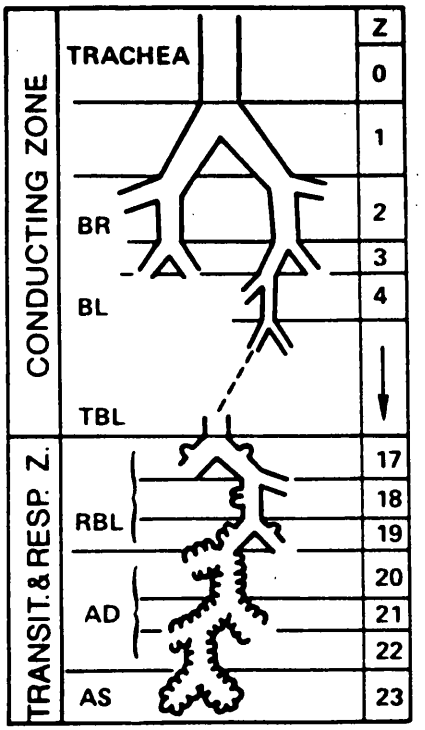


Figure 1.1

Idealization of the human lower respiratory tract. The first 16 generations (Z) make up the conducting airways and the last 7 the respiratory and transitional zones. BR; bronchus, BL; bronchiole, TBL; terminal bronchiole, RBL; respiratory bronchiole, AD; alveolar duct, AS; alveolar sac.

Adapted from Levitsky, 1995.

1.3 AIRWAY SMOOTH MUSCLE

1.3.1 INTRODUCTION

As stated above, the airways can be thought of as a series of branching tubes responsible for the conduction of air in and out of the gas-exchanging regions of the lungs. Contraction of airway smooth muscle is one of the main determinants of airway diameter and therefore of the resistance to airflow (Pedley *et al.*, 1970). According to Poiseuille's law, resistance is related to the fourth power of the radius, therefore small changes in airway smooth muscle tone can have a marked effect on airway resistance.

Poiseuille's Equation: $\text{Resistance} = \frac{8\eta l}{\pi r^4}$ where:

$\pi/8$ = constant of proportionality
r = radius of the tube
l = length of the tube
η = viscosity of the fluid

1.3.2 PHYSIOLOGY OF AIRWAY SMOOTH MUSCLE

1.3.2.1 Structure

All smooth muscle cells contain the contractile proteins actin, myosin, tropomyosin and caldesmon (Stull, 1980; Kamm & Stull, 1985a; Kamm & Stull, 1989). They function by forming into parallel filaments which slide past each other. Actin represents the major contractile protein of smooth muscle, being 10-20 times more abundant than myosin. Actin filaments are composed of two linear polymers of a 42-kilodalton (kDa) globular protein wrapped together in a helical configuration. Myosin filaments are thick, bipolar and arranged asymmetrically in a hexameric structure. Myosin comprises one pair of heavy chains (each of 200kDa) and two pairs of light chains (one pair of 17kDa known as the "essential light chain" and the other of 20kDa referred to as the "regulatory light chain"). The myosin molecule can be divided into the long 'tail' section and the globular 'head.' The globular head section of myosin contains the binding sites for attachment to actin as well as the enzymatic (ATPase) sites which cleave ATP to provide the energy necessary for the binding reactions to take place (Adelstein & Eisenberg, 1980). The two sets of myosin light chains are also located at the head of the myosin molecule and are considered to be essential in the regulation of the contractile process.

1.3.2.2 Regulation of Myosin by Ca^{2+}

When the concentration of free intracellular calcium ($[Ca^{2+}]_i$) is elevated from basal levels to between 0.5 and 1.0μm, the free Ca^{2+} binds to calmodulin, a low molecular weight binding protein with a high degree of specificity for Ca^{2+} (Cheung, 1980;

Manalan & Klee, 1984). Each calmodulin molecule has the capacity to bind up to a maximum of four molecules of Ca^{2+} and it is generally accepted that at least three binding sites must be occupied for activation to occur. The activated calcium-calmodulin complex stimulates myosin light chain kinase (MLCK), which in turn phosphorylates the 20kDa light chains of myosin on a specific serine residue. This phosphorylation step is regarded as a prerequisite for the activation of actin-dependent myosin ATPase and hence, the initiation of contraction (Stull, 1980; DeLanerolle & Stull, 1980; DeLanerolle *et al.*, 1982). Decreases in cytosolic $[Ca^{2+}]_i$ result in the dissociation of calmodulin from MLCK and conversion of the kinase to the inactive state in which it normally exists in resting smooth muscle cells (Dabrowska *et al.*, 1977). With MLCK inactivation, myosin light chain is dephosphorylated by a myosin light chain phosphatase (MLCP) localized to the contractile elements (Kamm & Stull, 1985b; Shimizu *et al.*, 1994; Shirazi *et al.*, 1994). Thus, the net extent of regulatory light chain phosphorylation, and hence the magnitude of smooth muscle contraction, is determined by the relative activities of MLCK and MLCP (see Kamm & Grange, 1996).

In addition to MLCK, myosin light chain can also be phosphorylated by protein kinase C (PKC) and cyclic AMP-dependent protein kinase A (PKA). Like MLCK, PKC is Ca^{2+} dependent, but its affinity for Ca^{2+} is enhanced by diacylglycerol (DAG, generated by agonist-activated receptors, as described below) such that it is fully active at basal levels of Ca^{2+} (Nishizuka, 1986; Collins *et al.*, 1992). Phosphorylation of myosin light chain by PKA occurs at such a slow rate that this mechanism is thought to be physiologically irrelevant with respect to mediating contraction (Walsh *et al.*, 1981), therefore, MLCK is accepted as being the primary regulatory mechanism in initiating smooth muscle contraction (Kamm & Stull, 1989)

1.3.2.3 Actin-Myosin interaction

The generation of contraction by actin and myosin is achieved by the cyclic attachment of the globular heads of the myosin molecules to actin (so-called crossbridge formation), a flexing change in the configuration of the myosin head with respect to actin, detachment of myosin from actin followed by subsequent re-attachment at another site further down the actin molecule. This rapid "crossbridge cycling" is responsible for the active force development in smooth muscle and is fuelled by the breakdown of ATP by actin-activated myosin ATPase. In the absence of ATP hydrolysis, i.e. when $[Ca^{2+}]_i$) is low and no activation of MLCK can occur, the orientation of the globular myosin heads is such that crossbridge formation with actin is prohibited. Thus, at rest, actin and myosin molecules can freely slide past each other and no active tension is developed.

1.3.2.4 Calcium Homeostasis

Given that contraction of airway smooth muscle (ASM) is inherently dependent upon the $[Ca^{2+}]_i$ within the cytoplasm of the cell, the regulation of $[Ca^{2+}]_i$ is a tightly controlled process. Sodium-calcium ion (Na^+-Ca^{2+}) exchangers (Bullock *et al.*, 1981) and Ca^{2+} efflux pumps (Bryson & Rodger, 1987) in the ASM cell membrane actively extrude Ca^{2+} from the cell, while a Ca^{2+} uptake mechanism in the sarcoplasmic reticulum sequesters intracellular Ca^{2+}. This results in a $[Ca^{2+}]_i$ of between 0.05-0.25μM in relaxed airway smooth muscle compared with a concentration of Ca^{2+} in the extracellular fluid of between 1-2mM. Since the cell membrane effectively partitions the intra- and extracellular environments, extracellular Ca^{2+} can only gain entry into the cell by the opening of Ca^{2+} channels in the plasmalemma, allowing Ca^{2+} to flow down the electrochemical and concentration gradient into the cell. Two types of Ca^{2+} channel have been proposed to exist in smooth muscle (a) voltage-dependent and (b) receptor-operated (Bolton, 1979).

1.3.2.4.1 Voltage-dependent Ca^{2+} Channels

These channels, referred to as "L-type Ca^{2+} channels" possess a Ca^{2+} conductance that is directly proportional to the potential difference that exists across the plasma membrane and are opened by membrane depolarization (Kotlikoff, 1988; Marthan *et al.*, 1989). Voltage-operated Ca^{2+} channels have been shown to exist in airway smooth muscle (Rodger, 1985; Giembycz & Rodger, 1987; Worley & Kotlikoff, 1990) and are responsible for generating the contractions evoked by potassium chloride (Kirkpatrick, 1975; Farley & Mills, 1977; Foster *et al.*, 1983). In addition, these channels are involved in mediating the effects of agents which activate or inhibit potassium (K^+) channels. K^+ channels are a heterologus family of membrane channels which are activated by various stimuli (Small *et al.*, 1991; Morley, 1994). Opening of K^+ channels allows K+ to leave the cell, resulting in membrane hyperpolarization. This hyperpolarization tends to inactivate the L-type Ca^{2+} channels, leading to a fall in $[Ca^{2+}]$i and hence a reduction in smooth muscle contractility. Agents which stimulate K^+ channels, including the so-called K^+ channel openers and β_2 adrenoceptor agonists, therefore tend to induce relaxation of smooth muscle.

1.3.2.4.2 Receptor-operated Ca^{2+} channels

Ion channels opened by a receptor for a particular ligand have been described in certain types of smooth muscle, including airway smooth muscle (Bolton, 1979; Murray & Kotlikoff, 1991; Murray *et al.*, 1993). Murray and Kotlikoff (1991) studied canine tracheal smooth muscle cells and demonstrated increases in cytosolic Ca^{2+} with histamine or bradykinin that were insensitive to dihydropyridines (antagonists such as nifedipine and verapamil which block L-type Ca^{2+} channels). Further evidence of dihydropryridine-insensitive Ca^{2+} pathways channels in airway smooth muscle has

been supplied (Croxton *et al.*, 1994; Vannier *et al.*, 1995), but the physiological role of these receptor-operated channels in airway smooth muscle remains to be established.

1.3.2.5 Excitation-contraction (E/C) Coupling

Essentially two forms of E/C coupling (electromechanical and pharmacomechanical) are recognized; electromechanical coupling depends either upon electrical depolarization of the plasmalemma, which opens voltage-operated Ca^{2+} channels, or on a voltage dependent release of Ca^{2+} from intracellular stores. In each case, the rise in $[Ca^{2+}]i$ is due to depolarization of the plasma membrane, whereas pharmacomechanical coupling mechanisms are voltage-independent. The latter may involve influx of extracellular Ca2+ via receptor-operated Ca^{2+} channels, release of Ca^{2+} from intracellular stores, or an increased sensitivity of the contractile proteins to Ca^{2+}.

The Ca^{2+} needed for smooth muscle contraction can come from extracellular sources or from internal stores such as the sarcoplasmic reticulum and the relative importance of each source depends upon the agent used to induce tone. Receptor stimulation by pharmacological agonists such as cholinomimetics, histamine etc., initiate contraction of airway smooth muscle via the release of Ca^{2+} from intracellular stores (Coburn, 1977). Entry of extracellular Ca^{2+} is required, however, for the maintenance of force in the continued presence of the agonist (Bourreau *et al.*, 1991; Murray & Kotlikoff, 1991). In contrast, the entry of extracellular Ca^{2+} is essential for both the initiation of the contraction and the maintenance of force induced by depolarizing agents such as potassium chloride (Coburn, 1977; Bourreau *et al.*, 1991). Thus, pharmacological agonists binding to specific cell surface receptors induce contraction of airway smooth muscle by liberating Ca^{2+} from intracellular stores. This has led to huge interest in the so-called "second-messengers" which transduce the signal from the stimulated receptor in the plasma membrane to the intracellular organelles involved in the contractile process.

1.3.3 SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN MEDIATING CONTRACTION OF AIRWAY SMOOTH MUSCLE

1.3.3.1 Receptor-G protein interactions

For the majority of physiological contractile agonists, activation of guanine nucleotide regulatory proteins (G proteins) is a critical intermediate step in the coupling of receptor-activation to second-messenger generation (for review see Raymond, 1995; Exton, 1997). G proteins are heterotrimers consisting of an α-subunit that binds guanine nucleotides and possesses intrinsic GTPase activity, and tightly associated β- and γ-subunits that anchor the α subunit to the cytoplasmic surface of the cell membrane (Neer & Clapham, 1988). In the resting state, G proteins exist as an αβγ holomer, with guanine diphosphate (GDP) bound to the α subunit (see Figure 1.2). Association of an agonist to its receptor promotes exchange of GDP for guanine triphosphate (GTP) in the α-subunit, which triggers the dissociation of the αβγ complex from the receptor and separation of α-GTP from the βγ-complex. The "active" α-GTP subunit is then able to interact with a particular effector mechanism to modulate second-messenger production or ion exchange. Hydrolysis of the bound GTP by an intrinsic GTPase activity in the α-subunit terminates its interaction with the effector and the α-GDP reassociates with the βγ-complex to form a heterotrimer ready for activation by another receptor molecule (Sternweis & Smrcka, 1992). There is considerable heterogeneity in the G proteins involved in signal transduction due to the existence of different forms of the α, β and γ subunits, although specificity of receptor-effector action is largely determined by structural and functional differences in the α-subunits. The G protein α subunits which are of most relevance to airway smooth muscle contractility are the subtypes $G_{\alpha s}$, $G_{\alpha i}$ (which stimulate and inhibit adenylyl cyclase, respectively) and $G_{\alpha q}$ which stimulates phospholipase C.

Figure 1.2 The G protein cycle

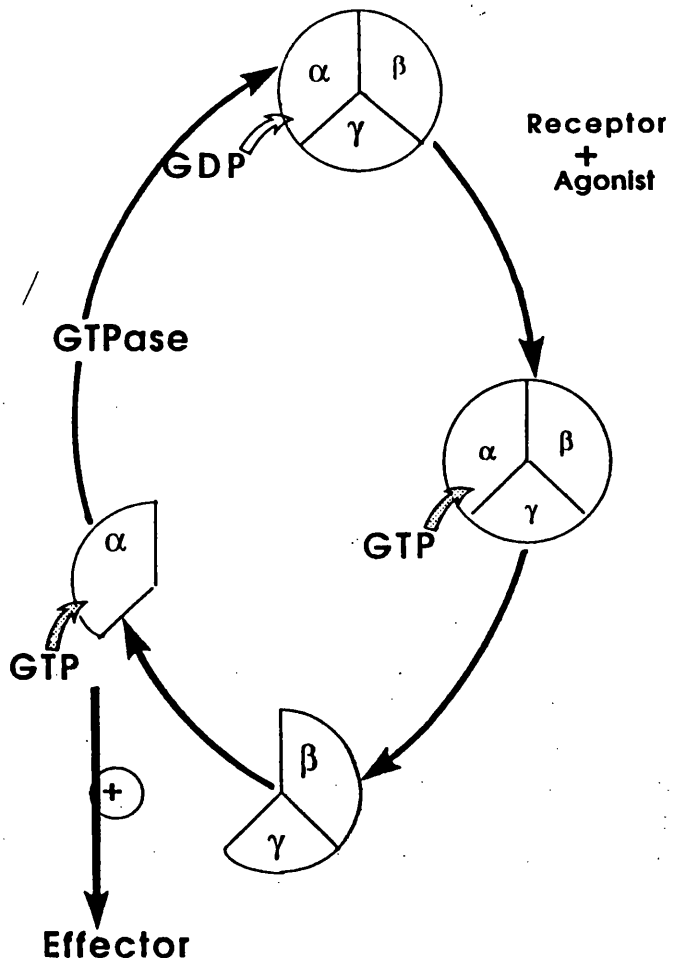


Figure 1.2
Diagrammatic representation of the G protein/GTP/GDP cycle that is regarded as critical in the receptor-mediated signal transduction process

1.3.3.2 Phosphatidylinositol 4,5-biphosphate (PIP_2) hydrolysis

The majority of agonists which evoke contraction of airway smooth muscle are believed to act via stimulation of phospholipase C (PLC). Stimulation of PLC results in the rapid breakdown of the membrane phospholipid, phosphatidylinositol 4,5-biphosphate (PIP_2), resulting in the generation of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).

1.3.3.2.1 Inositol 1,4,5-trisphosphate (IP_3)

Following the hypothesis of Berridge and Irvine (1984) that IP_3 is the "missing link" between plasma membrane receptors and internal Ca^{2+} stores, several papers demonstrated that IP_3 acts by mobilizing Ca^{2+} from intracellular pools (Berridge and Irvine; 1989; Carsten & Miller, 1990; Coburn & Baron, 1990; Somlyo & Somlyo, 1992). Reports that IP_3 is rapidly generated in airway smooth muscle after contractile agonist stimulation (Chilvers *et al.*, 1989; Langlands *et al.*, 1989; Chilvers *et al.*, 1991) and the findings that IP_3 accumulation correlates directly with airway smooth muscle contraction (Meurs *et al.*, 1988; Meurs *et al.*, 1989) are compelling evidence that IP_3 production is directly linked to the initiation of airway smooth muscle contraction.

1.3.3.2.2 Diacylglycerol (DAG)

By contrast, DAG is not implicated in the intracellular Ca^{2+} release process, and hence the initial generation of contraction. Instead, it has been suggested that DAG may be more intimately involved in the maintenance of smooth muscle contraction via stimulation of protein kinase C (PKC, Park & Rasmussen, 1986a; Rasmussen *et al.*, 1987).

PKC represents a family of related kinases which are of central importance in intracellular signalling pathways. At least 11 distinct isozymes of PKC have been described, several of which exist in airway smooth muscle (Webb *et al.*, 1997). Stimulation of PKC by phorbol esters generally produces slowly developing, sustained contraction of airway smooth muscle, in some cases without raising $[Ca^{2+}]_i$ (Dale & Obianime, 1987; Park & Rasmussen, 1986a; Obianime *et al.*, 1988).

It is thought that DAG increases the sensitivity of PKC to Ca^{2+} such that the kinase becomes active at $[Ca^{2+}]_i$ which are normally below the threshold required to induce contraction (Nishizuka, 1984; Nishizuka, 1986; Collins *et al.*, 1992). The precise mechanism by which PKC evokes contraction remain elusive, but it is proposed that they increase the sensitivity of the contractile elements to Ca^{2+}, either by phosphorylating MLCK directly (Nishikawa *et al.*, 1983; Singer & Baker, 1987; Fulginiti *et al.*, 1993) or by phosphorylating an intermediate kinase such as mitogen activated protein (MAP) kinase (see Singer, 1996).

1.3.3.3 Tyrosine kinase pathways

The role of tyrosine kinase-linked signal transduction pathways has been examined largely in the context of long-term responses such as cell proliferation (see section 1.5). Given that tyrosine kinase activity is frequently associated with elevations in $[Ca^{2+}]_i$, it has recently been suggested that this pathway may play an important role in regulating smooth muscle contraction (see Hollenberg, 1994; Di Salvo *et al.*, 1996). Growth factors such as epidermal growth factor and platelet-derived growth factor, which activate membrane receptor tyrosine kinase, have been shown to evoke contraction of various smooth muscle preparations, including airway smooth muscle (Nasuhara *et al.*, 1996). Furthermore, contractions evoked by angiotensin II and thrombin in gastric smooth muscle can be blocked by tyrosine kinase inhibitors (Yang *et al.*, 1993; Hollenberg *et al.*, 1993), suggesting that these G protein-coupled receptor agonists may act via tyrosine kinase activation rather than the generally accepted IP_3/DAG pathway (Griendling & Alexander, 1990). It should be apparent, however, that considerably more work must be conducted in this area before tyrosine kinase activation can be accepted as being an important component of the signal transduction pathways leading to smooth muscle contraction.

1.3.3.4 Mediator release

A number of agents induce contraction or relaxation of airway smooth muscle via the secondary release of mediators such as histamine, cyclo-oxygenase metabolites and leukotrienes (see section 1.4.3).

1.3.3.5 Phospholipase D activation

It has recently been suggested that activation of phospholipase D (PLD) may be a novel signal transduction pathway leading to contraction of airway smooth muscle. This enzyme hydrolyses phosphatidylcholine, forming phosphatidic acid (PA), which is believed to lead to the formation of DAG (Exton, 1990). A number of agonists, both G protein-coupled receptor agonists and tyrosine kinase receptor agonists, have been shown to stimulate PLD (Liscovitch *et al.*, 1993; Plevin *et al.*, 1994), however the mode of coupling between agonist receptors and PLD activation is poorly understood. It is generally accepted that PKC plays a pivotal role both as a mediator and as a modulator of PLD activity (for review see Exton, 1990; Kiss, 1990), although the precise pathways remain to be elucidated.

Figure 1.3 Mechanisms involved in excitation-contraction coupling in airway smooth muscle

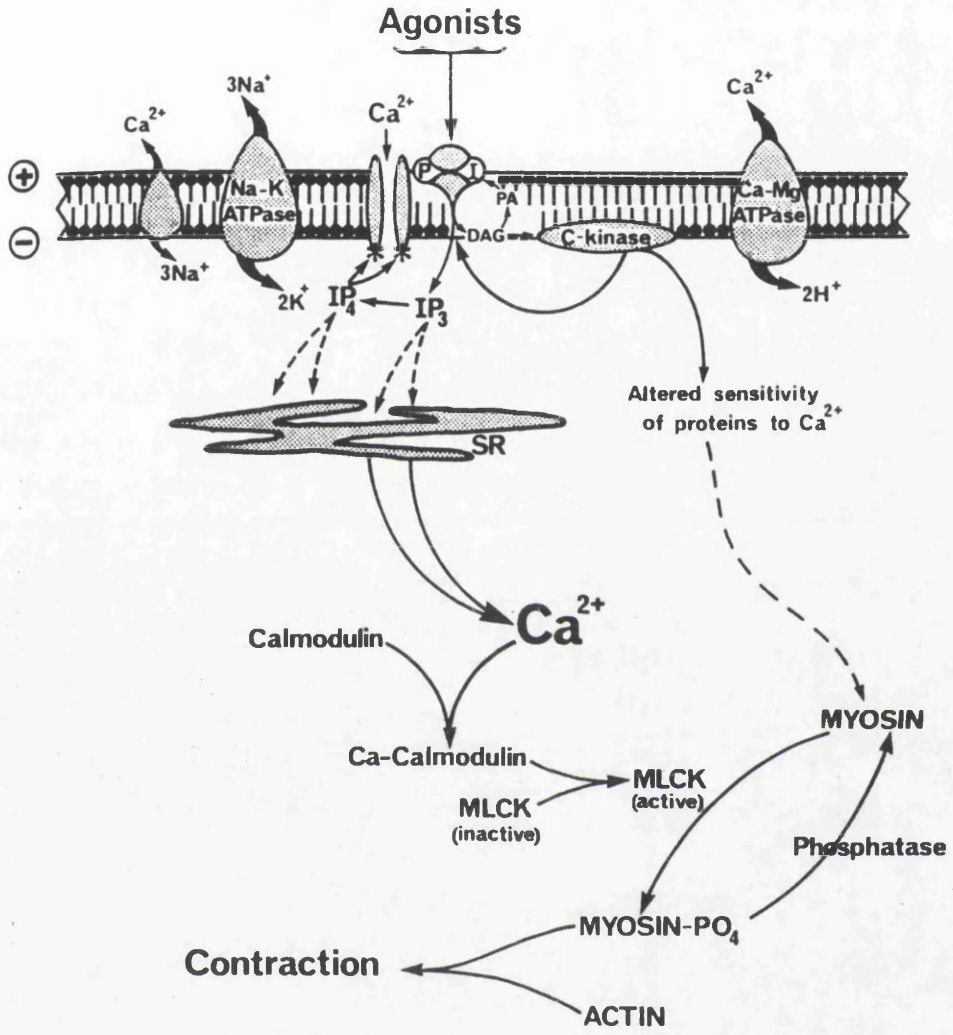


Figure 1.3

Diagrammatic summary of the sequence of events thought to be involved in contraction of airway smooth muscle. The cell membrane contains the sodium-calcium exchange system, the sodium pump (Na-K ATPase), the Ca2+ efflux pump (Ca-Mg ATPase), PKC (C-kinase) and a receptor complex coupled to the phosphatidylinositol (PI) system. Agonist-stimulation of the receptor activates, via G protein linkage, phospholipase C, resulting in the formation of IP_3 and DAG. The former evokes release of Ca2+ from the sarcoplasmic reticulum (SR), which then activates the myosin light chain kinase pathway.

Adapted from Leff, 1988.

1.3.4 SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN MEDIATING RELAXATION OF AIRWAY SMOOTH MUSCLE

The predominant second messengers involved in agonist-induced relaxation of airway smooth muscle are the cyclic nucleotides adenosine 3', 5'-cyclic monophosphate (cAMP) and guanosine 3', 5'-cyclic monophosphate (cGMP), which are formed by the activities of adenylyl cyclase and guanylyl cyclase, respectively.

1.3.4.1 Adenosine 3', 5'-cyclic monophosphate (cAMP) generation

Elevation of intracellular cAMP levels is closely correlated with relaxation of airway smooth muscle (Niewoehner *et al.*, 1979), indeed, exogenously administered cell-permeable analogues of cAMP induce bronchial smooth muscle relaxation (Triner *et al.*, 1977). Intracellular cAMP levels may be elevated by a number of mechanisms:

(a)An increase in the activity of adenylyl cyclase following stimulation of membrane receptors (for example, β-adrenoceptors, Katsuki & Murad, 1977; Lau & Lum, 1983; Rinard *et al.*, 1983) linked to G proteins of the Gs class.

(b)Direct activation of adenylyl cyclase via a receptor-independent mechanism, for example by forskolin (Seamon *et al.*, 1981; Insel *et al.*, 1982)

(c)Inhibition of phosphodiesterases (PDEs), which are responsible for the rapid hydrolysis of cAMP (Cortijo *et al.*, 1993).

1.3.4.2 Guanosine 3', 5'-cyclic monophosphate (cGMP) generation

Guanylyl cyclase exists in two forms, a soluble form (GCs) found in the cytosol and a particulate form (GCm) located in the plasma membrane. GCs is activated by nitric oxide (NO) and NO donors (Gruetter *et al.*, 1989; Ignarro *et al.*, 1981; Feelisch & Novak, 1987), whereas GCm is a plasma membrane receptor for the natriuretic peptides and related hormones (Ishii & Murad, 1989; Wong & Garbers, 1992). Like cAMP, cGMP is inactivated by phosphodiesterases (Torphy & Undem, 1991).

1.3.4.3 Mechanisms underlying cyclic nucleotide-mediated relaxation

Agents which elevate cAMP, such as β_2 adrenoceptor agonists and phosphodiesterase inhibitors, are the most commonly used bronchodilators in the treatment of asthma. Despite this, the signalling pathways involved in the mediation of cyclic nucleotide responses are still unclear. It was originally believed that cAMP produced relaxation by stimulating cyclic AMP-dependent protein kinase, which then phosphorylated MLCK and subsequently inhibited the binding of calmodulin (Conti & Adelstein, 1981). It is now proposed that both cAMP and cGMP may induce smooth muscle relaxation via stimulation of cyclic GMP-dependent protein kinase (PKG, Francis *et al.*, 1988; Lincoln *et al.*, 1990).

Given that contraction of smooth muscle is associated with an increase in $[Ca^{2+}]_i$, it was proposed that PKG evoked a lowering of $[Ca^{2+}]_i$ to induce relaxation. This may occur through a number of mechanisms:

1.3.4.3.1 Sequestration of intracellular Ca^{2+}

Early studies showed that cGMP could activate Ca^{2+} pumps in vascular smooth muscle, leading to a fall in $[Ca^{2+}]_i$ (Lincoln *et al.*, 1988; Furakawa and Nakamura, 1984; Vrolix *et al.*, 1988). It was subsequently found that PKG phosphorylated the Ca^{2+} uptake mechanism which exists in the sarcoplasmic reticulum of smooth muscle (Huggins *et al.*, 1989; Cornwell *et al.*, 1991; Karczewski *et al.*, 1992). This mechanism could therefore attenuate the contractions evoked either by entry of extracellular Ca^{2+} (through VOC) or by mobilization of intracellular Ca^{2+} (via IP_3 generation).

1.3.4.3.2 Activation of Ca^{2+}-activated K^+ channels

Large-conductance, calcium-activated potassium (BK) channels are found in virtually every type of smooth muscle (Nelson, 1993; Nelson & Quayle, 1995). In vascular smooth muscle, cGMP increases the activity of these channels (Tare *et al.*, 1990; Chen & Rembold, 1992) leading to membrane hyperpolarization and hence, relaxation. PKG is known to increase the opening probability of BK channels (Robertson *et al.*, 1993; Archer *et al.*, 1994), however, the role of these channels in PKG-mediated relaxation remains controversial, since many studies indicate that PKG can induce relaxation via mechanisms independent of BK channel opening (Hamaguchi *et al.*, 1991; Bolotina *et al.*, 1994).

1.3.4.3.3 Inhibition of PLC and IP_3

Several studies suggest that cGMP and PKG inhibit agonist-evoked PLC stimulation, leading to a fall in IP_3 production (Rapoport, 1986; Hirata *et al.*, 1990; Ruth *et al.*, 1993). Alternatively, more recent studies indicate that PKG may inhibit the ability of IP_3 to release Ca^{2+} from intracellular stores (Koga *et al.*, 1994; Komalavilas & Lincoln, 1994).

Thus, it would appear that cAMP and cGMP, acting via PKG, may regulate, simultaneously, various mechanisms involved in the reduction of $[Ca^{2+}]_i$ within the smooth muscle cell.

While it is now believed that both cAMP and cGMP act via PKG, it has also been suggested that high concentrations of cGMP may be capable of stimulating, and acting via, PKA (Francis *et al.*, 1988; Cornwell *et al.*, 1994), indicating that some "cross-over" exists between the cyclic nucleotides.

1.3.5 Overview

Given that elevation of cyclic nucleotide levels is associated with decreases in $[Ca^{2+}]_i$ and smooth muscle relaxation, it is unsurprising that many contractile agents such as α_2-adrenergic agonists, platelet-activating factor and various prostaglandins are associated with producing reductions in cyclic nucleotide levels, particularly cAMP. Indeed, in airway smooth muscle, activation of muscarinic M_2 receptors can produce contraction via inhibition of adenylyl cyclase (Candell *et al.*, 1990). Stimulation of PLC remains the predominant mechanism by which agents produce contraction of airway smooth muscle however, with inhibition of adenylyl cyclase thought to be responsible for modulation of relaxant activity rather than initiation of contraction *per se* (see Eglen *et al.*, 1994).

1.3.6 MAINTENANCE OF CONTRACTION IN AIRWAY SMOOTH MUSCLE

While it is generally accepted that the initiation of smooth muscle contraction is due to the Ca^{2+}-calmodulin-dependent phosphorylation of myosin light chains (as outlined above), there is still considerable debate regarding the mechanisms involved in the sustained phase of contraction. This is largely based on the finding that myosin phosphorylation may actually decline while developed contractions are maintained (Gerthoffer & Murphy, 1982; Kamm & Stull, 1985b). Two hypotheses, although not mutually exclusive, have been proposed to account for this; firstly the "latch-bridge" model and secondly, that PKC regulates the sustained phase of contraction.

1.3.6.1 The Latch-bridge theory

This model proposes that after the initiation of contraction, the rapidly cycling actin-myosin cross bridges are replaced by dephosphorylated myosin cross bridges which cycle slowly or not at all (Aksoy *et al.*, 1982; Hai & Murphy, 1989). This model accounts for the fact that during maintenance of contraction, energy consumption is much lower than during tension development. The maintenance of these latchbridges is Ca^{2+} dependent, suggesting that during the tonic phase of contraction, when $[Ca^{2+}]_i$ is low, there must be some mechanism in operation which increases the sensitivity of the contractile apparatus to Ca^{2+}. Precisely how this is achieved is unknown although a possible role for PKC has been proposed.

1.3.6.2 PKC activation

One of the strongest pieces of evidence in support of PKC being involved in maintaining tone is the finding that, during the sustained phase of agonist-induced and phorbol ester (which stimulate PKC)-induced contractions, the changes in patterns of protein phosphorylation are very similar (Park & Rasmussen, 1986b; Takuwa *et al.*, 1988). PKC is believed to increase the sensitivity to Ca^{2+} by phosphorylating specific residues on certain contractile proteins (Rasmussen & Barret, 1984; Rodger, 1986).

Thus there appear to be two separate, although in all likelihood interdependent, pathways which control contraction of airway smooth muscle. The first, a calmodulin/myosin light chain kinase pathway is thought to be responsible for the initial rapid phase of contraction. The second pathway, via PKC-mediated phosphorylations is postulated to adapt the cell to accommodate sustained muscle contraction with a minimum energy expenditure.

1.4 CONTROL OF AIRWAY SMOOTH MUSCLE TONE

1.4.1 NEURAL CONTROL

As early as the 17th century it was recognised that the airways are innervated (Bartholinus, 1663). Physiological experiments subsequently demonstrated that stimulation of the vagus nerve caused bronchoconstriction in dogs, an effect which could be blocked by the cholinergic receptor antagonist, atropine (Roy & Brown, 1885). Although the dominant role of airway innervation is believed to be direct control of airway smooth muscle contractility, the autonomic nervous system controls many other aspects of airway function. Indeed, the innervation of submucusal secretory cells, the pulmonary vascular bed, the epithelium, mast cells and other inflammatory cells may influence airway smooth muscle tone indirectly. Human airways are innervated by three distinct neural pathways; two of these pathways, the cholinergic and the adrenergic have been established for some time (Larsell & Dow, 1933; Gaylor, 1934). The third, termed nonadrenergic, noncholinergic (NANC) is more poorly understood due to lack of knowledge of the neurotransmitter(s) involved (Richardson, 1979; Richardson, 1981).

1.4.1.1 Cholinergic Innervation

The parasympathetic nervous system is the dominant neural control mechanisms in all mammals, including humans. In many animal species, section of the vagus nerve or administration of atropine causes a marked bronchodilation, indicating that cholinergic innervation contributes significantly to resting bronchomotor tone. While it is more difficult to study cholinergic nerve function in human airways, the bronchodilator response to atropine and other anticholinergic drugs in normal subjects does suggest a degree of resting vagal tone in human airways (Vincent *et al.*, 1970; de Troyer *et al.*, 1979). In addition, inhalation of the cholinesterase inhibitor edrophonium causes bronchoconstriction in normal subjects, confirming tonic release of acetylcholine in the airways (Quigley *et al.*, 1985).

Cholinergic efferent nerves arise in the vagal nuclei of the brainstem and pass down the vagus nerve to synapse in ganglia situated in the airway wall. Short, postganglionic fibres proceed from the ganglia to airway smooth muscle cells where they release the excitatory neurotransmitter acetylcholine from axon varicosities. Histochemical staining for acetylcholinesterase indicates that cholinergic nerve fibres innervate human airway smooth muscle from the trachea to the terminal bronchioles (Partanen *et al.*, 1982; Sheppard *et al.*, 1983). As is the case in many other species, the density of cholinergic innervation is believed to decrease in smaller airways, so that there are very few cholinergic fibres in terminal bronchioles and none in the alveolar walls. In contrast, a study by Daniel and co-workers in human airways (1986a) found that the smooth muscle of smaller bronchi (4th-7th order) was ten times more densely

innervated than trachealis muscle. Results from physiological and pharmacological studies, however, would suggest that cholinergic innervation does indeed decline in peripheral airways. For example, vagal stimulation in dogs causes marked bronchospasm in intermediate sized bronchi (1-5mm resting diameter), with relatively little effect in bronchioles with a resting diameter less than 0.5mm (Nadel *et al.*, 1971). In humans, the bronchodilator effects of anticholinergic drugs are greater in large compared to small airways (Ingram *et al.*, 1977; Hensley *et al.*, 1977), while in isolated human airways, the cholinergic effects are less pronounced in bronchioles than bronchi (Palmer *et al.*, 1986). It is possible, therefore, that cholinergic innervation increases descending from the trachea to the intermediate bronchi and then decreases from the bronchi to the bronchioles. Alternatively, the apparent disparity between the study by Daniel *et al* and other workers in this field may reflect differences in methodology, since Daniel and co-workers assumed that the nerves being studied were cholinergic on the basis of the varicosities containing small agranular vesicles. Unfortunately there is no direct method to show that these vesicles actually contain acetylcholine and some may contain other mediators such as neuropeptides.

1.4.1.1.1 Cholinergic Receptors

Acetylcholine released from preganglionic vagal fibres activates nicotinic cholinergic receptors in airway ganglia, whereas postganglionic neurotransmission is mediated via muscarine-sensitive cholinergic receptors. At least four subtypes of muscarinic acetylcholine receptors have now been cloned which vary in their tissue locations and pharmacological effect (for review, see Eglen *et al.*, 1994). M_1-receptors, which are excitatory, are present in airway parasympathetic ganglia of many animal species. Their function remains uncertain, but they may be involved in filtering the signal passing through the ganglia. Similar receptors may occur in humans since the M_1 receptor antagonist, pirenzepine, inhibits vagally-mediated bronchoconstriction at doses that do not inhibit the direct effect of cholinergic agonists on airway smooth muscle (Lammers *et al.*, 1989). The cholinergic receptors which mediate bronchoconstriction are located post-junctionally on airway smooth cells and are thought to be of the M_3 subtype (see Eglen *et al.*, 1994). Activation of these receptors evokes, via G proteins of the Gq class, stimulation of phosphoinositide turnover and generation of IP_3 and DAG (Caulfield, 1993). As is the case with many other types of smooth muscle, airway smooth muscle appears to co-express both M_2 and M_3 receptor subtypes postjunctionally (Chilvers *et al.*, 1990; Challiss *et al.*, 1993; Thomas *et al.*, 1993). Activation of M_2 receptors may also lead to contraction, via inhibition of adenylyl cyclase (Candell *et al.*, 1990), although the role of M_2 receptors appears to be modulation of β-adrenoceptor-mediated relaxation. Consistent with this hypothesis is the finding that M_2 receptor antagonists augment the relaxant effect of the β-adrenoceptor agonist isoprenaline in isolated airways from guinea-pig (Watson & Eglen, 1994), dog (Fernandes *et al.*, 1992) and rabbit (Schramm *et al.*, 1995). Furthermore, in the absence of prevailing relaxant tone,

M_2 receptor activation appears to have little or no effect (Thomas *et al.*, 1993). The physiological implications of these findings remain unclear, but it would appear that the M_3 receptors are the dominant subtype in terms of vagally-induced bronchoconstriction, whereas the M_2 receptors may become more important under conditions of high sympathetic activity.

In addition to excitatory M_2 receptors located postjunctionally, there are also prejunctional M_2 receptors which act to inhibit release of acetylcholine from the nerve endings. These inhibitory 'autoreceptors' appear to be responsible for feed-back inhibition and may serve to limit vagal bronchoconstriction (Figure 1). Such receptors have been demonstrated in human bronchi *in vitro* (Minette & Barnes, 1988) and *in vivo* (Ind *et al.*, 1989).

1.4.1.1.2 Cholinergic Innervation in Airway Disease

Anticholinergic drugs have been shown to have significant therapeutic benefit in the treatment of acute severe asthma (Ward *et al.*, 1981) and nocturnal asthma (Coe & Barnes, 1986). There is, however, no direct evidence that cholinergic mechanisms contribute to the airway hyperresponsiveness which is characteristic of asthma. Cholinergic mechanisms may play a role, however, in chronic obstructive pulmonary disease (COPD) and cystic fibrosis, indeed, anticholinergics are the most effective bronchodilators in the treatment of COPD (Crompton, 1968; Klock *et al.*, 1975).

Figure 1.4

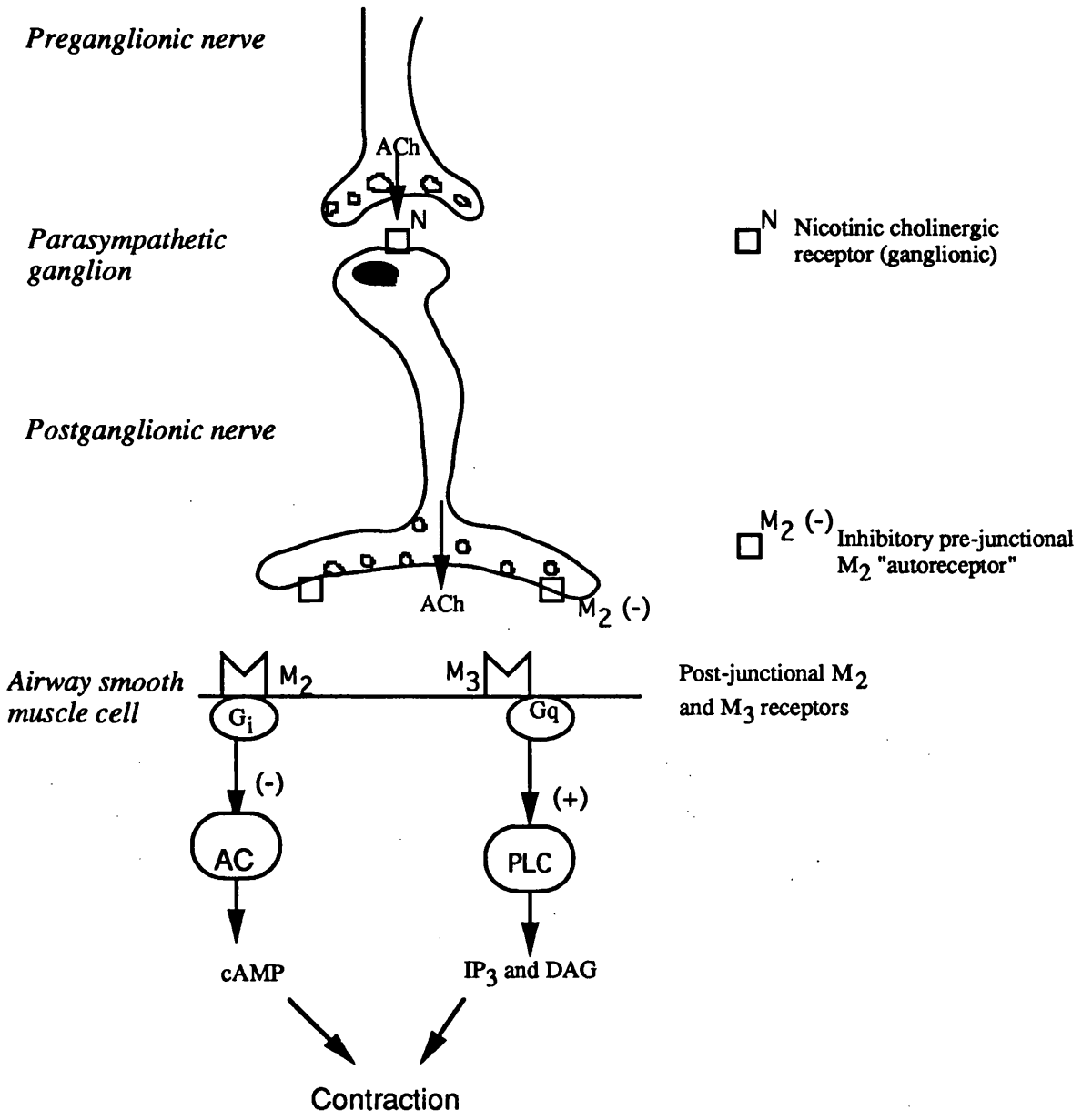


Figure 1.4
Acetylcholine (ACh) released from preganglionic nerves binds to and stimulates nicotinic cholinergic receptors ($\square^N$) in the parasympathetic ganglion. ACh released from postganglionic nerves evokes contraction of airway smooth muscle predominantly via activation of muscarinic M_3 receptors but also via M_2 receptors. M_3 receptors are coupled by G-proteins of the G_q class to phospholipase C and hence to production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). Stimulation of M_2 receptors inhibits, via G-proteins of the Gi class, adenylate cyclase, leading to a fall in cellular cyclic AMP (cAMP) levels. Activation of prejunctional M_2 "autoreceptors" inhibits the release of ACh from the postganglionic nerve. (See text for more detail).

1.4.1.2 Adrenergic Innervation

Catecholamine-containing nerve endings have been found in human airways although, in contrast to the dense parasympathetic nerve supply, sympathetic innervation is generally sparse (El-Bermani, 1978; Partanen *et al.*, 1982). Adrenergic nerve fibres have been found in close association with submucosal glands (Partanen *et al.*, 1982) and bronchial arteries (Doidge & Satchell, 1982), but very few have been shown to innervate airway smooth muscle (Richardson & Beland, 1976; Sheppard *et al.*, 1983). Stimulation of sympathetic nerves in guinea pigs evokes a marked bronchodilation (Ainsworth *et al.*, 1981), despite there being no direct sympathetic innervation of airway smooth muscle in this species (O'Donnell *et al.*, 1978). This suggests that adrenergic nerves may influence bronchomotor tone indirectly. In dogs, stimulation of the sympathetic nerves also causes bronchodilation, but the magnitude of this effect is dependent on the degree of pre-existing vagal tone (Cabezas *et al.*, 1971). Furthermore, the demonstration of adrenergic nerves within parasympathetic ganglia (Richardson & Ferguson, 1979) indicates that the sympathetic nerves may play a role in modulating cholinergic neurotransmission at the ganglionic level.

1.4.1.2.1 Adrenoceptor subtypes

Noradrenaline released from sympathetic nerve endings activates α and β-adrenoceptors on target cells in the airways (Barnes, 1984a). The sympathetically-induced bronchodilation mentioned above in guinea pigs and dogs can be abolished by β-adrenoceptor antagonists (Doidge & Satchell, 1982; Cabezas *et al.*, 1971), indicating that activation of β–receptors leads to relaxation of airway smooth muscle. α-adrenoceptor-mediated contraction of airway smooth muscle has been demonstrated in a number of species including man (Simonsson *et al.*, 1972; Kneussl & Richardson, 1978), although only under certain experimental conditions. For example, α-adrenoceptor-mediated contraction can only be shown in canine airways if there is a high degree of β-adrenoceptor blockade (Leff *et al.*, 1986), or after pretreatment with histamine or serotonin (Kneussl & Richardson, 1978; Barnes *et al.*, 1983). In humans, no α-adrenergic-induced contraction is found in normal airways, however, in certain disease states (chronic bronchitis, bronchopneumonia or after exposure to endotoxin), α-adrenoceptor contractile responses may be exhibited (Simonsson *et al.*, 1972; Kneussl & Richardson, 1978). Since there appears to be no direct sympathetic innervation of human airway smooth muscle, it is likely that α-adrenoceptor activation induces bronchoconstriction indirectly. Interestingly, α-adrenoceptor agonists have been shown to facilitate histamine release from human lung fragments *in vitro*, suggesting the presence of α-receptors on lung mast cells (Kaliner *et al.*, 1972), although this remains to be confirmed.

1.4.1.2.2 Adrenergic Innervation In Disease States

β-adrenoceptor antagonists have no effect on resting bronchomotor in normal humans, indicating an absence of any tonic sympathetic bronchodilator tone (Zaid & Beall, 1966; Tattersfield *et al.*, 1973). In contrast, asthmatic subjects develop bronchoconstriction after administration of β-blockers, suggesting an increased adrenergic drive to the airways in asthma (McNeill, 1964; McNeill & Ingram, 1966).

The finding that α-adrenoceptor responses may be "uncovered" by inflammatory mediators *in vitro* supports the idea that there may be enhanced α-adrenergic activity in asthma (Reed, 1974; Szentivanyi, 1968). For example, the density of α-adrenoceptors is greater in lungs from patients with airway obstruction than from normal subjects (Barnes *et al.*, 1980a). In addition, asthmatic subjects bronchoconstrict with inhaled α-adrenoceptor agonists (Snashall *et al.*, 1978), even in the absence of β-blockade (Black *et al.*, 1982), whereas normal subjects are unaffected. In general, however, current research suggests that sympathetic mechanisms are involved to a limited degree in controlling airway tone in health and disease states.

1.4.1.3 Nonadrenergic-noncholinergic Innervation

In addition to the classical sympathetic and parasympathetic innervation of the airways, neural mechanisms that are neither adrenergic nor cholinergic have been described (Richardson, 1981; Barnes, 1984b). NANC mechanisms are responsible for several different responses within the airways, including bronchoconstriction, bronchodilation and mucus secretion. It is generally believed that more than one neurotransmitter may be involved.

1.4.1.3.1 Inhibitory NANC nerves

Relaxation of airway smooth muscle by inhibitory NANC nerves has been demonstrated *in vitro* in a number of species, including humans (Richardson & Beland, 1976; Taylor *et al.*, 1984). In human airway smooth muscle, this nerve system is the only direct neural bronchodilator pathway, since there is no functional sympathetic innervation. NANC inhibitory nerves have also been demonstrated *in vivo* in feline airways by electrical stimulation of the vagus nerve after cholinergic and adrenergic blockade (Diamond & O'Donnell, 1980). Determination of the neurotransmitter(s) responsible for inhibitory NANC responses has proved difficult, although evidence favours neuropeptides, particularly vasoactive intestinal peptide (VIP).

This peptide produces a prolonged and potent relaxation of airway smooth muscle *in vitro* which is unaffected by adrenergic or cholinergic blockers (Ito & Takeda, 1982; Cameron *et al.*, 1983; Altiere & Diamond, 1984). Nerve terminals staining for VIP (Laitenen *et al.*, 1985) and VIP receptors (Palmer *et al.*, 1986) are present in large and intermediate bronchi but not bronchioles, which is consistent with the finding that NANC bronchodilation is predominantly seen in large, but not peripheral airways (Matsumoto *et al.*, 1985). Activation of VIP receptors on airway smooth muscle

stimulates adenylyl cyclase and hence increases cellular cAMP levels (Lazarus *et al.*, 1986), indicating that VIP could have a direct effect on airway smooth muscle. VIP nerves are often distributed with cholinergic nerves and VIP may coexist in the same nerve terminals as acetylcholine, suggesting that VIP may be cotransmitted with ACh (Laitenen *et al.*, 1985).

In addition to VIP, there is some evidence to suggest that nitric oxide mediates a component of the inhibitory NANC response in porcine (Kannan & Johnson, 1992), guinea pig (Li & Rand, 1991) and human airways (Belvisi *et al.*, 1992; Bai & Bramley, 1993).

1.4.1.3.2 Excitatory NANC nerves

Electrical stimulation of guinea pig bronchi and trachea *in vitro* produces a contractile component which is not blocked by atropine (Anderson & Grundstrom, 1983). A similar NANC response has been reported in human isolated airways, but is inconsistent and non-reproducible (Lundberg *et al.*, 1983). As with the inhibitory NANC response, the neurotransmitter involved in the excitatory response is thought to be a neuropeptide. Substance P (SP) remains a possible candidate since the *in vitro* excitatory NANC responses can be mimicked by SP and inhibited by SP antagonists. SP contracts human airway smooth muscle *in vitro* (Lundberg *et al.*, 1983) and produces bronchoconstriction in animals *in vivo* (Andersson & Persson, 1977) but has little effect on human airways *in vivo* (Fuller *et al.*, 1986). Autoradiographic studies in guinea pig and human lungs indicate that SP-receptors are found in high density in airway smooth muscle from the trachea to the peripheral bronchioles (Carstairs & Barnes, 1986), however, the role of SP as the excitatory NANC neurotransmitter remains to be confirmed.

1.4.1.3.3 NANC Responses In Disease States

It remains to be established whether abnormalities in NANC neurotransmission contribute to airway diseases. In a cat model of asthma there is a reduced inhibitory NANC response (Miura *et al.*, 1990) and post-mortem examination of asthmatic lungs revealed an apparent lack of VIP innervation (Ollerenshaw *et al.*, 1989). While this would suggests that impaired inhibitory NANC responses may contribute to asthma, there is no evidence of a reduced NANC bronchodilator effect in patients with mild asthma (Lammers *et al.*, 1989).

Despite considerable interest in the possibility that NANC abnormalities contribute to airway disease, there remains a lack of understanding regarding the role of neuropeptides in the control of airway smooth muscle tone. This is probably due to the large number of postulated neurotransmitters and the lack of specific agonists and antagonists which can be given safely to man.

Many blood-borne mediators have the potential to influence airway smooth muscle tone, the most notable being catecholamines, natriuretic peptides and "classical" hormones.

1.4.2.1 Catecholamines

Human airway smooth muscle is potently relaxed by β-adrenoceptor agonists *in vitro* and *in vivo* (Davis *et al.*, 1980; Davis *et al.*, 1982; Goldie *et al.*, 1986; van Koppen *et al.*, 1989). The apparent absence of direct functional adrenergic innervation (see section 1.4.1.2) suggests that circulating catecholamines might be important in regulating airway tone, indeed, adrenaline has been used in the treatment of asthma since 1900. Noradrenaline in the plasma is derived almost entirely from overspill of sympathetic nerve activity (Brown *et al.*, 1981), whereas adrenaline is secreted by the adrenal medulla and functions as a circulating hormone (Cryer, 1980). Infusion of noradrenaline within its physiological concentration range has no significant effect on airway function in normal or asthmatic subjects (Berkin *et al.*, 1985; Larssen *et al.*, 1986). In contrast, adrenaline is a potent bronchodilator in normal (Warren & Dalton, 1983) and asthmatic subjects (Barnes *et al.*, 1982a) and protects against histamine-induced bronchoconstriction in non-asthmatics (Warren *et al.*, 1984).

The lack of a bronchoconstrictor effect of β-adrenoceptor antagonists in normal subjects suggests that basal concentrations of circulating adrenaline are probably not important in the regulation of resting bronchomotor tone (Tattersfield *et al.*, 1973). In some asthmatics, however, β-blockers evoke a bronchoconstriction (McNeil, 1964; Richardson & Sterling, 1969) and may increase bronchial hyperresponsiveness (Townley *et al.*, 1976), indicating that circulating adrenaline may reduce airway tone in asthma. Strenuous exercise induces an increase in adrenaline levels to concentrations (Berkin *et al.*, 1988) that can cause bronchodilation in normal and asthmatic subjects (Barnes *et al.*, 1980b; Berkin *et al.*, 1985; Warren & Dalton, 1983), however, basal catecholamine levels are no higher in asthmatics than in normal subjects, nor is there any relationship between plasma catecholamine levels and the severity of bronchoconstriction (Barnes *et al.*, 1982b). Even during acute asthmatic attacks, there is no elevation in plasma adrenaline levels (Ind *et al.*, 1985), indicating that normal circulating levels of adrenaline may be sufficient to protect asthmatics against bronchoconstrictor influences.

It is not fully understood how adrenaline can exert this protective effect at the low concentrations found in plasma, since these concentrations of adrenaline exert little effect on airway smooth muscle directly. It is possible that adrenaline may influence airway mast cells, since it has an inhibitory effect on I_gE-induced histamine release from human lung fragments (Butchers *et al.*, 1980). Alternatively, anticholinergic drugs have been shown to prevent and reverse β-blocker-induced bronchoconstriction (Grieco

& Pierson, 1971), suggesting that adrenaline may exert an inhibitory effect on cholinergic ganglia.

1.4.2.2 Natriuretic Peptides

The natriuretic peptides; atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and urodilatin are produced primarily in the heart but are also released from other tissues such as the central nervous system and the lungs (Gutkowska & Nemer, 1989). Autoradiographic studies indicate that ANP receptors are present in the lungs, notably on airway smooth muscle cells (Van Schroeder *et al.*, 1985). ANP relaxes isolated airways from a number of species, including man, and provides varying degrees of protection against subsequent agonist challenge (O'Donnell *et al.*, 1985; Angus *et al.*, 1994; Nally *et al.*, 1994). Intravenous infusion of ANP produces a significant bronchodilator response in normal and asthmatic subjects (Hulks *et al.*, 1989; Hulks *et al.*, 1990) and attenuates the bronchoconstrictor response to inhaled histamine (Hulks *et al.*, 1991) and to fog challenge (McAlpine *et al.*, 1992). The relaxant effects of ANP appear to be mediated by stimulation of particulate guanylyl cyclase and subsequent generation of cGMP (Ishii & Murad, 1989), although a more recent study indicated that the opening of membrane-bound potassium channels may also be involved (Nally *et al.*, 1995). In addition to a direct effect on airway smooth muscle, there is evidence that ANP performs a neuromodulatory role on the autonomic nervous system, which may alter airway tone indirectly. For example, Robichaud and colleagues (1993) found that ANP enhanced cholinergic nerve transmission and attenuated sympathetic nerve transmission in rabbit airways, which, in contrast to its relaxatory action on airway smooth muscle, would be expected to increase bronchomotor tone.

While it appears unlikely that physiological concentrations of circulating ANP contribute to basal bronchomotor tone in normal humans (Hulks *et al.*, 1990), it is possible that ANP produced and released within the lungs may exert a local autocrine or paracrine effect on the airways.

1.4.2.3 "Classical Hormones"

1.4.2.3.1 Cortisol

Corticosteroids are widely used in the treatment of asthma and administration of inhaled or oral corticosteroids results in a significant reversal of airflow obstruction and improvement in lung function in this disease. The mechanisms by which corticosteroids act are incompletely understood, but their therapeutic success in asthma is ascribed to their anti-inflammatory actions. In normal humans, however, pharmacological doses of intravenous cortisol have no short term effects on airway calibre (Ramsdell *et al.*, 1983), indicating that endogenous cortisol is unlikely to have an important effect on airway tone under normal conditions. In asthmatics, the role of physiological concentrations of circulating cortisol is unclear. Some groups have postulated that

reduced plasma cortisol may contribute to nocturnal asthma since the lowest point on its circadian cycle occurs four hours before maximal bronchoconstriction (Reinberg *et al.*, 1963; Soutar *et al.*, 1975). Infusion of hydrocortisone to eliminate the fall in plasma cortisone at night however, does not prevent the nocturnal airway obstruction (Soutar *et al.*, 1975), indicating that the circulating cortisol is not the only factor in determining nocturnal asthma.

1.4.2.3.2 Thyroid Hormones

The relationship between asthma and thyroid disease provides indirect evidence for a role for thyroid hormones in maintaining airway function. Asthmatics who develop hyperthyroidism frequently undergo a deterioration in clinical condition, but subsequently improve after treatment of the thyrotoxicosis (Elliot, 1929; Ayers & Clark, 1981; Lipworth *et al.*, 1988). Conversely, the occurrence of hypothyroidism in asthma may be associated with an improvement in the clinical management of asthma which relapses following subsequent thyroxine replacement (Bush *et al.*, 1977).

It is unclear how thyroid hormones may influence airway smooth muscle tone and responsiveness, although a number of mechanisms have been postulated. Firstly, β adrenergic airway responsiveness has been reported to be inversely related to thyroxine levels both *in vitro* in guinea pig trachea (Taylor, 1983) and *in vivo* in non-asthmatic subjects (Harrison & Tattersfield, 1984). Following treatment of hyperthyroidism, airway β responses return to normal levels (Harrison & Tattersfield, 1984). It is unlikely that alterations in β adrenergic activity are due to changes in circulating catecholamine levels (Coulombe *et al.*, 1976) or β adrenergic receptor numbers (Scarpace & Abrass, 1981), but it is possible that thyroxine acts at a postreceptor site within the smooth muscle. Alternatively, thyroxine may alter the metabolism of arachidonic acid since prostaglandin breakdown has been shown to be reduced in hyperthyroid rats (Hoult & Moore, 1978).

1.4.2.3.3 Sex Hormones

It has been postulated that progesterone may influence airway smooth muscle tone, either by potentiating the effect of catecholamines (Foster *et al.*, 1983) or through its immunosuppressive properties. Progesterone levels and airway responsiveness do not show a clear relationship during either pregnancy or the menstrual cycle, although changes in the levels of other hormones may obscure an effect of progesterone on the airways (Juniper *et al.*, 1978; Juniper *et al.*, 1989). It is of interest that intramuscular progesterone has a beneficial effect in some women with severe premenstrual asthma (Beynon *et al.*, 1988).

Oestrogen possesses both immunostimulatory and immunosuppressive properties and causes increased acetylcholine activity in animal lungs (Abdul-Karim *et al.*, 1970), which could result in an increase or decrease in airway responsiveness. A recent report suggested that oestrogen treatment may have steroid-sparing effects in postmenoposal

asthmatics (Celedon *et al.*, 1995) although, conversely, hormone replacement therapy has been associated with an increased risk of developing asthma (Troisi *et al.*, 1995).

1.4.2.4 Summary

In conclusion, humoral factors appear to play a minor role in the physiological regulation of airway tone in normal individuals. Circulating adrenaline is the only hormone known to influence bronchomotor tone and it is only during strenuous exercise that concentrations are raised sufficiently to cause bronchodilation. Circulating hormones play a more important role in the regulation of airway tone in respiratory disease states such as asthma and possibly in other disorders such as congestive heart failure and thyroid diseases. For many hormones, however, little is known about their effects on airway tone or on other functions of the airways.

1.4.3 LOCAL CONTROL

It is now known that the epithelium is a metabolically active tissue system which, through release of various mediators, regulates and maintains a chemical homeostasis necessary for the functional integrity of the underlying airway smooth muscle. In addition to the epithelium, mediators may be released from a number of other cell types within the airways, notably the mast cells, macrophages, and eosinophils. Indeed, many factors are released by the airway smooth muscle cells themselves and act in an autocrine fashion to influence airway reactivity.

Mast cells and macrophages are widely distributed throughout the human respiratory tract and are found in large numbers in the walls of the alveoli and airways. Eosinophils are circulating cells which are normally found at relatively low concentrations within the airways. During an inflammatory reaction of the airways, however, eosinophils infiltrate the bronchial mucosa in great numbers and this rise in eosinophilia correlates closely to the severity of the reaction (Burrows *et al.*, 1980; Honsinger *et al.*, 1972). The array of different mediators secreted by these cells can stimulate or inhibit the surrounding airway smooth muscle cells, either directly or indirectly. Some of the more important mediators are listed below.

1.4.3.1 Eicosanoids

The release of arachidonic acid (AA) from cell membrane phospholipids, through the action of phospholipase A_2, leads to the production of a wide variety of mediators which may be relevant to airway smooth muscle function (see figure 1.5). The metabolism of AA by the enzyme cyclooxygenase produces the prostaglandins (PGD_2, PGE_2 and $PGF_{2\alpha}$), prostacyclin (PGI_2) and the thromboxanes (TxA_2 and TxB_2), while the metabolism of AA by 5-lipooxygenase produces the leukotrienes (LTB_4, LTC_4, LTD_4 and LTE_4). These metabolites, collectively termed eicosanoids, appear to be synthesised *de novo* on demand, rather than being preformed and stored in resting cells. Experimental data generally shows that the prostaglandins PGD_2 and $PGF_{2\alpha}$, the thromboxanes and the leukotrienes are all contractile agents for airway smooth muscle (Sweetman & Collier, 1968; Gardiner & Collier, 1980; Dahlen *et al.*, 1980; Jones *et al.*, 1982), while PGE_2 and PGI_2 are both relaxatory (Sheard, 1968; Hutas *et al.*, 1981).

Figure 1.5

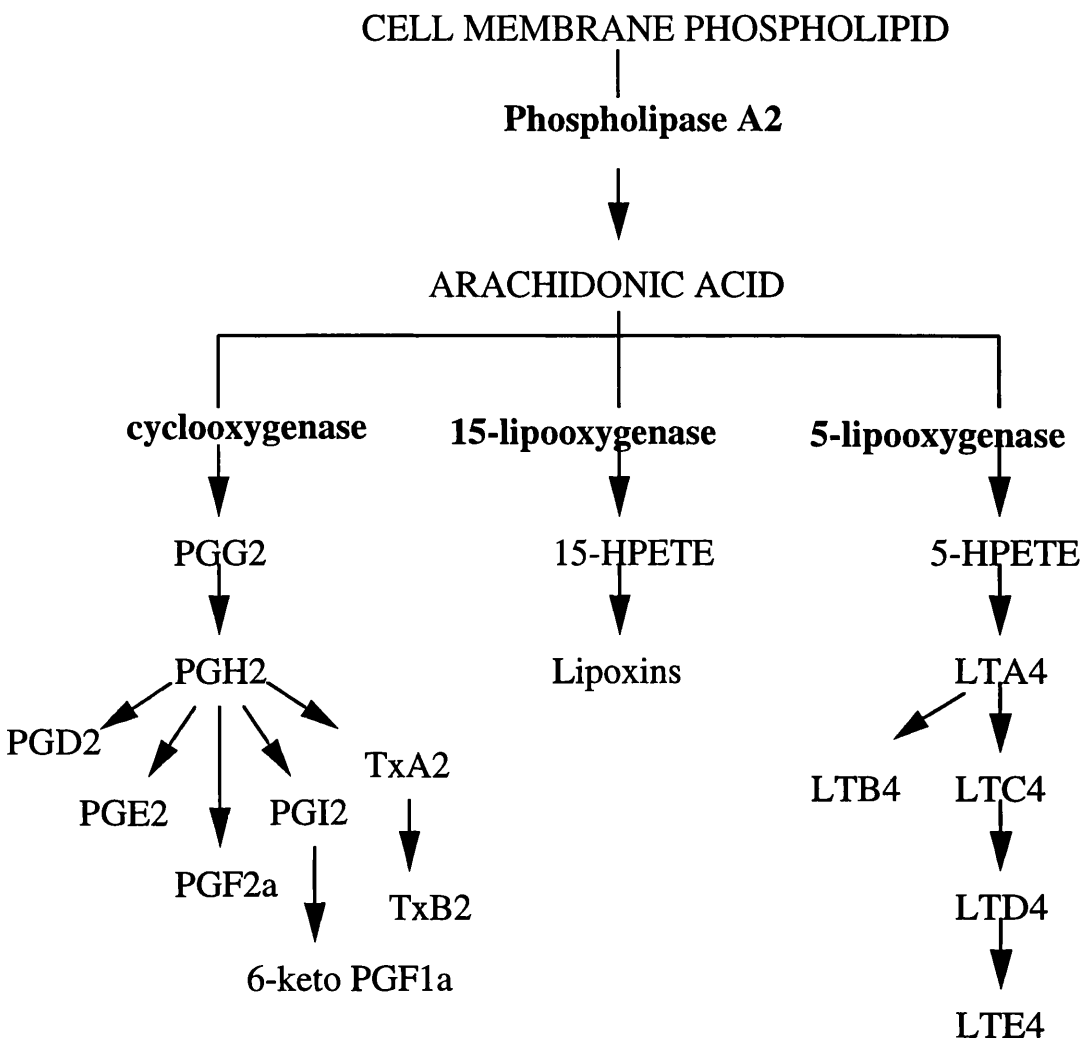


Figure 1.5
An overview of some of the eicosanoids produced as a consequence of arachidonic acid metabolism. The cyclooxygenase pathway leads to the production of the prostaglandins, the thromboxanes and prostacyclin. The 5-lipooxygenase pathway leads to formation of the leukotrienes, whereas the 15-lipoxygenase pathway produces the lipoxins

1.4.3.1.1 Prostanoids

Both PGD_2 and $PGF_2\alpha$ cause bronchoconstriction in human subjects, either through a direct effect on airway smooth muscle receptors or indirectly through stimulation of cholinergic-nerves (Beasley *et al.*, 1987). The activity profile of PGE_2 and PGI_2 is varied, although in general they relax and inhibit the responsiveness of airway smooth muscle, possibly via inhibiting acetylcholine release from parasympathetic nerves (Walters *et al.*, 1984). Interestingly, release of PGE_2 and PGI_2 may be responsible for the tachyphylaxis (a decreased response to repeated stimulation) exhibited by histamine and acetylcholine in airway smooth muscle both *in vitro* (Anderson *et al.*, 1977) and *in vivo* (Shore & Martin, 1985; Manning *et al.*, 1987).

Inhibitors of prostanoid production, such as the cyclooxygenase inhibitor, indomethacin, relax basal tone in guinea pig isolated airways, indicating that locally produced prostanoids contribute to the maintenance of basal tone (Farmer *et al.*, 1972). This finding may be unique to the guinea pig, however, since human airways produce prostanoids, yet do not relax *in vitro* when treated with cyclooxygenase inhibitors (Steel *et al.*, 1979; Brink *et al.*, 1980; Haye-Legrand *et al.*, 1986). Indomethacin does not alter baseline lung function in healthy or asthmatic subjects and furthermore, does not alter airway responsiveness to histamine or methacholine (Smith, 1975; Ogilvy *et al.*, 1981), therefore the involvement of cyclooxygenase metabolites in controlling airway function in humans is questionable.

1.4.3.1.2 Leukotrienes

The leukotrienes evoke a pronounced contraction in isolated airway smooth muscle (Dahlen *et al.*, 1980) and a sustained bronchoconstriction in healthy (Holroyde *et al.*, 1981) and asthmatic patients (Griffin *et al.*, 1983), being, on a molar basis, between 50 and 1000 times as potent as histamine. As stated earlier, cyclooxygenase products appear to be responsible for generating basal tone in guinea-pig trachea (Farmer *et al.*, 1972). In human bronchi, however, spontaneous tone can be abolished by lipooxygenase inhibitors (Ito *et al.*, 1985; Honda & Tomita, 1987; Watson *et al.*, 1997), indicating that basal production of leukotrienes is at least partly responsible for the generation of spontaneous tone in human airways. Further investigation into the role of leukotrienes in airway function awaits the development of selective leukotriene receptor antagonists, although a "first-generation" LTD_4 receptor antagonist significantly improved lung function in asthmatic patients (Cloud *et al.*, 1989). This clinical improvement took approximately 6 weeks to manifest, suggesting that in addition to their acute bronchoconstricting effect, the leukotrienes may have a chronic effect on the responsiveness of airway smooth muscle. This is supported by the findings that inhaled leukotrienes induce a prolonged hyperresponsiveness to the spasmogens methacholine and histamine in normal subjects (Kaye & Smith, 1990) and asthmatics (Arm *et al.*, 1988), with the effects persisting for up to a week.

1.4.3.2 Histamine

Histamine is associated predominantly with degranulation of mast cells (Riley & West, 1953), although it may also be released from basophils (Henderson, 1990) and nerve terminals (Ishikawa & Sperelakis, 1987). The direct effect of histamine on airway smooth muscle is dependent upon the prevailing pattern of histamine receptors which varies between species and even between different sites on the bronchial tree (Chakrin & Krell, 1980). In most species the predominance of "excitatory" H_1-receptors over "inhibitory" H_2-receptors ensures that histamine induces bronchoconstriction, as is the case in man. In addition to its direct effects, histamine may also influence airway tone indirectly by stimulating or inhibiting cholinergic nerves (White *et al.*, 1987), increasing epithelial permeability (Braude *et al.*, 1984), or by modulating mucus production (Shelhamer *et al.*, 1980).

1.4.3.3 Nitric oxide

The endothelial cells of the vasculature produce and release a substance which relaxes the vascular smooth muscle and acts as a functional antagonist for vasoconstrictor agents. This endothelial-derived relaxant factor (EDRF) was subsequently identified by Palmer *et al.* (1987a) and Ignarro *et al.* (1987) as being nitric oxide (NO). The enzyme responsible for the production of NO, nitric oxide synthase, is present in great abundance in the airway epithelium of a number of species, including humans (Shaul *et al.*, 1994; Asano *et al.*, 1994). Thus, NO may be the putative epithelial-derived relaxant factor (EpDRF), thought to be the equivalent of EDRF in blood vessels, although this remains unresolved.

Inhaled NO has a significant bronchodilator effect in guinea pigs *in vivo* (Dupuy *et al.*, 1992), indeed, NO donors have been used in the treatment of bronchospasm for more than a century. NO is also released from a number of inflammatory cells and there is compelling evidence that inhibitory NANC responses in guinea pig (Tucker *et al.*, 1990) and human (Belvisi *et al.*, 1992; Bai & Bramley, 1993) airways are mediated, at least partly, by release of NO from nerve terminals (see section 1.4.1.3.1).

1.4.3.4 Endothelin

The endothelins (ET-1, ET-2 and ET-3) are a family of structurally related peptides originally isolated from vascular endothelial cells (Yanagisawa *et al.*, 1988), but subsequently shown to be synthesised and released by a number of cell types within the airways, including the epithelium, macrophages and neuroendocrine cells (Mattoli *et al.*, 1990; Ehrenreich *et al.*, 1990; Seldeslagh & Lauweryns, 1993). The endothelins act as autocrine and paracrine factors in the airways, evoking responses such as increased epithelial secretion (Tamaoki *et al.*, 1991), histamine release from mast cells (Uchida *et al.*, 1992), contraction of airway smooth muscle (Advenier *et al.*, 1990), modulation of cholinergic nerve transmission (Henry & Goldie, 1995; Henry *et al.*, 1996) and

mitogenesis of epithelial cells (Murlas *et al.*, 1995) and airway smooth muscle cells (Glassberg *et al.*, 1994).

Two distinct endothelin receptors, termed ET_A and ET_B, have been cloned and characterised (Hosoda *et al.*, 1992; Arai *et al.*, 1993) although many reports suggest the existence of further subtypes. The ET_B receptor is the predominant subtype in human airway smooth muscle, with the ratio of ET_A to ET_B being approximately 15:85 (Goldie *et al.*, 1995). The contractile effect of the endothelins in airway smooth muscle is due, at least in humans, mainly to activation of ET_B receptors (Hay *et al.*, 1993), whereas their mitogenic actions appear to be mediated by ET_A receptors (Panettieri *et al.*, 1996). In a number of animal species, endothelin-induced bronchoconstriction is mediated to a large extent by the release of eicosanoids and indeed, activation of ET_A receptors in human airways induces the release of prostaglandins (Hay *et al.*, 1993). Current studies, however, indicate that, in humans, secondary-release of cyclooxygenase products does not contribute to the bronchoconstrictor effect of the endothelins and that contraction of human airway smooth muscle is due to a direct effect of ET receptor activation (McKay *et al.*, 1991; Hay *et al.*, 1993).

Activation of ET receptors can stimulate various signal transduction pathways, such as phospholipase A_2 or phospholipase C activation, adenylyl cyclase stimulation or inhibition, activation of tyrosine and threonine kinases and modulation of ion exchangers (for review, see Pollock *et al.*, 1995). In airway smooth muscle, endothelin-induced contraction is most commonly mediated by phospholipase C activation, leading to an elevation in intracellular free $[Ca^{2+}]$ via production of the second-messengers IP_3 and DAG. Through their numerous and widespread effects, the endothelins may contribute not only to the normal homeostasis of the airways but also to several lung diseases, including asthma, pulmonary fibrosis and pulmonary hypertension (see Michael & Markewitz, 1996, for review).

1.4.3.5 Summary

It is clear that a number of locally produced factors contribute to airway smooth muscle contractility under both basal and stimulated conditions. The precise role performed by each of these mediators is poorly understood and is further complicated by the finding that certain factors, such as the endothelins, the leukotrienes and histamine, may evoke their responses by inducing the secondary-release of other mediators.

A number of other mediators present within the lungs have the potential to modulate airway smooth muscle function, for example eosinophils and other inflammatory cells release various cytokines including the interleukines, eosinophil cationic protein and platelet-activating factor. The acute and chronic effect of these mediators on airway smooth muscle responsiveness remains to be established, however.

Table 1.1

	Cell Type			
Mediator	Epithelium	Macrophage	Mast Cell	Eosinophil
Prostaglandins	✓	✓	✓	✓
Thromboxanes		✓	✓	✓
Leukotrienes		✓	✓	✓
Histamine			✓	
Nitric Oxide	✓	✓	✓	✓
Endothelins	✓	✓	†	

Table 1.1: Some of the mediators which may influence airway smooth muscle function and the cell types which synthesize and release them.

Certain mediators, for example nitric oxide and prostaglandins are released from a number of cell types within the airways. Others, such as histamine, originate from a specific cellular source. † -denotes that endothelin has been shown to be released from murine mast cells (Ehrenreich *et al.*, 1992), although it is not yet known if this applies to other species. Nitric oxide is produced by the enzyme nitric oxide synthase, of which constitutive NOS (cNOS) is the subtype expressed by epithelial cells, whereas inducible nitric oxide synthase (iNOS) is expressed by inflammatory cells such as eosinophils.

1.5 AIRWAY SMOOTH MUSCLE PROLIFERATION

1.5.1 INTRODUCTION

It is widely recognised that airway remodelling contributes significantly to the airflow obstruction and bronchial hyperreactivity of asthma. Pathological findings in post mortem tissues from asthmatic patients have shown a marked thickening of the airway wall, largely as a result of a significant increase in the mass of the airway smooth muscle layer (Dunnill *et al.*, 1969; Hossain, 1973; Heard & Hossain, 1973). It has been proposed that there is sufficient thickening of the airway wall in asthmatic patients to explain the major part of the bronchial hyperresponsiveness characteristic of this disease (James *et al.*, 1989; Wiggs *et al.*, 1992). This suggestion is based on a modelling of the relationship between resistance and radius, in that the same degree of smooth muscle shortening in a thickened airway wall and a normal airway wall results in amplification of airway narrowing in the thickened airway (James *et al.*, 1989; Wiggs *et al.*, 1992; Pare, 1993).

This increase in the mass of airway smooth muscle may be due to an increase in either the size (hypertrophy) or the number (hyperplasia) of individual smooth muscle cells (Heard & Hossain, 1973; Ebina *et al.*, 1993; Pare, 1993), although such adaptive responses are not mutually exclusive and often occur simultaneously (Taubman *et al.*, 1989; Gabella, 1979).

The mechanisms involved in the control of airway smooth muscle proliferation are still poorly understood (Hirst & Twort, 1992; Stewart *et al.*, 1993; Stewart *et al.*, 1995a), although several factors with mitogenic properties have been implicated in the remodelling process. Smooth muscle cell mitogens fall into three broad categories: (1) growth factors linked to activation of receptors with intrinsic tyrosine kinase activity; (2) contractile agonists which mediate their effects through receptors coupled to G protein and (3) cytokines released from inflammatory cells.

The intracellular signal transduction pathways leading to cell proliferation are complex and incompletely defined. A number of events may be involved in the signalling process, including activation of phospholipase C (PLC), stimulation of protein kinase C (PKC) and the phospholipases D and A_2 and activation and elevation of intracellular Ca^{2+} (Huang & Ives, 1987; Rozengurt, 1989; Pouyssegur & Seuwen, 1992; Stewart *et al.*, 1995a). These and other signals lead to activation of immediate early response genes such as c-fos, c-myc and c-jun (Curran & Franza, 1988; Nambi *et al.*, 1989; Simons *et al.*, 1992). The protein products of these proto-oncogenes play a critical role in transducing growth signals from the cell surface to the nucleus and in regulating gene transcription. More recently, the central role of mitogen-activated protein (MAP) kinase has been described (Meloche *et al.*, 1992; Whelchel *et al.*, 1997). This enzyme phosphorylates c-jun and other targets and its on-going activation appears to be required for cell division (Lenormond *et al.*, 1993).

1.5.2 MECHANISMS INVOLVED IN SMOOTH MUSCLE CELL PROLIFERATION

1.5.2.1 Extracellular Receptors

1.5.2.1.1 Growth factors receptors with intrinsic receptor tyrosine kinase activity

Growth factors are defined as polypeptides that stimulate cell proliferation through binding to specific high-affinity cell membrane receptors (Paris & Pouyssegur, 1993) and are thought to play a central role in the regulatory mechanisms controlling cell growth. In smooth muscle, epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin-like growth factor (IGF) induce proliferation by binding to receptors with intrinsic tyrosine kinase (RTK) activity (Ross *et al.*, 1974; King *et al.*, 1985; Banskota *et al.*, 1989; Bornfeldt *et al.*, 1990). Indeed, these growth factors are among the most potent smooth muscle mitogens.

Several sub-classes of RTK exist, but all share a similar molecular structure consisting of a large extracellular ligand-binding region, a single hydrophobic transmembrane segment, a cytoplasmic portion containing the tyrosine kinase catalytic domain and lastly, a carboxy-terminal regulatory region (see Cardenn & Gill, 1992; Panettieri, 1996). Activation of the RTK is necessary for transduction of the growth factor-mediated response. Although the precise mechanisms by which the ligand activates the RTK is unknown, studies suggest that the ligand binding to the receptor may induce oligomerization of receptor monomers or may form a receptor-ligand complex which is then internalized (see Cardenn & Gill, 1992). The next step, autophosphorylation of tyrosine residues on the receptor, is critically important in transducing the extracellular signal into activation of an intracellular pathway. Autophosphorylation of the receptor removes inhibitory substrates and creates high-affinity sites containing phosphotyrosine residues. The substrates that bind to these autophosphorylated tyrosine residues contain particular binding sites, termed SH2 domains.

Substrates with SH2 domains are responsible for coupling activated growth factor receptors to intracellular pathways involved in the control of a variety of cellular functions such as proliferation and gene expression. Proteins with one or more SH2 domains include phospholipase C, phosphatidylinositol 3-kinase (PI 3-kinase), Ras and other cytoplasmic kinases (Carpenter, 1992). Many of the proteins that posses SH2 domains also contain a distinct sequence of approximately 50 amino acid residues termed the SH3 domain. Recent studies suggest that SH3 domains may modulate protein-protein interactions through the recognition of short peptide sequences that do not require phosphorylation. Signalling complexes based on the formation of SH2/SH3 interactions are then followed by activation of downstream effector proteins which involve, for example, nonreceptor tyrosine kinases and MAP kinases (Satoh & Kaziro, 1992; L'Allemain *et al.*, 1991; Meloche *et al.*, 1992).

1.5.2.1.2 Receptors coupled to G proteins

It is now recognised that several agonists which evoke contraction of airway smooth muscle can also induce myocyte proliferation. Contractile agonists typically bind to receptors coupled to G proteins, which are in turn coupled to various signal transduction pathways. G proteins are composed of three distinct subunits (see section 1.3.3.1). Initially, the α subunits were considered to be the most important component in mediating down-stream signalling events, however, recent studies suggest that the βγ complex is involved in activating various signal transduction cascades.

1.5.2.1.3 Cytokine receptors

Cytokine receptors differ from RTKs in that the receptor is not a tyrosine kinase itself, but rather, upon activation, stimulates an associated protein, a Janus kinase (JAK), to phosphorylate tyrosine residues on both itself and the receptor. This provides docking sites for various proteins including signal transducers and activators of transcription (STATs) and other signalling molecules such as phospholipase C and PI3 kinase (see Denhardt, 1996).

1.5.2.2 Intracellular signal transduction cascades involved in mitogenesis

1.5.2.2.1 Mitogen-activated protein (MAP) kinase pathway

Activation of the MAP kinase, also known as extracellular regulated kinase (ERK), pathway is believed to play an important role in cell growth and proliferation. MAP kinase is initially located within the cytoplasm (Northwood *et al.*, 1991), however following activation, MAP kinase translocates to the nucleus (Chen *et al.*, 1992; Sanghera *et al.*, 1992) and initiates the activation of transcription factors such as c-jun (Pulverer *et al.*, 1991) and c-myc (Seth *et al.*, 1991). Thus, MAP kinase provides a physical link in the signal transduction pathway from the cytoplasm to the nucleus.

Cloning studies indicate the existence of several isoforms of MAP kinase, a 42 and 44-kilodalton version encoded by the ERK1 and ERK2 genes, respectively, with a third, 54-kilodalton version having approximately 50% sequence similarity with its counterparts (Boulton *et al.*, 1990; Boulton *et al.*, 1991). Expression of both the 42 and 44-kilodalton isoforms appears to be essential for agonist-induced mitogenesis in various cell types (Pages *et al.*, 1993; Cowley *et al.*, 1994; Mansour *et al.*, 1994). The activation of MAP kinase requires phosphorylation of both tyrosine and threonine residues (Anderson *et al.*, 1990) by the dual specificity tyrosine/threonine kinase MAP kinase kinase, MEK1 (MacDonald *et al.*, 1993; Yan & Templeton, 1994). Raf-1 is currently thought to be the only physiologically relevant activator of MEK, although there is evidence for the existence of other MEK kinases (MAP kinase kinase kinases, Gupta *et al.*, 1992; Lange-Carter, 1993). Raf is in turn activated by the membrane-bound small guanine-nucleotide binding protein Ras (McCormick, 1993), which is

thought to be linked to RTKs by intermediate binding proteins such as SOS and Grb (Bonfini *et al.*, 1992; Bowtell *et al.*, 1992; Egan *et al.*, 1993).

The MAP kinase pathway has historically been associated with growth factor receptors which display intrinsic tyrosine kinase activity (Fantl *et al.*, 1993; Egan *et al.*, 1993), however it is now clear that signals from G protein-coupled receptors can also impact on the Ras/MAP kinase signal transduction cascade (DeVivo & Iyengar, 1994).

The intracellular pathways that link G protein-coupled receptors to MAP kinase activation are poorly understood, but for many agonists, stimulation of PKC appears to be a critical step (Granot *et al.*, 1993; Bogoyevitch *et al.*, 1994). Recent evidence, however, suggests that the $\beta\gamma$ subunit complex from individual G proteins may be able to stimulate Ras (Faure *et al.*, 1994; Crespo *et al.*, 1994), thus leading to MAP kinase activation.

In airway smooth muscle cells, MAP kinase is activated by a number of mitogenic factors including the growth factors PDGF, EGF and IGF (Kelleher *et al.*, 1995) and the G protein-coupled agonists bradykinin (Malarkey *et al.*, 1995), thrombin, endothelin-1 (Shapiro *et al.*, 1996) and 5-hydroxytriptamine (Kelleher *et al.*, 1995). As in other cell types, the duration of MAP kinase appears to be critical in evoking proliferation of airway smooth muscle. Sustained activation of MAP kinase is associated with mitogenesis of airway smooth muscle cells, whereas agonists which stimulate only a transient activation of MAP kinase failed to induce a proliferative response (Malarkey *et al.*, 1995; Kelleher *et al.*, 1995).

1.5.2.2.2 Protein Kinase C (PKC)

PKC is a family of multiple isoenzymes with different biochemical characteristics, substrates and co-factor requirements (Hug & Sarre, 1993). Activation of PKC by DAG is an important signalling event in the proliferation of many cell types (Adamo *et al.*, 1986). DAG is primarily produced by hydrolysis of PIP_2 via the action of PLC. (In the case of G protein-coupled receptors, phosphoinositide-specific PLC, PLC-β_1, is activated rather than PLC-γ, which is activated by RTK-dependent receptors (Panettieri, 1996). Furthermore, with G proteins of the Gq family, it is the α subunits that activate PLC-β_1, whereas with G proteins of the Gi class, activation is mediated through the $\beta\gamma$ subunits (Exton, 1996; Lee & Rhee, 1995)). Recent evidence, however, suggests that the production of DAG from the phospholipase D-mediated hydrolysis of phosphatidylcholine (Daniel *et al.*, 1986b; Takuwa *et al.*, 1987; Grillone *et al.*, 1988; Griendling *et al.*, 1986; Konishi *et al.*, 1991) may contribute greatly to the regulation of PKC.

While RTK-mediated cell proliferation is both PKC-dependent and independent (Stumpo & Blackshear, 1983), agonist-induced mitogenesis is thought to be almost exclusively PKC-dependent. Indeed, activation of PKC is believed to be the primary mechanism by which signals from G protein-coupled receptors impinge on the MAP kinase pathway (Granot *et al.*, 1993; Bogoyevitch *et al.*, 1994). Recent evidence,

however, suggests that G protein-linked agonists may activate MAP kinase via a PKC-independent manner (van Corven *et al.*, 1993; McLees *et al.*, 1995; Malarkey *et al.*, 1995), possibly via a Gi-type G protein-linked pathway.

While it is clear that PKC activation is an important constituent of many signalling pathways, the critical targets of the various PKC isoforms remain, for the most part, to be discovered.

1.5.2.2.3 PI3 kinase

Phosphatidylinositol 3-kinase (PI3 kinase) is a dimer of an 85kDalton α subunit, which contains an SH2 domain and therefore associates with growth factor RTKs, and a 110kDalton β subunit (Kazlauskas & Cooper, 1989; Escobedo *et al.*, 1991). Growth factor-stimulated PI3 kinase activation, leading to the formation of phosphatidylinositol (3,4,5) trisphosphate, was originally demonstrated by Cantley and co-workers (Whitman *et al.*, 1988). As with other tyrosine kinase pathways, G protein-coupled receptor agonists have also been shown to activate the PI3 kinase pathway (Kumagai *et al.*, 1993; Stephens *et al.*, 1993). PI3 kinase has been proposed to mediate a number of intracellular events involved in mitogenesis such as activation of PKC (Nakanishi *et al.*, 1993; Liu, 1996), stimulation of a ribosomal kinase (rsk, Lane *et al.*, 1993) and activation of the MAP kinase pathway (Rodriguez-Viciana *et al.*, 1994).

1.5.2.2.4 PLD

Regulation of phospholipase D (PLD) has received considerable attention since hydrolysis of the membrane phospholipid, phosphatidylcholine, by this enzyme produces, phosphatidic acid, a potential second-messenger in many cell types (see Exton, 1994). The activity of PLD in a wide variety of cells is influenced by many hormones, neurotransmitters, growth factors and other agonists linked to heterotrimeric G proteins or tyrosine kinases (Exton, 1994; Exton, 1997). The mechanisms by which G protein-coupled receptors and RTK are linked to PLD are not well understood but PKC activation appears to play a major role in many cell types (Eldar *et al.*, 1993; Balboa *et al.*, 1994). Since the majority of agonists that promote PLD activation also stimulate PLC, a logical mechanism is that PLD activation is secondary to PKC stimulation due to production of DAG from PIP_2 (Exton, 1997). At present, the mechanism(s) by which PKC activates PLD is undefined, indeed the role for PLD in early mitogenic signalling is controversial, with both supporting and conflicting evidence (see Boarder, 1994).

1.5.2.2.5 PLA_2

The hydrolysis of phosphatidylcholine by phospholipase A_2 (PLA_2) was one of the earliest phospholipase pathways to be described. The "classical" role of PLA_2 activation is to generate arachidonic acid, which is the rate limiting precursor of prostaglandin and leukotriene biosynthesis (see section 1.4.3.1). It is probable that in inflammatory states this is the major function of arachidonate, however in mitogenic signal transduction, other pathways may be important. These alternative pathways include the activation of certain isoforms of PKC (Asaoka *et al.*, 1992) as well as PLD stimulation (Wang *et al.*, 1992). Although PLA_2 is activated by many agonists linked to heterotrimeric G proteins, there is no compelling evidence that G proteins interact directly with this enzyme (Clark *et al.*, 1995). RTK growth factors such as PDGF and EGF can stimulate PLA_2 activity (Goldberg *et al.*, 1990), indeed it has been demonstrated that PLA_2 can be phosphorylated and activated by MAP kinase (Lin *et al.*, 1993). Since growth factors and contractile agonists can both stimulate MAP kinase, it is possible that both RTK-linked growth factors and G protein-coupled receptor agonists control the activity of PLA_2 through MAP kinase activation.

In rabbit airway smooth muscle, endothelin-1 induces cell proliferation by activating PLA_2, leading to the generation of thromboxane A_2 and leukotriene D_4 (Noveral *et al.*, 1992; Panettieri *et al.*, 1991a).

1.5.2.2.6 SAP kinases

As their name suggests, stress activated protein kinases (SAP kinases) are activated by agents which induce cellular stress, such as UV light and the cytokines, tumour necrosis factor α and interleukin-1 (Kolesnick & Goldie, 1994). The SAP kinases include additional members of the MAP kinase family (a 38-kilodalton isoform, Han *et al.*, 1994) and the c-jun N-terminal kinases (Jun kinases) which are distant relatives of the MAP kinases (Kyriakis *et al.*, 1994), but are thought to exist in signal transduction cascades distinct from MAP kinase. There is evidence, however, for the existence of cross-talk between the two pathways since RTK-linked growth factors (Minden *et al.*, 1994) and G protein-coupled receptor agonists (Dalton & Treisman, 1992) can also activate the SAP kinases. A recent study in airway smooth muscle cells showed that Jun kinase was activated by the G protein-linked receptor agonists endothelin-1 and thrombin (Shapiro *et al.*, 1996), indicating that contractile agonists may induce proliferation of airway smooth muscle via both the MAP kinase and SAP kinase pathways.

1.5.2.2.7 Jak STATS

One of the most recently identified tyrosine kinase signalling cascades involves the activation of a novel class of tyrosine kinases, comprising Janus kinase (JAK) and the tyrosine phosphorylation of cytoplasmic proteins called signal transducers and activators of transcription (STATs). As stated earlier (see section 1.5.2.1.3), this pathway is activated primarily by cytokines such as interferons (Schindler *et al.*, 1992; Shuai *et al.*, 1992), interleukin-3 (Silvennoinen *et al.*, 1993) and growth hormone (Winston & Hunter, 1995). JAKs do not contain SH2 or SH3 domains, but cytokine stimulation leads to the association of these kinases with the receptor and their subsequent tyrosine phosphorylation and activation (Velazquez *et al.*, 1992). Recently, RTK-linked growth factors (Fu & Zhang, 1993) and G protein-coupled receptors (Marrero *et al.*, 1995) have been shown to activate components of this cascade and may represent an additional pathway through which these agents can induce mitogenesis.

1.5.2.3 Intracellular signal transduction cascades involved in inhibiting mitogenesis

Recognition that airway smooth muscle proliferation is important in the pathogenesis of asthma has focused attention on identifying cellular and molecular mechanisms that inhibit smooth muscle cell growth. An understanding of these mechanisms is not only critical in preventing cell growth, but also in addressing whether the loss of inhibitory signals may induce proliferation.

Cytokines, activation of cyclic nucleotides and alterations in extracellular matrix proteins have all been reported to inhibit myocyte proliferation (Stewart *et al.*, 1995b; Panettieri, 1996).

1.5.2.3.1 Alterations in extracellular matrix proteins

Heparin and related molecules, which are components of the extracellular matrix, potently inhibit proliferation of vascular smooth muscle (Clowes & Karnovsky, 1977), although the molecular mechanisms underlying this inhibition are unresolved. Pukac *et al* (1992) suggest that specific receptor signals transduce the antiproliferative effects of heparin, whereas others propose that the antiproliferative effect is mediated by its intracellular action (Castellot *et al.*, 1985). After binding to high affinity surface receptors, the heparin-receptor complex is internalized and then localizes near the perinuclear membrane where it may interact with proteins or proto-oncogenes that modulate gene transcription (Castellot *et al.*, 1985).

In cultured airway smooth muscle cells, growth factor-induced mitogenesis is inhibited by heparin (Page, 1991; Johnson *et al.*, 1994), but not by chondroitin sulphate, another extracellular matrix protein (Panettieri *et al.*, 1990). Furthermore, although extracellular matrix proteins have been shown to inhibit proliferation, these proteins may prevent degradation of certain growth factors and thereby potentiate mitogenesis (Border *et al.*,

1992; Paris & Pouyssegur, 1993). Taken together, these results suggest that heparin and other matrix proteins may modulate smooth muscle cell growth in a complex manner.

1.5.2.3.2 Cytokines

Smooth muscle cells stimulated by growth factors have been shown to release cytokines which modulate myocyte proliferation in an autocrine manner (Hajjar *et al.*, 1992). Transforming growth factor β (TGF β), a cytokine secreted by many cell types including smooth muscle, may either enhance (Majack *et al.*, 1990) or inhibit (Majack *et al.*, 1987) growth factor-induced mitogenesis of vascular smooth muscle cells.

The inflammatory cytokine tumour necrosis factor-α (TNFα) has been detected in increased levels in broncheoalveolar lavage from symptomatic asthmatic patients (Broide *et al.*, 1992) and alveolar macrophages cultured from asthmatic subjects produce higher levels of TNFα (Gosset *et al.*, 1991). TNFα inhibits the mitogenic effect of agents such as thrombin and EGF in airway smooth muscle cells, but at very low concentrations, TNFα has a small stimulatory effect on these cells (Stewart *et al.*, 1995b). The signalling pathways which transduce the cellular effects of cytokines are largely unknown.

1.5.2.3.3 Cyclic nucleotides

cyclic Adenosine Monophosphate (cAMP)

Several studies now support the idea that elevation of intracellular cAMP levels inhibits the proliferation of airway smooth muscle cells (Panettieri *et al.*, 1990; Tomlinson *et al.*, 1994; Tomlinson *et al.*, 1995). There is circumstantial evidence, however, that transient elevation of cAMP may be a mitogenic stimulus (Lew *et al.*, 1992). Elevation of cAMP activates cAMP-dependent protein kinase A (PKA, see section 1.3.4), which inhibits agonist-induced cell growth (Panettieri *et al.*, 1991b; Panettieri *et al.*, 1993; Shapiro *et al.*, 1996). Interestingly, PKA activation appears to selectively inhibit mitogens which induce airway smooth muscle cell growth through PKC-dependent pathways, whereas mitogenesis induced by RTK-activating growth factors are less sensitive to PKA-mediated inhibition (Panettieri *et al.*, 1991b; Panettieri *et al.*, 1993).

The mechanism by which PKA inhibits mitogenesis remain unclear, but in various cell lines PKA has been shown to block activation of Raf by Ras and thereby inhibit the MAP kinase cascade (Cook & McCormick, 1993; Wu *et al.*, 1993).

cyclic Guanosine Monophosphate (cGMP)

It has also been reported that pretreating cells with cGMP-producing vasodilators, such as atrial natriuretic peptide, or with cGMP analogues inhibits proliferation of vascular smooth muscle cells (Garg & Hassid, 1989; Itoh *et al.*, 1990). As with cAMP, the intracellular mechanisms by which cGMP inhibit mitogenesis are poorly understood, but, as mentioned earlier (see section 1.3.4.3), under certain conditions, cGMP appears to be able to stimulate PKA.

1.5.2.3.4 Other inhibitory mechanisms

Anti-inflammatory steroids do reduce airway smooth muscle cell proliferation directly, but the magnitude of the effect is dependent on the stimulant - EGF and the thromboxane A_2 mimetic U46619 are relatively insensitive, whereas the effects of thrombin are almost abolished by dexamethasone (Stewart *et al.*, 1995a).

1.5.3 SUMMARY

When a receptor is activated, a number of signalling pathways is typically stimulated to a varying degree. The fact that some of the proteins in a pathway become multiply phosphorylated raises the possibility that more than one signal may be transmitted simultaneously via the same pathway. If this phenomenon, known as multiplex signalling (Denhardt, 1996), exists, it adds another layer of complexity to the signalling process involved in mitogenesis.

The relationship between the extent of the airway remodelling and the severity of the asthmatic condition (Kuwano *et al.*, 1993) suggests that this is a progressive feature of the disease. As such, early intervention with appropriate agents may arrest or reverse the development of these structural changes. Identification of the critical cellular and nuclear mechanisms by which airway smooth muscle proliferation is controlled is therefore of obvious clinical importance.

1.6 HYPOXIA AND THE AIRWAYS

1.6.1. INTRODUCTION

1.6.1.1 Historical perspective

It has long been recognised that air is essential for life. In the 17th century, Robert Boyle noted that neither a flame nor an animal would survive in a confined chamber (Boyle, 1668). Although oxygen was first discovered in the early 1770s by Priestley, it was an essay by Lavoisier in 1775, entitled "Experiments on the respiration of animals and on the changes affecting air in its passage through the lungs," which gave rise to the concept that metabolic processes of the body are dependent upon a constant supply of oxygen (see Fishman & Richards, 1964).

1.6.1.2 Classifications of hypoxia

Hypoxia is a physiological term for the condition in which the oxygen supply to cells, tissues, organs or whole animals is insufficient to maintain a normal function. The physiological classifications of hypoxia are based on the original description by Barcroft (1920), in which he divided anoxaemia into three types: low oxygen in the blood (anoxic hypoxia), reduced functional haemoglobin (anaemic hypoxia) and an insufficient blood supply to tissues (stagnant hypoxia). The classification was expanded by Peters and van Slyke (1932) to include a fourth class (histotoxic hypoxia) caused by exposure to poisons that retard the oxidation process.

More recently, anoxic and anaemic hypoxia have been grouped under the term "simple hypoxia," in which the major change is a decrease in the oxygen concentration of the blood (Silver, 1977; Jones, 1986). In contrast to stagnant hypoxia, simple hypoxia occurs without blood flow-dependent limitations in nutrient supply and removal of waste products. Stagnant hypoxia occurs in acute ischaemia, caused by cardiac insufficiencies, blood vessel occlusion or blood stasis, whereas simple hypoxia results from anaemia, low inspired oxygen or respiratory disorders involving impaired ventilation or alveolar gas exchange.

The focus of this chapter is the effect of hypoxia on airway smooth muscle function. *In vivo*, airway smooth muscle could be subjected to hypoxia classified as simple, stagnant or even histotoxic hypoxia. From the following sections, however, it should become clear that the term 'hypoxia' in this case refers to alveolar hypoxia and would therefore be classified as 'simple hypoxia'.

1.6.1.3 The hypoxic ventilatory response

Considerable attention has been focused on the ventilatory response to hypoxia. In several mammalian species, including humans, the hypoxic ventilatory response is described as biphasic and consists of an initial increase in ventilation followed by a decline that may return to or below the control level (see Fisher *et al.*, 1987). The

increase in ventilation is thought to be due to an increase in the respiratory rate rather than in tidal volume (Haldane *et al.*, 1919; Rebuck *et al.*, 1976).

Although most authors agree that the initial ventilatory rise is due to the hypoxic activation of the peripheral chemoreceptors, the mechanisms behind the subsequent decline in ventilation have been, and continue to be, the object of many publications. The most obvious hypothesis is that rapid adaptation of the peripheral chemoreceptors or central filtering of the chemoreceptors input occurs, although experimental evidence appears to suggest otherwise (Eldridge & Millhorn, 1986; Vizek *et al.*, 1987). Alternatively, the initial increase in ventilation may lead to hypocapnia, which would therefore lead to a reduction in ventilatory drive. Maintaining CO_2 levels, however, does not significantly change the biphasic ventilatory response (Easton & Anthonisen, 1988). Indeed, even a modest hypercapnia does not eliminate the secondary decline in ventilation (Georgopoulos *et al.*, 1989). Further studies indicate that hypoxia-induced elevations in airway resistance are not likely to be responsible for the ventilatory decline (Fisher *et al.*, 1987).

In short, acute hypoxia evokes an initial increase, followed by a decline, in ventilation although the mechanisms underlying the subsequent fall remain unknown.

1.6.1.4 Introducing the effects of hypoxia on the airways

Alveolar hypoxia, and subsequent hypoxaemia, can often occur in respiratory diseases such as acute severe asthma, chronic obstructive airway disease, and bronchial dysplasia. Despite this the effects of hypoxia on airway smooth muscle function are controversial and poorly understood. In isolated airway preparations, reducing the oxygen tension generally impairs contractile responses (Stephens & Chui, 1970; Stephens & Kroeger, 1970; Paterson *et al.*, 1988) and reverses induced tone (Twort & Cameron, 1986; Gao & Vanhoutte, 1989; Fernandes *et al.*, 1993), whereas *in vivo*, hypoxia has been reported to induce bronchoconstriction (Nadal & Widdicome, 1962; Sterling, 1968; Saunders *et al.*, 1977; Teague *et al.*, 1988), bronchodilation (Wetzel *et al.*, 1992; Julia-Serda *et al.*, 1993) or have no effect (Goldstein *et al.*, 1979; Tam *et al.*, 1985). The conflicting results among the various reports may reflect variations in the design of the studies, since differences in the species studied, the intensity and duration of the hypoxic stimulus, the method used to measure changes in airway tone and the anaesthetic technique employed may all have important effects on the resulting response. A key point in the methodology is the control, or otherwise, of other relevant stimuli, such as carbon dioxide levels, which are known to have potent effects on airway tone. Since hypercapnia is commonly associated with hypoxia in many physiological and pathophysiological conditions, combined hypoxia/hypercapnia would thus provide a more relevant characterization of the effects of hypoxia on the respiratory system. Unfortunately, it also limits the general applicability of the results from each study and makes the task of synthesizing the data more difficult.

1.6.2 THE EFFECT OF HYPOXIA ON AIRWAY SMOOTH MUSCLE TONE *In vitro*

As stated above, the general response of airway smooth muscle to hypoxia *in vitro* is an impairment of contraction. Hypoxia-induced pulmonary vasoconstriction plays a major role in the crucial physiological function of ventilation (V) /perfusion (Q) matching by diverting blood flow away from hypoxic areas of the lung (Sylvester *et al.*, 1986). Theoretically, hypoxia-induced bronchodilation could improve V-Q matching by decreasing airway resistance and facilitating gas flow to the hypoxic regions of the lungs.

In contrast to the extensive study of hypoxic pulmonary vasoconstriction, there is a paucity of data regarding the mechanisms of hypoxia-induced impairment of contractile responses in airway smooth muscle.

1.6.2.1 Postulated mechanisms of hypoxia-induced relaxation

1.6.2.1.1 Depletion of ATP levels

Early work by Stephens and co-workers (Stephens & Chui, 1970) proposed that the impairment of airway smooth muscle tone during hypoxia was due to depletion of the energy stores needed for phosphorylation of myosin. During moderately severe hypoxia, however, intracellular ATP concentrations are in the millimolar range (Zhou *et al.*, 1991), whereas the amount of ATP required by smooth muscle to maintain contraction is in the micromolar range (Paul, 1980).

1.6.2.1.2 Release of inhibitory factors

Others have postulated that hypoxic relaxation is secondary to the release of inhibitory agents within the airways. A likely source of relaxant factors is the airway epithelium and indeed, Gao and Vanhoutte (1989) found that hypoxia-induced relaxation of canine bronchi was dependent upon the presence of the epithelial layer. In contrast, however, a more recent study in porcine bronchi found that removing the epithelium did not alter the ability of hypoxia to induce relaxation (Fernandes *et al.*, 1993). Furthermore, indomethacin, methylene blue and propranolol each failed to inhibit the hypoxic response, indicating that cyclooxygenase products, cGMP or β-adrenergic agonists are unlikely to mediate hypoxic relaxation of airway smooth muscle (Fernandes *et al.*, 1993).

Alternatively, several groups have postulated that cells known as pulmonary neuroendocrine cells (PNEC) act as airway receptors involved in controlling bronchomotor tone in response to hypoxia. Groups of PNEC, termed neuroepithelial bodies (Lauweryns & Peuskens, 1972), are found in the epithelial layer and mucosa throughout the respiratory tract (Hung, 1984). PNEC are known to secrete a number of bioactive molecules such as serotonin (Lauweryns *et al.*, 1973), bombesin/gastrin

related peptide (Wharton *et al.*, 1978) and calcitonin related peptide (Cutz *et al.*, 1981). Rather than secreting their products into the airway lumen, their polarity of secretion is directed towards structures underlying the basement membrane such as nerve fibres, pulmonary blood vessels and airway smooth muscle cells (Hung, 1984). The association of PNEC with nerve fibres has long suggested a "receptor" role for these cells (Lauweryns & Peuskens, 1972), indeed, acute airway hypoxia or hypercapnia both cause release of secretory products from these cells (Lauweryns *et al.*, 1978; Keith & Will, 1982), implying a role as 'intra-airway oxygen sensors'. Interestingly though, the products secreted by these cells are all reported as inducing contraction of airway smooth muscle (Zucker & Cornish, 1980; Palmer *et al.*, 1987b; Belvisi *et al.*, 1991), therefore one would expect hypoxia to evoke a contractile response in the airways. The disparity between this and the hypoxia-induced reversal of tone in isolated airways may be due to the fact that PNEC are concentrated in smaller conducting airways whereas the preparations used for *in vitro* studies tend to be tracheal or upper bronchus. Many authors now propose that the primary role of PNEC is in regulating airway differentiation and lung development.

Thus, there is a lack of evidence supporting the hypothesis that hypoxic relaxation of airway smooth muscle is due to release of an inhibitory factor within the airways.

1.6.2.1.3 Inhibition of entry of extracellular Ca^{2+}

More recent studies suggest that calcium availability may be the limiting factor during hypoxia. As mentioned in section 1.3.2, the calcium needed for smooth muscle contraction can come from extracellular sources or internal calcium stores. Fernandes *et al* (1993) showed that hypoxia did not inhibit the initial contractile response to carbachol, whereas the maintained phase of the carbachol-induced contraction was inhibited. In addition, both the initial and the sustained phase of contractions evoked by potassium chloride were impaired (Fernandes *et al.*, 1993). This strongly suggests that hypoxia attenuates the contractility of airway smooth muscle by inhibiting the entry of extracellular calcium, whereas the release of calcium from intracellular stores appears to be unaffected. Entry of extracellular calcium into airway smooth muscle cells occurs almost exclusively through voltage dependent Ca^{2+} channels, although there is indirect evidence for the existence of receptor-operated Ca^{2+} channels in this tissue (Croxton *et al.*, 1994; Murray & Kotlikoff, 1991). Vannier *et al* (1995) recently showed that hypoxia limits entry of Ca^{2+} predominately via voltage operated channels, although a small component of the hypoxia-induced relaxation may be mediated by inhibition of receptor-operated Ca^{2+} channels.

1.6.2.2 Postulated mechanisms of Ca^{2+} influx inhibition

1.6.2.2.1 K+ channel opening and membrane hyperpolarization

A possible mechanism by which hypoxia inhibits entry of extracellular Ca^{2+} is hyperpolarization of the cell membrane. Hyperpolarization would reduce the open-state probability of voltage-operated Ca^{2+} channels, leading to a decrease in Ca^{2+} entry, a decrease in free Ca^{2+} and a reduction in airway tone. Physiological hyperpolarization is often accomplished by opening potassium (K^+) channels on the cell membrane, indeed, Lindeman *et al* (1994) demonstrated that opening of ATP sensitive K^+ (K_{ATP}) channels is involved in the relaxatory responses of airway smooth muscle to hypoxia. The mechanisms by which K_{ATP} channels are activated during hypoxia are not yet known. Reductions in oxygen tension could (1) decrease availability of ATP required to directly inhibit the channels, (2) redistribute intracellular ATP required for K_{ATP} channel phosphorylation, or (3) reduce intracellular pH. Indeed, these factors could also affect VOC channels directly, without the involvement of K_{ATP} channels. For example, VOC channel activity is inhibited by reductions in the availability of ATP in vascular smooth muscle cells (Ohya & Sperelakis, 1989) and by intracellular acidosis in ventricular myocytes (Kalibara & Kameyama, 1989). The importance of these regulatory mechanisms on Ca^{2+} channel activity has not been established in airway smooth muscle, however a recent study, outlined below, by Croxton *et al* (1995) investigated the role of intracellular pH changes in the relaxatory response of isolated airway smooth muscle to hypoxia.

1.6.2.2.2 Intracellular pH changes

Given that both hypercapnia, which decreases active force generation in bronchial rings (Stephens *et al.*, 1968) and relaxes precontracted airway smooth muscle (Duckles *et al.*, 1974; Twort & Cameron, 1986), and hypoxia evoke a fall in intracellular pH, it was postulated that hypoxia may inhibit VOC channel activity in airway smooth muscle cells via intracellular acidosis. It was found however, that while hypercapnia-induced relaxation was indeed associated with intracellular acidosis, hypoxia evoked substantial relaxation without altering intracellular pH (Croxton *et al.*, 1995). This demonstrates that changes in intracellular pH may be an important regulator of airway smooth muscle tone, but that intracellular acidosis does not mediate the rapid relaxatory response to hypoxia. The possibility remains that changes in intracellular pH may occur in airway smooth muscle during more prolonged hypoxia and that these changes may have additional effects on airway tone.

1.6.2.3 Summary

In summary, in isolated airway preparations, hypoxia relaxes precontracted tissues and attenuates active tension generation. Inhibition of VOC channels appears to play a key role in the hypoxic response, but the cellular mechanisms underlying this remain elusive.

1.6.3 THE EFFECT OF HYPOXIA ON AIRWAY SMOOTH MUSCLE TONE *In vivo*

The effects of alveolar hypoxia on the mechanical function of the airways *in vivo* have been recognised since the report by Roy and Brown in 1885 that asphyxia can both increase and decrease bronchial tone in dogs (Roy and Brown, 1885). Einthoven extended the observation in 1892, showing that alveolar hypercapnia and hypoxia can each independently produce a bronchoconstrictive response in dogs (Einthoven, 1892). Since these early publications, a large number of investigators have approached the effects of hypoxia on the airways from various angles to reach conclusions that are not always uniform. Aside from the differences in methodology and study design, a likely explanation for such discrepancies is the fact that the response of the upper airways to hypoxia may differ from the response of the lower airways.

1.6.3.1 Response of the upper airways to hypoxia

There is little question that alveolar hypoxia stimulates the contraction of the muscles that dilate all three segments of the upper airway: the nose, pharynx and larynx (van Luteren, 1991). Breathing a hypoxic or hypercapnic gas mixture stimulates the alae nasi muscles (Strohl *et al.*, 1982; Mezzanotte *et al.*, 1992) resulting in nasal flaring, a classic sign of respiratory distress (Strohl *et al.*, 1980). Activation of these muscles can reduce nasal resistance by nearly 25% (Maltais *et al.*, 1991), a significant figure if one considers that the nose can contribute up to 40% of the total respiratory resistance in humans (Span & Hyatt, 1971). The pharyngeal (Martin *et al.*, 1990; Okabe *et al.*, 1993) and laryngeal (Bartlett, 1979; Wheatley *et al.*, 1991) dilator muscles are also selectively recruited during alveolar hypoxia, resulting in a further decrease in resistance to airflow (Maltais *et al.*, 1991).

1.6.3.2 Response of the lower airways to hypoxia

The idea that the smooth muscle of the trachea and the bronchi can respond to changes in the composition of the inspired gas has intrigued physiologists for over a century. In addition to the classic observations of Roy and Brown (1885) and Einthoven (1892), highlights in the quest to characterize this response include the demonstrations: by

Houssay and Cruciani (1929) and Daly *et al* (1953), that perfusion of the brain with anaemic blood or oxygen-desaturated blood causes bronchoconstriction; by Loofbourrow *et al* (1957) that asphyxia, hypoxaemia and hypercapnia contract the trachealis muscle; and by Nadel and Widdicombe (1962) that either carotid denervation or vagotomy abolish the constrictive effects of alveolar hypoxia and hypercapnia on the trachea and bronchi.

While many subsequent studies confirm that hypoxia produces bronchoconstriction (Sterling, 1968; Saunders *et al.*, 1977; Fisher *et al.*, 1987), there are other showing that it has no measurable effect on the airways (Goldstein *et al.*, 1979; Tam *et al.*, 1985). Indeed, two separate groups have found a bronchodilatory response to hypoxia *in vivo* (Wetzel *et al.*, 1992; Julia-Serda *et al.*, 1993).

As stated earlier, the apparent confliction in results may be due to methodological reasons. While the trachealis muscle is accessible to direct force recordings, the bronchial smooth muscle cannot be studied conveniently without disturbing its innervation or its mechanical anchoring on the surrounding tissue. The traditional strategy is to rely on indirect measurements of airway calibre, such as by measuring flow-volume and pressure-flow relationships of the respiratory system on the assumption that they will reflect changes in airway diameter. Unfortunately, these measurements, or the total lung and respiratory resistances calculated from them do not provide a proportional representation of all airways. Moreover, they cannot easily distinguish the contributions of airways and lung or chest wall tissues to the flow-dependent properties of the system. Consequently, they may be influenced by changes unrelated to airway diameter, such as changes in lung volume. Furthermore, invasive procedures such as nasal and pharyngeal catheters or fibre-optic laryngoscope may stimulate upper airway reflexes and hence modify airway resistance. Interestingly, two of the more non-invasive techniques, computed tomography and acoustic reflection (Wetzel *et al.*, 1992; Julia-Serda *et al.*, 1993, respectively) recorded a bronchodilation during hypoxia.

After taking into account the methodological considerations outlined, the bulk of the existing information still points to a bronchoconstrictor response to hypoxia, a response which appears to be dependent on a reflex loop that includes the peripheral chemoreceptors and the parasympathetic motoneurones of the vagus nerve (Nadel & Widdicombe, 1962; Green & Widdicombe, 1966). The response is due to peripheral chemoreceptor stimulation and can be eliminated by section of either the glossopharyngeal nerves, which represent the afferent pathway of the reflex, or the vagus nerves which supply the efferent innervation to the airway smooth muscle (Nadel & Widdicombe, 1962). As mentioned in section 1.4.1.1, the parasympathetic nerves provide the dominant excitatory innervation of the airway smooth muscle. These neurones are connected with areas of the brain stem known to receive inputs from the peripheral and central chemoreceptors and lung receptors (Haxihiu *et al.*, 1994) and are

also functionally coordinated with the central respiratory generator (Mitchell *et al.*, 1985).

While a bronchoconstrictor response to hypoxia, leading to an increase in airflow resistance, may at first seem to be self-limiting, it is part of a homeostatic reflex and must be considered in conjunction with the overall ventilatory response to hypoxia (see section 1.6.1.3). The elevated expiratory efforts caused by hypoxia place increased transmural pressures on the central airways, thereby limiting flow by dynamic compression (Fry & Hyatt, 1960). Contraction of the airway smooth muscle makes the airways stiffer, thereby reducing the likelihood of deformation. Thus the balance between decreased airflow resistance and susceptibility to collapse tends to favour an increased stiffening of the lower airways in an effort to prevent deformation during the hypoxia-induced increase in ventilation.

1.6.3.3 Complicating factors

The effect of hypoxia on airways *in vivo* may be complicated by a number of factors. For example, the direct response of the airways to hypoxia may lead to alterations in lung volumes and CO_2 levels or to activation of lung receptors, all of which may exert a modulatory role on airway calibre.

1.6.3.3.1 Responses to CO_2 in the airways

The effects of hypo- and hypercapnia on the bronchi are just as complex as those of hypoxia. The available information indicates that isolated arterial hypocapnia relaxes bronchial smooth muscle by decreasing cholinergic tone (Ingram, 1975). In contrast, isolated alveolar hypocapnia may evoke bronchoconstriction (Ingram, 1975), probably by a local action of CO_2 on the muscle cells (Stephens *et al.*, 1968). These apparently contradictory effects may reflect the presence of two regulatory mechanisms: one triggered primarily by increases in arterial P_{CO2} and directed at limiting airway deformation during periods of hyperpnea, and the other initiated by decreases in alveolar P_{CO2} and directed at limiting gas flow to regions of the lung where blood flow is reduced.

As with hypoxia-induced bronchoconstriction, chemoreceptor stimulation by arterial hypercapnia evokes a bronchoconstrictor response via a reflex pathway involving stimulation of the efferent vagus nerve (Nadel & Widdicombe, 1962; Green & Widdicombe, 1966). In contrast to hypoxia, however, the response to hypercapnia remains intact after section of the glossopharyngeal nerves, indicating that the response to CO_2 is mediated via central, rather than peripheral chemoreceptors (Nadel & Widdicombe, 1962; Green & Widdicombe, 1966). In addition, cooling of the ventral surface of the medulla, an area of presumed chemoreceptor function (see section 1.1.3.1.2), blocks the cholinergically mediated constriction of the airways to hypercapnia (Deal *et al.*, 1986).

This increase in bronchomotor tone during hypercapnia may represent, in a manner similar to the bronchoconstrictive response to hypoxia, a protective mechanism to reduce airway deformation during periods of increased ventilation.

1.6.3.3.2 SAR activation

Airway slowly adapting stretch receptors (SAR, see section 1.1.3.2.1) increase their activity during contraction of airway smooth muscle (Bartlett *et al.*, 1976), leading to a reflex bronchodilation (Widdicombe & Nadal, 1963). Since both hypoxia and hypercapnia evoke a bronchoconstriction, SAR activity would be expected to increase during inhalation of these gas mixtures. Indeed, in awake dogs, the increase in ventilation due to hypoxia stimulates SAR and produces a bronchodilation which can completely offset the hypoxia-induced bronchoconstriction (Sorkness & Vidruk, 1986). Fisher *et al* (1983) have proposed that the stimulation of SAR may be a physiological mechanism for limiting the bronchoconstriction induced by hypoxia and hypercapnia.

1.6.3.3.3 The effect of lung volumes

Alveolar hypoxia has been reported to affect the elastic recoil of the lungs and therefore to alter lung volumes (Green & Widdicombe, 1966; Saunders *et al.*, 1977). This may have an important effect on airway calibre, indeed it has been postulated that the hypoxic-bronchoconstriction may be mediated in part via changes in lung volume (Watney *et al.*, 1988). The majority of studies report an increased functional residual capacity and residual volume of the lungs in hypoxia. The mechanism may involve delayed lung emptying by the increase in airway resistance, or a decrease in the tone of the intercostal muscle (Saunders *et al.*, 1977; Garfinkel & Fitzgerald, 1978). Perhaps more likely, however, is a reduction in the elastic recoil of the lungs, which has been reported to occur during acute hypoxia (Saunders *et al.*, 1977; Gautier *et al.*, 1982). This may be caused by relaxation of smooth muscle in the lung parenchyma, either due to a direct effect of hypoxia or an increase in circulating catecholamine concentrations (Saunders *et al.*, 1977; Gautier *et al.*, 1982). Considered in terms of its effects on the mechanical behaviour of the respiratory system, the increase in lung volume produced by hypoxia may counteract the contraction of the airway smooth muscle which, in isolation, would tend to increase lung recoil.

1.6.3.3.4 Hypoxic depression of central ventilatory centres

Another factor which should be considered is the ability of hypoxia to depress the central neural systems that control ventilation (Lee & Milhorn, 1975). If the bronchomotor centre, like the ventilatory control centre, is depressed by hypoxia, one would expect that, via this mechanism, hypoxia would reduce airway smooth muscle tone. Whether this effect occurs or not, or whether the level of the hypoxic stimulus in these studies is low enough to produce central depression, is unknown.

1.6.3.4 The effect of chronic hypoxia on airway smooth muscle tone *in vivo*

The effects of chronic alveolar hypoxia on lung mechanics have not been well studied. Chronic hypoxia is associated with airflow limitation, possibly resulting from a structural encroachment of the airways by the thickened pulmonary arteries (Bancalari *et al.*, 1977; De Troyer *et al.*, 1977). Experimental studies, conducted almost exclusively in rats, have shown that sustained alveolar hypoxia during development causes an increase in the lung size, the number and size of alveolar spaces (Burri & Weibel, 1971; Mortola *et al.*, 1986) and a decrease in total lung resistance (Okuba & Mortola, 1989). Furthermore, chronically hypoxic rats have more compliant lungs (Okuba & Mortola, 1989), possibly due to a decrease in elastic recoil generated by surface tension in the larger alveoli.

It is unclear how this relates to the response of humans to chronic hypoxia, since Brody *et al* (1977) found an increase in lung resistance in Andean natives. Furthermore, a more recent study in calves exposed to chronic hypoxia also found an increase in lung resistance (Inscore *et al.*, 1990), indicating that the effects of chronic hypoxia on lung mechanics remain to be established.

1.6.4 THE EFFECT OF HYPOXIA ON AIRWAY RESPONSIVENESS

1.6.4.1 Acute hypoxia

In addition to the rapid effect of hypoxia on airway tone, several authors have reported that hypoxia enhances bronchial reactivity to various spasmogens *in vivo* (Ahmed & Marchette, 1985; D'Brot & Ahmed, 1988; Vidruk & Sorkness, 1985). The mechanisms underlying this alteration in airway responsiveness are not well understood. For example, Vidruk and Sorkness (1985) demonstrated that histamine-induced reflex tracheal constriction in anaesthetized dogs was enhanced by hypoxia and that this response was abolished by denervation of the trachea. The authors proposed that the potentiating effect of hypoxia on reflex bronchoconstriction is caused by an interaction between lung sensory receptors (possibly rapidly adapting receptors or C-fibre receptors) and carotid body chemoreceptors (Vidruk & Sorkness, 1985). In contrast, Ahmed and co-workers suggest that hypoxia-induced bronchial hyperreactivity is due to local release of mediators which "prime" the airway smooth muscle. In support of this theory, sodium cromoglycate, a mast cell membrane-stabilizing agent, prevents hypoxia-induced degranulation of mast cells as well as enhancement of bronchial reactivity after hypoxia in awake sheep (Ahmed *et al.*, 1982; Ahmed & Marchette, 1985), suggesting a central role for mast cell mediators. Other studies implicate metabolites of arachidonic acid as being involved. For example, leukotriene C_4 concentrations in broncheoalveolar lavage fluid have been shown to be elevated during alveolar hypoxia (Morganroth *et al.*, 1984) and furthermore, a leukotriene antagonist abolishes hypoxia-induced airway hyperreactivity in sheep (D'Brot & Ahmed, 1988). A more recent study, however, showed that cyclooxygenase metabolites are not involved since indomethacin, a cyclooxygenase inhibitor, failed to attenuate the enhanced responsiveness (D'Brot & Ahmed, 1991). Thus it is possible that hypoxia stimulates the 5-lipoxygenase-linked cascade without affecting the cyclooxygenase pathway.

Interestingly, it appears that the hypoxic enhancement of airway reactivity is abolished by breathing a hyperoxic gas mixture. While hyperoxia per se has no effect on resting tone (Vidruk & Sorkness, 1985) or airway responsiveness (D'Brot & Ahmed, 1991), it attenuated the hypoxic-enhancement of reflex bronchoconstriction in dogs (Vidruk & Sorkness, 1985) and the hypoxic-enhancement of histamine and carbachol-mediated bronchoconstriction in sheep (D'Brot & Ahmed, 1991). The effect of hyperoxia on airway responsiveness in humans is unclear, with both an inhibition (Inoue *et al.*, 1989) and lack of effect of hyperoxia on methacholine responsiveness being reported (Beckett & Wong, 1988).

1.6.4.2 Chronic hypoxia

To the best of my knowledge, only one study has examined the effect of chronic hypoxia on airway responsiveness. Inscore *et al* (1990) exposed neonatal calves to 14 days of hypobaric hypoxia and subsequently measured airway responsiveness both *in vivo* and *in vitro*. Chronic hypoxia evoked a marked remodelling process in these animals, manifested as a significant increase in airway fibrous tissue and airway smooth muscle. Compared with control calves, however, responses to methacholine either *in vivo* or *in vitro* were not altered by chronic hypoxia, although contractility to potassium chloride was significantly increased in airways from hypoxic calves *in vitro*.

In contrast to the lack of study of chronic hypoxia on airway responsiveness, there are many reports, conducted almost exclusively on rats, concerning the effects of chronic hyperoxia on airway reactivity. Chronic exposure to hyperoxia results in airway hyperresponsiveness, both *in vivo* and *in vitro*, to contractile agents such as 5-hydroxytryptamine and methacholine (Szarek, 1989; Hershenson *et al.*, 1992a and b). Most groups report that this increase in airway reactivity correlates closely with the thickening of the airway wall which develops in chronically hyperoxic rats (Hershenson *et al.*, 1992a and b), indicating that airway remodelling is largely responsible for the airway hyperresponsiveness in these animals. More recently, however, Szarek *et al* (1995) showed that the development of airway hyperreactivity in chronically hyperoxic rats occurs before the remodelling process, suggesting that other mechanisms are also involved. Removal of the epithelial layer or treatment with indomethacin abolishes the hyperreactivity to acetylcholine in chronically hyperoxic rats (Hershenson *et al.*, 1994), implying that epithelial-derived prostanoids may contribute to the hyperoxia-induced hyperresponsiveness in rats. In contrast, Burghardt *et al* (1996), found that a 5-lipoxygenase inhibitor prevented the development of airway hyperreactivity and airway remodelling in chronically hyperoxic rats, indicating a possible role for leukotrienes.

1.6.5 SUMMARY

There is no doubt that the airways respond to acute and chronic changes in alveolar oxygen tension. The manner of the response is less well defined, partly due to the difficulty in interpreting the conflicting results from various studies. As stated above, it is technically very difficult to study the direct effect of hypoxia on airway function without altering other variables, for example CO_2 levels, lung volumes, lung receptors and airway reflexes, which may have a significant influence on the end response.

While there remains a fair amount of controversy among various groups, the majority of studies report that hypoxia inhibits contractility of airway smooth muscle *in vitro*, perhaps reflecting the direct effect of hypoxia on airway smooth muscle function, and dilates the upper airways *in vivo*. In contrast, hypoxia tends to induce bronchoconstriction in the lower airways, a response that would at first appear to be maladaptive in that it would increase resistance to airflow and hence, impair alveolar ventilation. This response, however, should be considered in terms of the overall response to hypoxia, an increase in respiratory rate, which would elevate the airway transmural pressures. Thus, the bronchoconstrictor response to hypoxia is proposed to be an attempt to stiffen the airways and hence avoid dynamic compression.

1.7 THE EFFECT OF HYPOXIA ON CELL PROLIFERATION

1.7.1 INTRODUCTION

As stated in section 1.5, excessive proliferation of airway smooth muscle cells leads to thickening of the airway wall and may contribute to airflow limitation. It is of obvious clinical relevance, therefore, to understand the physiological and pathophysiological factors which are involved in regulating mitogenesis. There is compelling evidence that the oxygen concentration in the surrounding environment has an important effect on cell proliferation.

Much of the work studying the effect of hypoxia on cell growth has been conducted upon pulmonary artery smooth muscle. This perhaps reflects the well defined response of the pulmonary arteries to hypoxia; namely an initial contraction followed by subsequent hypertrophy and hyperplasia (Hales, 1985). This remodelling process occurs in pulmonary hypertension, a clinical condition associated with sustained alveolar hypoxia.

Exposing animals, mainly rats or mice, to sustained hypoxia is the most commonly used model for pulmonary hypertension. Rats subjected to chronic hypoxia (either normobaric or hypobaric, see section 2.1.7.4) exhibit significant pulmonary hypertension and develop morphological changes in the pulmonary vascular bed that are similar to those observed in human pulmonary hypertension (Hislop & Reid, 1976; Rabinovitch *et al.*, 1979).

1.7.2 THE EFFECT OF HYPOXIA ON THE MITOGENESIS OF PULMONARY CELLS

1.7.2.1 *In vivo*

The histological changes occurring in the pulmonary arterial wall in response to chronic hypoxia have been extensively described and several cell types appear to be involved. DNA synthesis by endothelial cells is detectable in the first 24 hours of hypoxic exposure (Meyrick & Reid, 1979), with hypertrophy and hyperplasia of these cells resulting in increased intimal thickness. In proximal pulmonary arteries, medial thickening caused by hypertrophy and hyperplasia of vascular smooth muscle occurs gradually and follows the early endothelial change (Meyrick & Reid, 1979), while in the distal pulmonary circulation, muscularization of previously nonmuscular vessels occurs. The earliest and most dramatic hypoxic growth occurs in the adventitial fibroblasts, especially in resistance-sized vessels (Meyrick & Reid, 1979).

1.7.2.2 *In vitro*

In cultured endothelial cells, hypoxia induces the formation of several growth-promoting peptides (Shweiki *et al.*, 1992; Kourembanas *et al.*, 1993), although proliferation of these cells has not been reported. In a similar manner, hypoxia does not stimulate proliferation of pulmonary vascular smooth muscle cells *in vitro*, indeed hypoxia may actually have an inhibitory effect in these cells (Dempsey *et al.*, 1991). Only the fibroblast proliferates directly in response to hypoxia *in vitro* (Storch & Talley, 1988), indicating that the effects of hypoxia *in vitro* are cell type-specific.

1.7.2.3 Mechanisms by which hypoxia affects mitogenesis

While hypoxia induces proliferation of pulmonary vascular smooth muscle cells *in vivo,* it is apparently without effect *in vitro*. This suggests that hypoxia may alter proliferation of smooth muscle cells indirectly, possibly via removing inhibitory mechanisms, via effects on signal transduction pathways or via changes in growth factor expression.

1.7.2.3.1 Removal of inhibitory mechanisms

An elaborate network of proliferative and antiproliferative mechanisms is thought to exist for most cell types *in vivo*. Hypoxia may alter this balance by impairing mechanisms which attenuate proliferation. For example, vascular endothelial cells secrete heparin sulphates (Benitz *et al.*, 1990) which directly inhibit smooth muscle and fibroblast growth (Castellot *et al.*, 1989; Das *et al.*, 1995). Release of this substance is attenuated by hypoxia (Benitz *et al.*, 1990), therefore removal of this inhibitory influence may induce or facilitate proliferation.

1.7.2.3.2 Effects on signal transduction pathways

Many different effects of hypoxia on protein synthesis, transport mechanisms and signal transduction pathways have been described in vascular cells. These events do not lead directly to growth in isolated smooth muscle cells (Dempsey *et al.*, 1991), but in other systems have been implicated in proliferative responses. For example, in pulmonary artery smooth muscle cells *in vitro*, hypoxia stimulates an increase in intracellular Ca^{2+} (Salvaterra & Goldman, 1993; Cornfield *et al.*, 1994), stimulates translocation of PKC (Dempsey *et al.*, 1996) and increases polyamine transport (Haven *et al.*, 1992). Although none of these hypoxia-induced changes stimulate vascular cell growth directly, they may have indirect proliferative effects. Specifically, they may enhance responsiveness to other growth-promoting stimuli such as locally produced growth factors.

1.7.2.3.3 Changes in growth factor expression

Hypoxia has been shown to increase the expression of various growth factors, such as PDGF (Katayose *et al.*, 1993), insulin-like growth factor (Perkett *et al.*, 1992) and ET-1 (Stelzner *et al.*, 1992), in whole lungs and also in isolated cells. To date, ET-1 is the mitogen most strongly implicated in the vascular remodelling found in chronic hypoxia-induced pulmonary hypertension (Bonvallet *et al.*, 1994; Zamora *et al.*, 1993).

1.7.3 SYNERGY BETWEEN SIGNAL TRANSDUCTION PATHWAYS AND MITOGENS

Stimuli which activate complementary signalling pathways can combine to yield dramatically increased cell proliferation. In pulmonary artery smooth muscle cells, activation of PKC by basic fibroblast growth factor augments the mitogenic response to insulin-like growth factor (Dempsey *et al.*, 1990). Furthermore, in human airway smooth muscle cells, epidermal growth factor-induced mitogenesis is potentiated by ET-1 (Panettieri *et al.*, 1996).

Activated signalling pathways can also increase expression of growth factors that promote further growth (Shubeita *et al.*, 1992). For example, ET-1 induces proliferation of rabbit airway smooth muscle by activating phospholipase A_2 and thereby generating thromboxane A_2 and LTD_4 (Noveral *et al.*, 1992; Panettieri *et al.*, 1993). Similarly, prostaglandin mediators (thromboxane A_2) and mitogens (angiotensin II) stimulate increased expression of specific complementary growth factors that lead to augmented growth (Ali *et al.*, 1993; Delafontaine & Low, 1993). Synergy between signal transduction pathways and growth factors may be an important means of augmenting hypoxia-initiated cell growth.

1.7.4 THE EFFECT OF HYPOXIA ON THE PROLIFERATION OF AIRWAY SMOOTH MUSCLE CELLS

While the effect of chronic hypoxia on the proliferation of pulmonary vascular smooth muscle cells has been extensively studied, the effect on airway remodelling has received little attention. As stated in section 1.6.3.4, the mechanical airflow limitation associated with exposure to hypoxia has previously been assumed to be due to a structural encroachment of the airways by the thickened pulmonary arteries (Bancalari *et al.*, 1977; Hordof *et al.*, 1977). Inscore *et al* (1990) showed that in neonatal calves exposed to chronic hypobaric hypoxia, the airway smooth muscle surrounding the terminal bronchioles and the large central airways was significantly thickened. This suggests that, in a manner similar to pulmonary arteries, chronic hypoxia induces

proliferation of airway smooth muscle *in vivo*, resulting in a remodelling process which may increase resistance to airflow.

1.7.5 THE EFFECT OF HYPEROXIA ON THE PROLIFERATION OF AIRWAY SMOOTH MUSCLE CELLS

In contrast to the paucity of data concerning the effect of chronic hypoxia on the proliferation of airway smooth muscle, there are many reports concerning the effects of chronic hyperoxia on this cellular response.

As stated in section 1.6.3.4, chronic alveolar hyperoxia induces remodelling of the respiratory tract in rats, characterized as a thickening of the airway smooth muscle and epithelial cell layers from the central conducting airways to the small peripheral airways (Hershenson *et al.*, 1992). Hyperoxic exposure also causes airway constrictor hyperresponsiveness in these animals, the severity of which correlates with the severity of airway wall thickening (Hershenson *et al.*, 1992). Thickening of the airway wall may theoretically arise from hyperplasia, hypertrophy or accumulation of collagen and elastin within the smooth muscle layer. Indeed, Hershenson *et al* (1994) have shown that hyperplasia of airway smooth muscle cells occurs in rats exposed to chronic hyperoxia and that this contributes to the remodelling process. In contrast, Absher *et al* (1994) found that hyperoxia inhibited proliferation of rat tracheal smooth muscle cells in culture, but induced a hypertrophic response in these cells.

1.7.5.1 Mechanisms underlying the effects of hyperoxia on airway smooth muscle

1.7.5.1.1 Growth factor release

The mechanisms underlying the potential effects of hyperoxia on airway remodelling have not been established, although several reports have implicated local release of growth factors such as PDGF. Chronic hyperoxia increases expression of PDGF B-chain mRNA in rat lungs (Fabisiak *et al.*, 1989) and furthermore, Davis and colleagues (1983) found increased PDGF bioactivity in the broncheoalveolar lavage fluid from humans who inspired a hyperoxic gas mixture for up to 18 hours.

1.7.5.1.2 Reactive O_2 species

Under hyperoxic conditions, reactive O_2 species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) are formed (Heffner & Repine, 1989). These active particles may have potent effects on cell growth and have been reported to either inhibit (Libby *et al.*, 1985) or to stimulate (Rao & Berk, 1992) proliferation in vascular smooth muscle. Delafontaine & Ku (1997) showed that reactive O_2 species increase the synthesis and release of insulin-like growth factor I in cultured rat aortic smooth muscle cells, suggesting that O_2 radicals may mediate their

cultured rat aortic smooth muscle cells, suggesting that O_2 radicals may mediate their proliferative effects via the secondary release of mitogenic agents. A recent study, however, demonstrated that superoxide anion can mediate cell proliferation directly via activation of the ras/Raf-1/MEK/MAP kinase pathway (Bhunia *et al.*, 1997).

1.7.6 THE EFFECT OF HYPOXIA ON GENE EXPRESSION

Within recent years, a number of genes have been identified that are regulated by oxygen tension. Most of our current understanding of the hypoxic regulation of gene control is derived from studies on erythropoietin (EPO). This protein hormone stimulates the production of red blood cells and is a crucial mechanism by which mammals respond to hypoxia. EPO mRNA and protein increase 10- to 100-fold in response to hypoxia (Goldberg *et al.*, 1991), partly through increased transcription and partly through increased stability of mRNA (Rondon *et al.*, 1991). A hypoxic responsive enhancer region exists in close proximity to the EPO gene (Semenza *et al.*, 1991) and recent studies show that a transcription factor, hypoxia-inducible factor (HIF-1), binds to this region (Semenza & Wang, 1992; Beck *et al.*, 1993). Binding of HIF appears to be critical for activation of the enhancer region (Semenza *et al.*, 1994), and hence gene transcription.

There are other nuclear transcriptional factors, such as AP-1 and NF-kB, which appear to be oxygen sensitive (Webster *et al.*, 1994; Schenk *et al.*, 1994), although involvement in hypoxia-induced gene expression is less well defined than HIF-1.

In spite of the wealth of information on the EPO gene, the initial steps in sensing oxygen tension and the intracellular elements that transduce the primary response to hypoxia remain unclear. At present, it is widely believed that the oxygen sensor is a haem-containing protein (Goldberg *et al.*, 1988) that exists in two reversible conformational states, deoxy or oxy, depending upon the available oxygen tension. It is proposed that the haem-group might serve as a ligand that binds directly to transcription factors such as HIF-1, or alternatively, that haem may function as an oxygen sensor within the transcription factor itself (for review, see Pitt *et al.*, 1996).

In addition to EPO, several other gene products, many involved in cell growth, have been shown to be elevated by hypoxia. Transcription of PDGF (Kourembanas *et al.*, 1990), ET-1 (Kourembanas *et al.*, 1991), interleukin-8 (Karakurum *et al.*, 1994), vascular endothelial cell growth factor (Shweiki *et al.*, 1992) and transforming growth factor-β1 (Falanga *et al.*, 1991) is increased by hypoxia in various cell cultures. It is noteworthy that several of these growth factors are increased in the lungs of hypoxic animals *in situ* (Elton *et al.*, 1992; Katayose *et al.*, 1993), suggesting that hypoxic-regulation of these, and other, gene products may contribute to the pathologies of various diseases associated with chronic hypoxia.

1.7.7 SUMMARY

As stated in section 1.7.2, the reported effects of hypoxia on smooth muscle proliferation differ between *in vitro* and *in vivo* experiments. This is also the case for hyperoxia, with an inhibitory effect of hyperoxia on proliferation of rat airway smooth muscle cells in culture (Absher *et al.*, 1994), compared to a stimulatory effect *in vivo* (Hershenson *et al.*, 1994). This suggests that the effect of changes in gas composition on airway smooth muscle proliferation may be indirect and may involve other factors or cell types which cannot be mimicked by studying isolated airway smooth muscle cells in culture. This makes the task of elucidating the mechanisms of hypoxia- and hyperoxia-mediated airway remodelling all the more difficult.

There appears to be a certain amount of confliction between the effects of hypoxia or hyperoxia on airway smooth muscle proliferation. On the one hand, hyperoxia induces proliferation of airway smooth muscle in rats *in vivo*, while chronic hypoxia also induces thickening of the airway smooth muscle layer, although in this case in calves (Inscore *et al.*, 1990). The most obvious explanation is that this represents a species difference between rats and cows, although the findings that pulmonary hypertension can be induced in rats by either chronic hypoxia or hyperoxia (Coflesky *et al.*, 1988), or that PDGF expression in rat lungs appears to be elevated by both hypoxia and hyperoxia is not consistent with this. It is possible therefore, that the two opposite ends of the spectrum, hypoxia and hyperoxia, produce the same end response, namely an increased proliferation of airway smooth muscle, although the mechanisms underlying this may be different in each case.

1.5 AIMS OF PROJECT

The main points I wished to address with my research were to:

1) Study the effect of acute changes in oxygen tension on airway smooth muscle function. In the first instance, using bovine bronchial rings, I set out to examine the effect of changes in oxygen tension on the responsiveness of airway smooth muscle to contractile and relaxatory agonists.

2) Examine the effect of chronic exposure to hypoxia on airway smooth muscle responsiveness. I compared contractile and relaxatory responses using tracheal rings isolated from either control rats or rats exposed to chronic hypobaric hypoxia.

3) Compare and contrast endothelin receptor-mediated responses in tracheal rings from both chronically hypoxic and control rats and to characterise the endothelin receptor subtypes involved in mediating these responses.

4) Examine the effect of hypoxia on the proliferation of cultured human airway smooth muscle cells and to determine if hypoxia alters the responsiveness of these cells to mitogenic agents.

5) Study the effect of changes in inspired oxygen tension on salbutamol-mediated bronchodilation and methacholine- and histamine-mediated bronchoconstriction in asthmatic patients *in vivo*.

6) Conduct a parallel study *in vitro*, examining the effect of changes in oxygen tension on salbutamol-, methacholine- and histamine-mediated response in human isolated bronchial rings.

CHAPTER 2

MATERIALS AND METHODS

2.1 TECHNIQUES FOR STUDYING ISOLATED AIRWAYS

2.1.1 ORGAN BATH SET UP FOR STUDYING ISOLATED AIRWAY PREPARATIONS

Standard organ bath procedures were used for studying responses in isolated airway preparations *in vitro*. Figure 2.1 shows a schematic diagram of the organ bath set up. Rings of trachea or bronchus were suspended between two stainless steel wires. The lower wire was anchored to a glass tissue holder and the upper was connected by cotton to a force displacement transducer (Grass FT03T). Isometric contractions were recorded via a transducer connected to a 6-channel chart recorder (Linseis L2005). Each airway preparation was mounted in a 5 ml organ bath containing modified Krebs-Henseleit solution (see section 2.1.8 for composition). Appropriate tension was then placed on the vessels (see experimental chapters) and the Krebs-Henseleit solution was maintained at 37± 0.5°C, by means of an insulating water jacket surrounding the bath. The Krebs-Henseleit solution was bubbled with an appropriate gas mixture (for exact gas details see section 2.1.5).

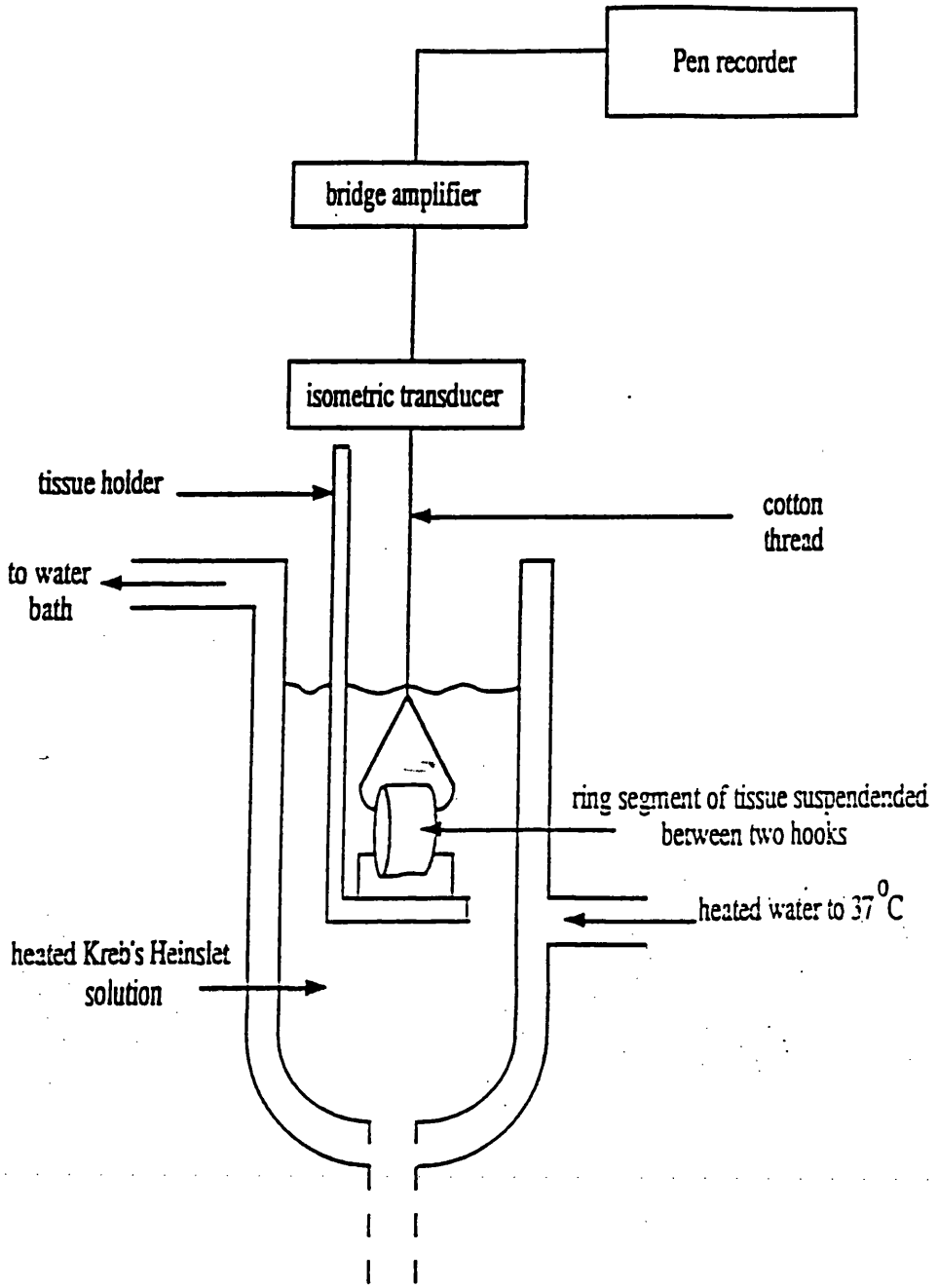


Figure 2.1.

Diagrammatic representation (not to scale) of the organ bath experimental apparatus used for studying larger diameter pulmonary arteries *in vitro*. Volume of the organ bath was 5 ml, gas bubbling apparatus is not shown on diagram.

2.1.2 DISSECTION OF AIRWAY PREPARATIONS

2.1.2.1 Rat trachea

Rats were killed by overdose of sodium pentobarbitone (60 mg/kg i.p.) and the trachea removed and immediately placed in ice cold Krebs-Henseleit solution. The trachea was carefully cleaned of connective tissue using a dissecting microscope and cut into rings without damage to the epithelium. Each ring preparation was approximately 4mm long with an internal diameter (i.d.) of ~2mm. Each rat normally yielded 4 such tracheal ring preparations. Where appropriate, removal of the epithelium was achieved by gentle rubbing of the intimal surface. These vessels were then placed in a vial of ice cold Krebs-Henseleit solution in preparation for mounting in the organ baths (see section 2.1.1).

2.1.2.2 Bovine bronchus

A lobe of bovine lung was placed on a dissecting tray with its visceral surface exposed. The main internal bronchus was identified and dissected free using large scissors. This exposed the branches of the next generation of bronchi. The branching pathway of this generation of bronchi was followed using fine scissors until an internal diameter of approximately 3-5mm was identified. This length of bronchus was then removed and cut into rings approximately 4mm long (i.d. 3-5mm) using sharp disposable blades. Each lobe of lung could yield more than 30 of these bronchial ring preparations. These bronchial rings were placed in a vial of ice cold Krebs-Henseleit solution. To avoid desiccation of the tissue during the course of the dissection, the tissue was regularly moistened with Krebs-Henseleit solution.

2.1.2.3 Human bronchus

The lung samples were placed in a petri dish containing Krebs-Henseleit solution. The size of the sample was very variable, but bronchi with internal diameters of approximately 3-5mm were identified and carefully dissected free using fine scissors or sharp disposable blades. The bronchus was cut into rings approximately 4mm long (i.d. 3-5mm) and cleaned of surrounding connective tissue before being placed in a vial of ice cold Krebs-Henseleit solution.

2.1.3 CALIBRATION OF EQUIPMENT

The transducers and chart recorders used in the organ bath set-up were calibrated daily using known weights (1 g wt). The temperature of the solution in the organ baths was routinely measured with a thermometer.

2.1.4 GENERAL PROCEDURE FOR ORGAN BATH EXPERIMENTS

The general procedure for *in vitro* organ bath studies is listed below, but varies between individual experiments. Exact procedures are therefore given in each experimental chapter.

1) After mounting the airway preparations in the organ bath and applying the appropriate degree of tone, vessels were allowed to equilibrate for 45 minutes. During this time, tension was reapplied where necessary.
2) The viability of the tissue was ascertained by the addition of a single concentration (10^{-4}M) of methacholine to evoke a reference contractile response. Once the response reached a plateau, the vessels were than washed 3 times with fresh Krebs-Henseleit solution and allowed to return to baseline tensions.
3) Tension was adjusted if necessary and the vessels were left for a further 45 minutes before the addition of any drugs.
4) A cumulative concentration-response curve (CCRC) was then conducted to the required agonist covering a range of concentrations which ensured that the threshold response and the maximum response (if possible) were included.
5) For agonists which reversibly bind to their receptor, vessels were washed 3 times with fresh Krebs-Henseleit solution following the initial CCRC. Tissues were left for 45 minutes to allow the tension to return to baseline levels and the antagonist/inhibitor was added and left for its required incubation period. Following this, a second CCRC to the agonist was conducted. On each experimental day, one tissue acted as a time control in that the second CCRC was conducted without the addition of the antagonist/inhibitor.
For agonists which irreversibly bind to their receptors, only one CCRC could be conducted in each preparation. In such cases, separate tissues were used to perform experiments using antagonists/inhibitors. In such experiments, the antagonist/inhibitor was added 45 minutes after the methacholine response and left for its required incubation period before the CCRC was performed.

2.1.5. GAS MIXTURES

The standard gas mixture used for *in vitro* studies of isolated tissues is 95% O_2/5% CO_2. This hyperoxic gas mixture was originally chosen to prevent areas of the tissue becoming hypoxic. 5% CO_2 is included to buffer the pH of the Krebs-Henseleit solution to 7.4. On certain experimental occasions, the O_2 content of the perfusing gas mixture was reduced from 95% to 20% or 0% O_2, by substituting N_2 for O_2. Bubbling the Krebs-Henseleit solution in the organ baths with gas mixtures containing 95, 20 or 0% O_2 gave final bath O_2 tensions of 520-525mm Hg, 142-145mmHg and 24-28mmHg, respectively. The O_2 tension in each organ bath was measured by means of a Jencons O_2 probe placed directly in the Krebs-Henseleit solution. In each of the experimental gas mixtures, 5% CO_2 was included to buffer the pH to 7.4. The pH in each organ bath was measured using a Mettler pH meter and remained within ± 0.1 of pH 7.4.

2.1.6 NOTES ON ET RECEPTOR AGONISTS

CCRCs conducted to the ET receptor agonists ET-1 and sarafotoxin S6c were normally taken to a maximum concentration of $3x10^{-7}$M. Addition of this final concentration, however, often produced a further contractile response and can therefore not strictly be considered "maximal." The two reasons for not increasing the concentrations of agonist further are that: (1)The relative solubility of the peptide yields a stock solution of 10^{-4}M therefore a large volume of stock solution must be added to 5ml baths to give final concentrations of 10^{-6}M and (2) ET-1 and sarafotoxin S6c are extremely expensive and we therefore could not afford to use large volumes of stock solution.

2.1.7 ANIMAL MODELS USED IN THESE STUDIES

As human tissue was only available in limited amounts, it was necessary to use tissue from various animal models.

2.1.7.1 Bovine

Bovine bronchial rings were used in some experiments as this tissue is reported as being a good model for human bronchi (for example, see Nally *et al.*, 1994b; Angus *et al.*, 1994). Bovine lungs were obtained on the day of experimentation from the local abattoirs (either Duke St., Glasgow or Sandyford, Paisley). Lungs were removed from freshly slaughtered cattle and were transported to the laboratory in a container filled with chilled Krebs-Henseleit solution.

2.1.7.2 Human

Whenever possible, studies were carried out using human bronchial tissue. Macroscopically normal sections of human lung were obtained from patients undergoing thoracic surgery at the Western Infirmary, Glasgow for bronchial carcinoma. Samples were placed in chilled Krebs-Henseleit solution as soon as possible (normally less than 15 minutes after being removed from the patient) and taken to the laboratory for dissection (see section 2.1.2.3). Human tissue was normally utilised as soon as possible after dissection, but in some cases was stored overnight in Krebs-Henseleit solution at 4°C. Published data has shown that overnight storage of this tissue does not alter its reactivity to contractile and relaxant agonists (Brink *et al.*, 1980; Nally *et al.*, 1994a). Details of individual patient histories are not known.

2.1.7.3 "In House" adult Wistar rat.

The adult Wistar rat from the "In House" breeding stock of the University of Glasgow's Central Animal Facility was used in several studies. The Wistar rat was used to produce the chronically hypoxic rat model (see section 2.1.7.4) and also acted as controls for the chronically hypoxic rats. Animals were maintained on a twelve hour light/dark cycle and allowed free access to standard diet and water.

2.1.7.4 Chronic hypobaric hypoxic rat

2.1.7.4.1 Introduction

Hypoxic animal models (mainly rats and mice) have been used since the 1920's to study various environmental conditions and disease states. The animals were exposed to hypoxic environments by the use of environmental chambers (normobaric hypoxia; a decreased inspired O_2 at normal atmospheric pressure, or hypobaric hypoxia; a decreased inspired O_2 due to decreased atmospheric pressure) or by actual relocation of the animals to altitude where the inspired O_2 levels are lower than at sea level (see Campbell, 1927a,b,c; Timiras *et al.*, 1957). These early investigations studied the acclimatisation of animals to altitude, or the O_2 tensions which would be experienced at altitude. Animals exposed to these hypoxic environments exhibited initial weight loss and alterations in certain internal organ weights (Campbell, 1935; Timiras *et al.*, 1957). The basic principles of normobaric and hypobaric hypoxia are described below.

2.1.7.4.2 Normobaric versus Hypobaric hypoxia

Normobaric hypoxia

The normobaric method of chronic hypoxia used by most investigators has been adapted from a chamber described by Cryer and Bartley (1974). The O_2 concentration within the chamber is reduced from the normal 21% to ~10% (160mm Hg to ~80mm Hg O_2) by intermittent infusion of N_2. To prevent the build up of CO_2, humidity and other gases, the air is circulated through specific chemical absorbers.

Hypobaric hypoxia

Hypobaric hypoxia reduces the inspired O_2 content of the environment by reducing the atmospheric pressure within the chamber. This is the equivalent of taking the animals to high altitude. As the atmospheric pressure decreases, the partial pressure of the gaseous components of air decreases. Therefore, while the percentage of the gaseous components of the air remains the same (O_2 ~ 21% and N_2 ~ 78%), the effective partial pressure of inspired O_2 declines. Hypobaric hypoxia is achieved by withdrawing air from the chamber by use of a pump until the pressure within the chamber is equivalent to ~0.5 atmospheres, which reduces the inspired O_2 pressure from 160mm Hg to 80mm Hg. The chamber is continuously flushed with room air to maintain conditions of low humidity and CO_2.

While normobaric and hypobaric chambers are both commonly used (mainly for the study of pulmonary hypertension), the hypoxic hypobaric chamber has proved to be more economical and it is this type which was used in my studies. The Royal Hallamshire Hospital Sheffield designed and manufactured the hypoxic hypobaric chamber for Dr Margaret MacLean and her Pulmonary Research Group based in the Institute of Biomedical and Life Sciences at the University of Glasgow. The chamber conforms to the high safety standards required by the Home Office.

2.1.7.4.3 Chamber Design

The chamber is designed to hold two standard rat cages, with up to four rats in each cage. The structure of the chamber is made from transparent high resistance Plexiglass. Air is continually removed from the chamber by the pump. Air constantly flows through the chamber at 45L/minute, ensuring that moisture and CO_2 do not build up. Temperatures are similar both inside and outside of the chamber.

2.1.7.4.4 Maintenance of animals

The chamber was housed in a specially designed environmental room which maintains temperature at approximately 21°C, humidity at 55%, gives 20 changes of filtered air per hour and maintains a 12 hour on/12 hour off light cycle. Animals used in the experiments were obtained from credited commercial suppliers, Harlan UK Ltd. The rats supplied were male specific pathogen free and ordered in at age 28-30 days (approximately 60g weight). Animals were allowed to acclimatise within the

environmental chamber for five days before being split into two groups of four. One group of animals was placed in the chamber and the other group remained in normal atmospheric conditions to act as age matched controls.

2.1.7.4.5 Production and maintenance of chronic hypoxic rats

(1) The chamber door was removed and the rat cage placed in the chamber with the food and water dispensers at the rear, thus allowing observation of the rats.

(2) The chamber door was replaced and the pump switched on.

(3) The chamber was taken down to the desired pressure (550mbar) in small steps by slowly closing the inlet valve and observing the pressure reading on the gauge. Once the stable experimental pressure was reached, rats were checked every 15 minutes for the following hour to ensure that the pressure remained stable and the animals were not in distress.

(4) The chamber could then be left to run for the experimental time required. Checks were made five times a day to ensure that :

(a) pressure readings were not fluctuating beyond desired levels

(b) temperature inside and outside the chamber remained between 20 and 23 °C

(c) animals were not showing signs of distress

(d) the pump was in good operating condition

(5) At weekends, the chamber was checked by a member of the Central Animal Facility staff, University of Glasgow.

(6) When the animals required fresh diet and water, usually every three days, the chamber was taken down to atmospheric pressure over two hours by opening the inlet valve. Once atmospheric pressure was reached, the pump was switched off and allowed to cool for half an hour while the rat cages were cleaned and animals given fresh food and water. Following this, the animals were placed back in the chamber, the pump switched on and the chamber taken back to 550mbar over a period of two hours.

(7) After 14 days in the chamber, two of the rats were removed and the remaining two rats were taken back to experimental pressure for a further two days. Of the two rats removed, one was immediately sacrificed and studied on that day along with an aged matched control. The second rat was left in room air to be studied no longer than 24 hours after removal from the chamber. The same procedure occurred on day 16 for the remaining two rats.

2.1.8 MATERIALS AND SOLUTIONS

The composition of the Krebs-Henseleit solution was as follows: (mM) NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1.
The following drugs and chemical reagents were used:

COMPOUND	SUPPLIER
ANP (α-human atrial natriuretic peptide 28 amino acid)	Bachem
BQ-788 (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulphonamide)	Peptide international
Endothelin-1	Novabiochem
FR 139317 (N-CO-L-Leu-D-1-Me-Trp-D-3 (2-Pyridyl) Ala-OH	Neosystems
Histamine	Sigma
Isosorbide dinitrate ("Isoket")	Schwarz Pharma Ltd.
Indomethacin	Sigma
L-NAME (N^{ω}-nitro-L-arginine methylester)	Sigma
methacholine chloride	Sigma
Phosphoramidon (N-(α-rhamnopyranosyloxyhydroxy phosphinyl)-L-leucyl-L-tryptophan	Sigma
potassium chloride	BDH
salbutamol	Sigma
Sarafotoxin S6c	Sigma
SB 209670 (1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methyleudioxy-phenyl)-5-(propyl-1-oxylindane-2-carboxylic acid)	Gift
sodium nitroprusside	Sigma

2.1.9 DATA ANALYSIS

2.1.9.1 Calculation of results

For measurements of isometric tension, data from preparations undergoing the same procedure were grouped together and expressed as the mean value ± the standard error of the mean (SEM). Data may be expressed as absolute contraction (mg wt), percentage of reference contraction to 10^{-4}M methacholine or as a percentage of its own maximum response (see experimental chapters).

2.1.9.2 Measurement of agonist potency

Values are given as the pD_2, the -log of the concentration of an agonist which produces 50% of the maximum response to that agonist. In cases where a maximum response was not achieved, values for the pEC_{200mg} or pEC_{400mg} are given, the -log of the concentration of an agonist which produces a response of 200mg wt or 400mg wt, respectively.

2.1.9.3 Statistical Analysis

Statistical comparisons between data points was made using Students t-test for paired or unpaired data where appropriate. Comparisons between data sets was tested by two-way analysis of variance (ANOVA). A value of $P<0.05$ was considered to be statistically significant.

2.2 STUDIES ON CULTURED HUMAN AIRWAY SMOOTH MUSCLE CELLS

2.2.1 PRIMARY CULTURE OF HUMAN AIRWAY SMOOTH MUSCLE CELLS

Human airway smooth muscle cells were harvested from bronchial segments obtained from thoracic surgery. Each segment was cleaned of adhering parenchyma, minced and resuspended in 10ml of serum free Dulbecco's Modified Eagle's Medium (DMEM, see section 2.2.6) containing 5mg/ml collagenase and 1mg/ml elastase. Enzymatic dissociation of the tissue was performed for 120 minutes in a shaking water bath at 37°C. Cells were collected by centrifugation (5 minutes, 200g, room temperature), washed in 10ml of DMEM supplemented with 20% foetal calf serum (FCS) and seeded in two 25 cm^2 culture flasks (5ml into each flask). The flasks were labelled with the date and kept in a humidified LEEC incubator (model number GA 25N) under an atmosphere of 5% CO_2 (to buffer the pH of the media to 7.4) and a temperature of 37°C.

Confluency (the cells covering the lower surface of the flask) normally occurred within 7-10 days. At this stage, cells were passaged and reseeded in 75 cm^2 culture flasks.

2.2.2 CELL PASSAGE

Under sterile conditions (within a Microflow laminar-flow hood, model number M25121/1), the media in each 25cm^2 flask was aspirated off. The cells were washed twice with 2ml trypsin (0.25% trypsin (w/v) in phosphate buffered saline (see section 2.2.6)) which was immediately aspirated off. The cells were incubated at 37°C for 3-4 minutes until the cells were seen to have lifted off the bottom of the flask. The cells were suspended in 10ml of DMEM supplemented with 10% FCS and transferred to a 75 cm^2 flask. Thereafter, cells were harvested weekly and passaged at a ratio of 1:3 in 75 cm^2 culture flasks. Cells between passage 3 and 7 were used as published data shows that over these intervals, they retain their native contractile protein expression (Panettieri *et al.*, 1989).

2.2.3 IMMUNOCYTOCHEMISTRY

The identity of the bronchial smooth muscle cells was confirmed by immunocytochemical staining using a smooth muscle specific α–actin mouse monoclonal antibody (DAKOM635) detected using rabbit anti-mouse HRP-linked antibody and 3-amino 9-ethyl-carbazole (Sigma). Cells were subcultured into 8-well glass tissue culture chamber slides and grown to 100% confluency before being washed three times in phosphate buffered saline. The cells were permeabilized for 5 minutes with cold methanol (-20°C) and washed a further three times in phosphate buffered saline. This was followed by exposure to the primary antibody (α–actin mouse antibody) for 1 hour at room temperature and then the second antibody (anti-mouse antibody conjugated to fluorescein isothiocyanate) for 1 hour at room temperature. The staining of these cells was then observed by fluorescence microscopy. Each of the cell lines used in these studies showed uniform staining for smooth muscle specific α–actin.

2.2.4 ASSESSMENT OF CELL PROLIFERATION

2.2.4.1 Introduction

Cell proliferation is a process fundamental to growth, development, adaptation to disease and neoplasia. Assessing proliferation is often a pivotal investigation in biomedical science and a range of techniques have evolved to quantify the process, both directly and indirectly.

2.2.4.2 The cell cycle

The concept of the cell cycle is central to cell proliferation. Recognition of the synthetic (S) phase, a period of intense DNA synthesis, combined with the mitotic (M) phase as two landmarks separated by two gaps led to the model of the cell cycle shown in Figure 2.2. M phase is followed by G_1, the post-mitotic gap, and S phase is followed by the post-synthetic gap G_2, which is in turn followed by a further M phase in progressively proliferating tissue. Cells not actively proliferating can temporarily occupy a fifth phase (G_0) after M phase but, with appropriate stimuli, may be recruited to re-enter the cycle.

Figure 2.2 The cell-cycle

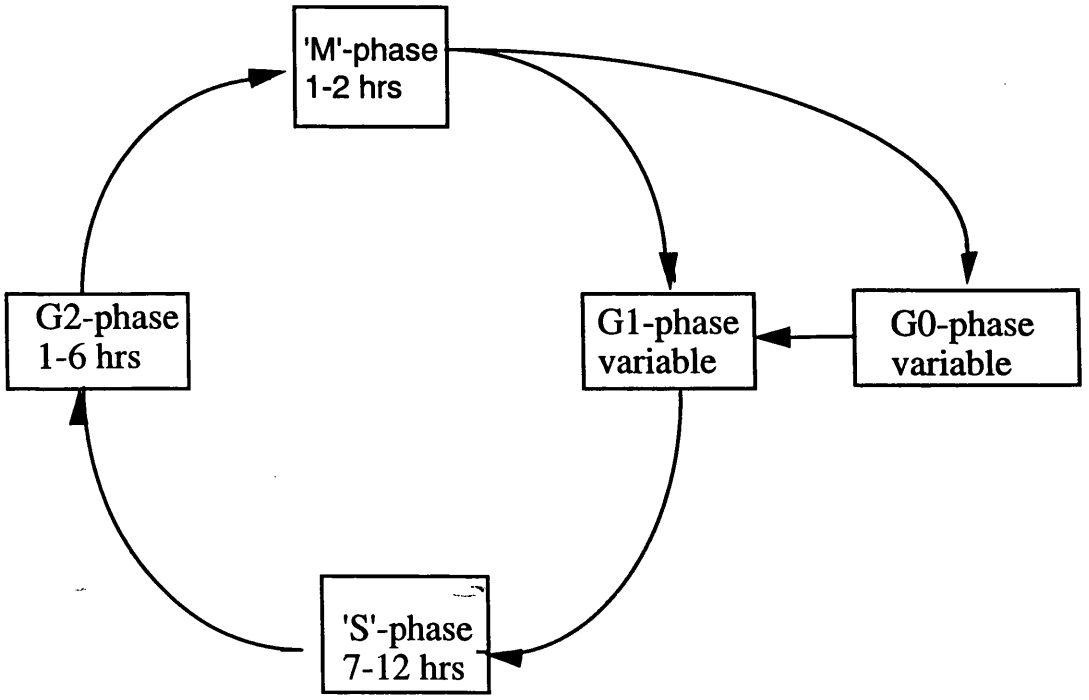


Figure 2.2

Cell growth can be conceptualised as occurring in distinct phases; the mitotic M phase and the synthetic S phase, each divided by a Gap phase (G1 or G2). Cells may temporarily occupy an additional G0, or "resting phase."

2.2.4.3 Assessment of DNA synthesis

In general, these techniques measure the assimilation of radiolabelled nucleotides such as [^{3}H] thymidine into newly synthesized DNA. Thymidine is commonly used as it is not incorporated into RNA. Most tissue cultures comprise cells at various stages of the cell cycle, so cells are typically growth-arrested in serum-free media. This synchronises cells to the G_0/G_1 phase of the cell cycle in which cells minimally incorporate [^{3}H] thymidine (Panettieri *et al.*, 1989).

There are a number of potential drawbacks in relating [^{3}H] thymidine uptake to DNA synthesis: Firstly, although most DNA synthesis occurs during cell replication, some of the DNA synthesis may be for ungoing reparative processes rather than for proliferation. Secondly, [^{3}H] may be incorporated into cellular macromolecules other than DNA, such as RNA and lipids. Despite these potential artefacts, measuring uptake of [^{3}H] thymidine remains one of the best and most widely used methods for assessing cell proliferation.

2.2.4.4 [^{3}H] Thymidine Incorporation Assay Used in Current Studies

Cells were grown to 60% confluency in 24 well plates and growth-arrested for 24 hours in serum free DMEM (see section 2.2.4.3). Cells were then stimulated with appropriate agonists and incubated for 24 hours, either in a normal CO_2 incubator (5% CO_2) or in a LEEC variable O_2 flow incubator (model number GA 156). In the variable O_2 flow incubator, the O_2 content of the atmosphere was reduced from 21% to 2% by flooding with N_2. Using a Jencons oxygen probe placed in the media inside the chamber, the O_2 tension of the culture medium under an environmental O_2 tension of 2% was estimated to be between 26 and 32mm Hg. In both normoxic and hypoxic incubators, 5% CO_2 was included to buffer the pH of the media to 7.4. Using a Mettler pH probe, the media from both the normoxic and hypoxic incubators was measured and found to be pH 7.4±0.1

For the remaining 4 hours of agonist stimulation (20 hours after addition of agonist), cells were labelled with [^{3}H] thymidine (0.1μCi/ml) to give an indication of DNA synthesis and hence, cell proliferation. The reaction was stopped by washing the cells twice in phosphate buffered saline. Proteins were precipitated by washing three times in 5% trichloroacetic acid and lipid fractions were solubilised by washing twice in 90% ethanol before the remaining cell contents were solubilised by a 30 min incubation in 0.3M NaOH. The contents of each well were transferred to scintillation vials, to each of which was added 3mls of Ecosint A scintillation fluid. Vials were vortexed thoroughly before radioactive counts were measured by scintillation counter. Results are expressed as disintegrations per minute (DPM).

2.2.5 NOTE ON STERILITY

Since the conditions (warm, humid and nutrient rich) necessary for culturing the cells are an ideal environment for promoting fungal and bacterial growth, extreme caution must be taken to avoid contamination. All steps such as making up solutions, changing media etc must be conducted under sterile conditions, that is, within a clean, laminar flow hood. The laminar flow hood was dismantled and cleaned regularly and before use each day was sprayed liberally with 70% (w/v) ethanol. Anything taken inside the flow hood (i.e. pipettes and reagent bottles) was also sprayed with ethanol and sterile gloves were worn throughout. Pipette tips and distilled water were sterilised using a Prestige Medical "Omega" autoclave (model number 220140). To avoid bacterial or fungal contamination of the culture medium, the DMEM was supplemented with penicillin/streptomycin at concentrations of 400iu/ml and 400μg/ml, respectively and 2.5μg/ml of amphotericine B. For the initial primary culture, these concentrations of penicillin/streptomycin and amphotericine B were doubled.

2.2.6 MATERIALS AND SOLUTIONS

All general purpose compounds were purchased from Sigma (Poole, Dorset, UK). All tissue culture flasks and media were purchased from Gibco (Paisley, Renfrewshire, UK). Fetal calf serum was purchased from Imperial Laboratories (Andover, Hants, UK). Antibodies were purchased from Affiniti Research Products Ltd. (Nottingham). [^{3}H] thymidine was purchased from DuPont (Stevenage, Hertfordshire, UK).

The cell culture media was composed of Dulbecco's Modified Eagles Medium (DMEM) supplemented with L-glutamine (27mg/ml), penicillin/streptomycin and amphotericine B (see section 2.2.5 for concentrations) and either 10 % or 20% fetal calf serum (see sections 2.2.1 and 2.2.2).

Phosphate buffered saline was composed of 100mM NaCl and 20mM NaH_2PO_4 and buffered to pH 7.4.

Drugs used in these cell culture experiments were:

BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu])	Peptide International
BQ-788 (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl] -benzenesulphonamide)	Peptide International
Endothelin-1	Novabiochem
Platelet-derived Growth Factor	Sigma
Sarafotoxin S6c	Sigma
Staurosporine	Sigma

2.2.7 STATISTICS AND DATA ANALYSIS

Proliferation assays were carried out in quadruplicate and in three cell lines derived from lung specimens from three individual patients. Results are expressed as mean values ± s.e.means for n=3 cell lines. Statistical significance between individual data points was assessed using Student's *t* test, while comparisons between data sets (concentration-response curves) was assessed by two way analysis of variance

(ANOVA). In each case, a probability level of $P<0.05$ was considered to be statistically significant.

2.3 STUDIES CONDUCTED UPON HUMAN SUBJECTS *IN VIVO*

2.3.1 RECRUITMENT

Asthmatic subjects were recruited into the study from databases held by the Asthma Research group, West Glasgow Hospitals University NHS Trust (see experimental chapters for precise details on group sizes, age range etc). The medical history of each patient was recorded, i.e smoker or non-smoker, bronchodilator therapy etc. All subjects gave informed written consent to the studies, which had the approval of the West ethics committee.

2.3.2 STUDY DESIGN

Protocols differed for the various studies (see experimental chapters), but a general protocol is listed below:

(1) At an initial screening visit, subjects performed baseline spirometry (see section 2.3.3.2), and underwent a bronchial provocation test to measure the responsiveness of the airways to the appropriate agonist (i.e salbutamol, methacholine or histamine). If the patients displayed airway responses within certain predetermined limits they were deemed eligible for the study.

(2) On study days, subjects using bronchodilator therapies were asked to discontinue inhaled β_2 agonists for 8 hours, salmeterol for 24 hours and oral theophyllines for 48 hours prior to attendance. Subjects using inhaled corticosteroids were asked to continue their medication as normal. All subjects were asked to refrain from caffeine containing products for 8 hours before each study.

(3) On study days, subjects were rested in a supine position for 30 minutes following which the patients were connected to closed breathing circuit. After a 10 minute period breathing air through the closed breathing circuit, baseline measurements of FEV_1 (see section 2.3.3.2), oxygen saturation (SaO_2%), heart rate (HR), respiratory rate (RR), inspired oxygen and carbon dioxide levels (insp O_2%, insp CO_2%) and end-tidal oxygen and carbon dioxide levels ($pETO_2$%, $pETCO_2$%) were made.

(4) Patients then received the study gas (FiO_2 1.0, FiO_2 0.21 or FiO_2 0.15) for the remainder of the experimental protocol. All gases were administered in a randomised, double-blind fashion by a second operator. Ten minutes after commencing the study gas for that day the measurements made at baseline were repeated. Subsequently at fifteen minute intervals, in a randomised double-blind fashion, patients received incremental doses of the appropriate agonist delivered via a nebuliser. Control subjects received nebulised saline. The MicroCirrus nebuliser was driven by the experimental

gas mixtures at a predetermined flow rate to produce a nebuliser output of 0.13 mls/min. Measurements were repeated thirteen minutes after each nebulisation until completion of the study day.

2.3.3 MEASUREMENTS

2.3.3.1 Heart rate, SaO_2, inspired and expired O_2 and CO_2 levels

Heart rate and oxygen saturation were measured using a pulse oximetry probe (Datex Division of Instrumentarium Corp, Helsinki, Finland). A side port on the face mask allowed continuous sampling of the inspired and expired gases and monitoring of respiratory rate. The gases were continuously analysed using an OSCARoxy TM multigas monitor (Datex Instrumentarium Corp, Helsinki, Finland). Recordings were made every ten seconds for one minute and automatically printed by a Hewlett Packard Think Jet printer in a blind fashion. Results were analysed after completion of the study.

2.3.3.2 FEV_1

Due to the practical problems of measuring airway calibre *in vivo*, changes in airway radius are normally assessed indirectly, for example by measuring resistance to airflow. The physiological meaning of total airways resistance is obscured by the complexity of the branching system of the airways. The resistance to airflow is reported to be greatest in the upper airways of the nose and larynx, which accounts for approximately half of the airways resistance. The trachea and major bronchi are believed to contribute 30% of the total resistance, with only 20% being sited in airways smaller than 2mm in diameter (Macklem & Mead, 1967). These early estimates may be too low (Hoppin *et al.*, 1978), but it is known that peripheral resistance is much higher in patients with chronic airflow obstruction (Hogg *et al.*, 1968).

The most accurate measure of airways resistance is whole body plethysmography, but for reasons of cost, time and ease, the most widely used method for measuring airflow is spirometry. Simply, a patient exhales forcibly to residual volume after a full inspiration to total lung capacity. The ratio of the forced expiratory volume $_1$ (FEV_1), the volume expired in the first 1 second of forced expiration, to the vital capacity is an important indicator of airways obstruction. Comparison of these measurements to tables of predicted values gives an important indication as to whether the airways are patent or narrowed. The measurements of FEV_1 and vital capacity are highly reproducible and normally form the starting point for any investigation of lung function.

In our studies, FEV_1 was measured using a dry wedge spirometer (Vitalograph S, Vitalograph, Buckingham, UK). The best of three attempts was taken for analysis.

2.3.3.3 Plasma catecholamines

In some of the *in vivo* studies, plasma catecholamine levels were measured. Five millilitre aliquots of venous blood were collected into lithium heparin tubes, stored on ice and spun within 90 minutes. Adrenaline and noradrenaline were later measured by radioenzymatic assay (Goldstein *et al.*, 1981) with both interassay and intra-assay variations of less than 10%.

2.3.4 OXYGEN BREATHING CIRCUIT

Study gases were generated by passing oxygen (G size cylinders 3400 litre capacity from British Oxygen Corporation, Medical Gases) and nitrogen (British Oxygen Corporation, Medical gases) contained in separate cylinders through a Quantiflex air/oxygen flowmixer (model A-O, Cyprane Ltd, Keighley, Yorkshire) which was connected via elephant tubing to a calibrated flow head and a five litre rebreathing bag. Elephant tubing then ran from the rebreathing bag to a two way breathing valve attached to an aircraft face mask (Thomas Respiratory Systems, London) from which the patient inspired the desired oxygen tension throughout the study day.

2.3.5 STATISTICAL ANALYSIS

For the *in vivo* studies, analysis of Variance (ANOVA) corrected for multiple comparisons was used to compare measurements made at baseline and following each dose of nebulised saline or agonist between study days.

2.3.6 DRUGS AND SOLUTIONS

The solutions of salbutamol, methacholine, histamine and saline were made up by the sterile unit of our pharmacy department, West Glasgow Hospitals University NHS Trust.

CHAPTER 3

THE EFFECT OF ACUTE CHANGES IN OXYGEN TENSION ON THE RESPONSIVENESS OF BOVINE ISOLATED BRONCHI TO CONTRACTILE AND RELAXATORY AGONISTS

3.1 INTRODUCTION

As stated in section 1.6, acute hypoxia impairs active tension generation in isolated airway preparations (Fernandes *et al.*, 1993). In contrast, hypoxia appears to enhance bronchial reactivity to spasmogens in animals *in vivo*. For example, bronchoconstrictor responses to agonists such as histamine in dogs (Vidruk & Sorkness, 1985) and carbachol and histamine in sheep (Ahmed & Marchette, 1985; D'Brot & Ahmed, 1988) are enhanced after acute exposure to hypoxia. Less is known, however, regarding the effect of acute hypoxia on airway reactivity *in vitro*.

In this study, we measured, in bovine isolated bronchial ring preparations, responses evoked by the standard bronchoconstrictor methacholine, a stable analogue of acetylcholine. Responses were measured in three different oxygen tensions, generated by perfusing the bathing medium with gas mixtures containing oxygen concentrations of 95% (hyperoxia), 20% (approximately normoxia) and 0% (hypoxia).

While most interest has concentrated upon the effect of oxygen tension on spasmogen-evoked responses in airway smooth muscle, the effect on bronchodilators has not received sufficient study. When investigating the effectiveness of a bronchodilator, two aspects of its performance are important: (1)the ability to relax tissue pre-constricted by a spasmogen and (2)the ability to protect against subsequent challenge with a bronchoconstrictor.

In this study, we compared the ability of four drugs to reverse methacholine-induced tone and also to confer protection against subsequent challenge with this agonist in hyperoxia, normoxia and hypoxia. The drugs used; salbutamol, atrial natriuretic peptide, sodium nitroprusside and isosorbide dinitrate were selected on the basis of their postulated mechanisms of action. Salbutamol, a $ß_2$-adrenoceptor agonist, is thought to act via stimulation of adenylyl cyclase leading to elevation of cAMP (Rinard *et al.*, 1983) as well as via K^+ channel opening (Miura *et al.*, 1992), while atrial natriuretic peptide evokes a rise in cyclic guanosine monophosphate (cGMP) via stimulation of particulate guanylyl cyclase (Ishii & Murad, 1989). Sodium nitroprusside and isosorbide dinitrate also induce an elevation in cGMP levels, in this case via stimulation of soluble guanylyl cyclase (Ignarro & Kadowitz, 1985; Feelisch & Novak, 1987).

We therefore sought to examine if changes in oxygen tension altered the effectiveness of the drugs used in this study and if each of the drugs was affected to a similar degree.

3.2 METHODS

3.2.1 Tissue Collection And Preparation

Rings of bovine bronchi were prepared and mounted in vertical organ baths as described in section 2.1.1.

3.2.2 Measurement Of Isometric Responses

Initial experiments (see section 3.3.1) indicated that the optimal level of applied tension in this tissue was 2g wt. In all subsequent experiments using bovine bronchial rings, therefore, a resting tension of 2g wt was applied. Tissues were allowed to equilibrate for 45 minutes, during which time tension was reapplied where necessary. The concentration of O_2 in the gas mixture was reduced from 95% to 20% or 0% by substituting O_2 with nitrogen. The oxygen tension of the solution was measured directly using a Jencons oxygen probe placed in the organ bath. Concentrations of O_2 reached steady state in the organ baths within 2-3 minutes. Gas mixtures containing 95%, 20% and 0% O_2, produced O_2 tensions of 524, 147 and 26 mm Hg, respectively. The pH was buffered to 7.4 by the inclusion of 5% CO_2. A Mettler pH meter was used to measure the pH of the solution in the organ baths. Lowering the oxygen tension from 95% to either 20 or 0% evoked a marked reduction in applied tension. Tension was reapplied until a stable baseline of approximately 2.0g wt was achieved.

3.2.3 Experimental protocol

3.2.3.1 Optimisation of applied resting tension

Under hyperoxic conditions, an initial resting tension of 0.5g wt was applied to each bronchial ring. Tissues were allowed to equilibrate for 15 minutes before addition of a single concentration of methacholine (10^{-4}M). This concentration was chosen as it evoked a near maximal contractile response. When the contraction had reached a plateau, the tissues were washed three times with fresh Krebs-Henseleit solution over a 15 min period, or until the tension returned to baseline. Tension was then removed before increasing the degree of initial tension to the next increment (1.0g wt).

3.2.3.2 Contractile responses to methacholine

Cumulative concentration-response curves to methacholine (10^{-9}-$3x10^{-4}$M) were constructed in each of the O_2 tensions. Results are expressed both as a % of the maximum response in 95% O_2 and also in absolute terms (mg wt).

3.2.3.3 Reversal of methacholine-induced tone

To study the effect of O_2 tension on responses evoked by the bronchodilators, tissues were preconstricted with methacholine ($3x10^{-6}M$, approximately the EC_{50} for methacholine in this tissue). In separate tissues, cumulative-concentration response curves were then constructed to salbutamol (10^{-8}-$3x10^{-6}M$), atrial natriuretic peptide (10^{-9}-$10^{-6}M$), sodium nitroprusside (10^{-8}-$10^{-5}M$) and isosorbide dinitrate (10^{-7}-$3x10^{-5}M$). Results are expressed in terms of the mean maximal inhibition. Whenever atrial natriuretic peptide was used, the neutral endopeptidase inhibitor, phosphoramidon ($3.7x10^{-5}M$), was included to prevent its rapid breakdown (Angus *et al.*, 1994a). Experiments were also conducted to ascertain if phosphoramidon altered methacholine-induced tone. In each case, one tissue acted as a time control to establish that the methacholine contraction was sustained.

3.2.3.4 Protection against subsequent methacholine challenge

Cumulative concentration-response curves to methacholine (10^{-9}-$3x10^{-4}M$) were constructed in each of the O_2 tensions, first in the absence then the presence of each dilator. Three concentrations of each dilator were used; ANP (10^{-7}, $3x10^{-7}$ and $10^{-6}M$), salbutamol (10^{-7}, $3x10^{-7}$ and $10^{-6}M$), SNP (10^{-6}, $3x10^{-6}$ and 10^{-5}) and ISDN (10^{-5}, $3x10^{-5}$ and $10^{-4}M$). These concentrations were based on the ability of each drug to reverse methacholine-induced tone in the experiments mentioned above, in which the approximate IC_{50} (the concentration which evoked 50% of the mean maximal inhibition) for each drug was: ANP; $10^{-7}M$, salbutamol; $10^{-7}M$, SNP; $10^{-6}M$ and ISDN; $10^{-5}M$.

As stated earlier, phosphoramidon ($3.7x10^{-5}M$) was included to prevent the rapid breakdown of ANP by neutral endopeptidases. Experiments were also conducted to ascertain if phosphoramidon had any effect on methacholine-induced responses. In each experiment, one tissue acted as a time control to establish that the methacholine contractions did not vary with time. Results are expressed as a % of the maximum response in the initial concentration-response curve to methacholine.

The sensitivity of the tissue to methacholine is expressed in terms of the pD_2 (the negative log of the concentration of methacholine which evokes 50% of the maximum response).

3.2.4 Materials

The following chemicals were used; atrial natriuretic peptide, isosorbide dinitrate, methacholine, salbutamol and sodium nitroprusside. See section 2.1.8 for a list of full chemical and suppliers names.

3.2.5 Analysis of Results

Number of observations (n) refers to the number of individual animals from which tissue was obtained (n=6 in each case, unless otherwise stated). Statistical analysis was performed using the Minitab package. Two-way analysis of variance was used for the different data sets generated by concentration response curves. Significance between data points was calculated by Student's *t* test. Results are expressed as a percentage ± standard error of the mean (s.e.m.). A probability of $P<0.05$ was considered to be statistically significant.

3.3 RESULTS

3.3.1 Optimisation Of Initial Resting Tension

Changing the degree of applied (passive) tension significantly altered the contractile (active) response evoked by a near maximal concentration of methacholine (10^{-4}M). The measured contractile response increased as the applied tension was increased from 0.5g wt to 2.0g wt, reaching a maximum contraction at 2.0g wt. When the applied tension was increased beyond 2.0g wt, there was no significant change in the contractile response to 10^{-4}M methacholine. The maximum response to methacholine, at a resting tension of 2.0g wt, 2561.6±435.9mg wt, was significantly greater ($P<0.05$ for data points) than the response to methacholine at a resting tension of 1.5g wt, 1728.6±242.8 mg wt. Number of observations (n) =6 in each case.

Figure 3.1

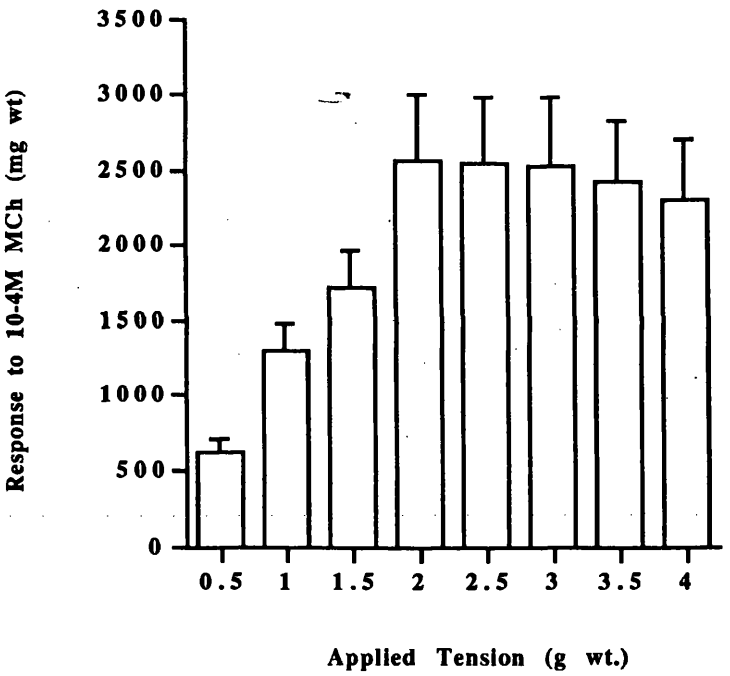


Figure 3.1

The effect of changing the degree of initial resting tension on contractile responses. Contractions evoked by a single concentration (10^{-4}M) of methacholine were measured in bovine bronchial rings in hyperoxia. Responses are expressed in milligrams weight (mg wt). Contractile responses were significantly ($P<0.05$ for data points) greater at a resting tension of 2.0g wt than at a resting tension of 1.5 g wt. Number of observations (n) = 6 in each case.

responses to methacholine were significantly altered throughout the concentration-response curve, changes in O_2 tension did not alter the sensitivity of the tissue to methacholine (methacholine pD_2 values: in 95%; 5.11±0.10, n=8, in 20%; 5.17±0.09, n=8 and in 0%; 5.24±0.12, n=8).

Expressing the results as either % of the maximum response in hyperoxia (Figure 3.3A), or in mg wt (Figure 3.3B) did not alter the pattern of responses. Furthermore, expressing the results in mg wt did not significantly alter the estimations of methacholine pD_2 values (methacholine pD_2 values from results expressed in absolute terms: in 95%; 5.34±0.21, n=8, in 20%; 5.43±0.32, n=8 and in 0%; 5.49±0.36, n=8).

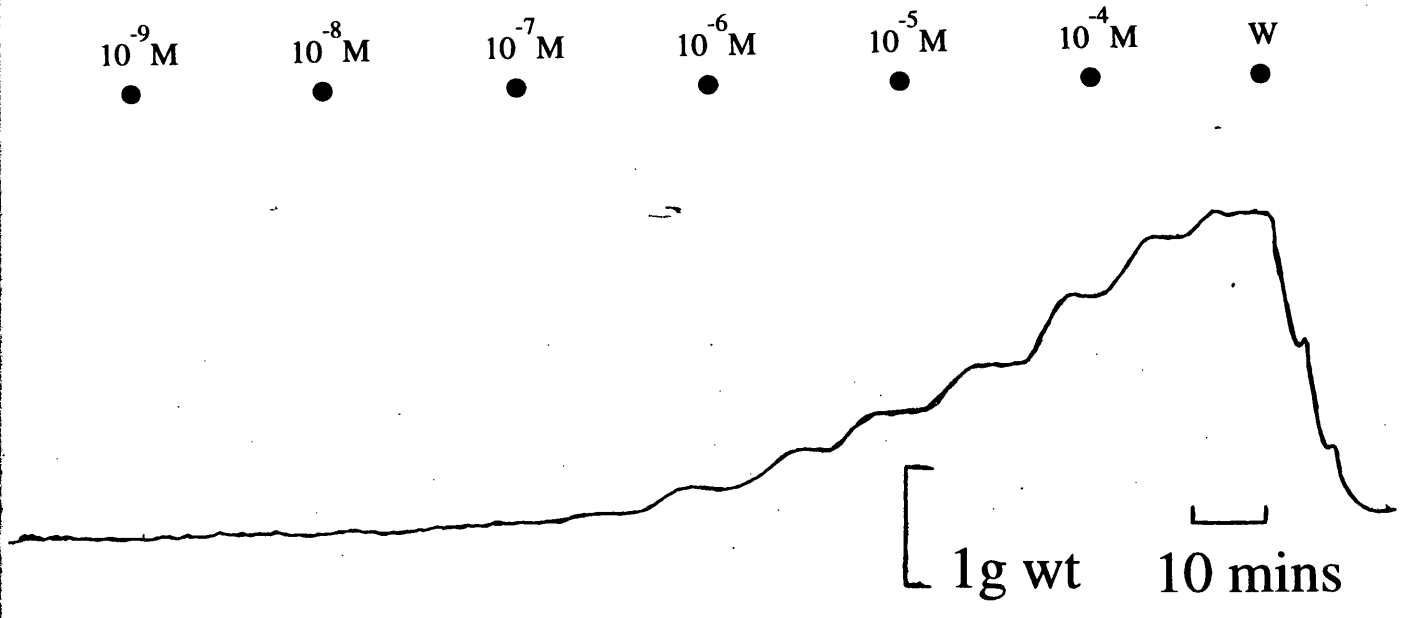


Figure 3.2

Representative trace depicting contractile responses evoked by methacholine in bovine bronchial rings. Methacholine was added cumulatively to give final bath concentrations of 10^{-9}M - $3x10^{-4}$M. Consecutive concentrations were added after the previous response had reached a plateau.

Figure 3.3

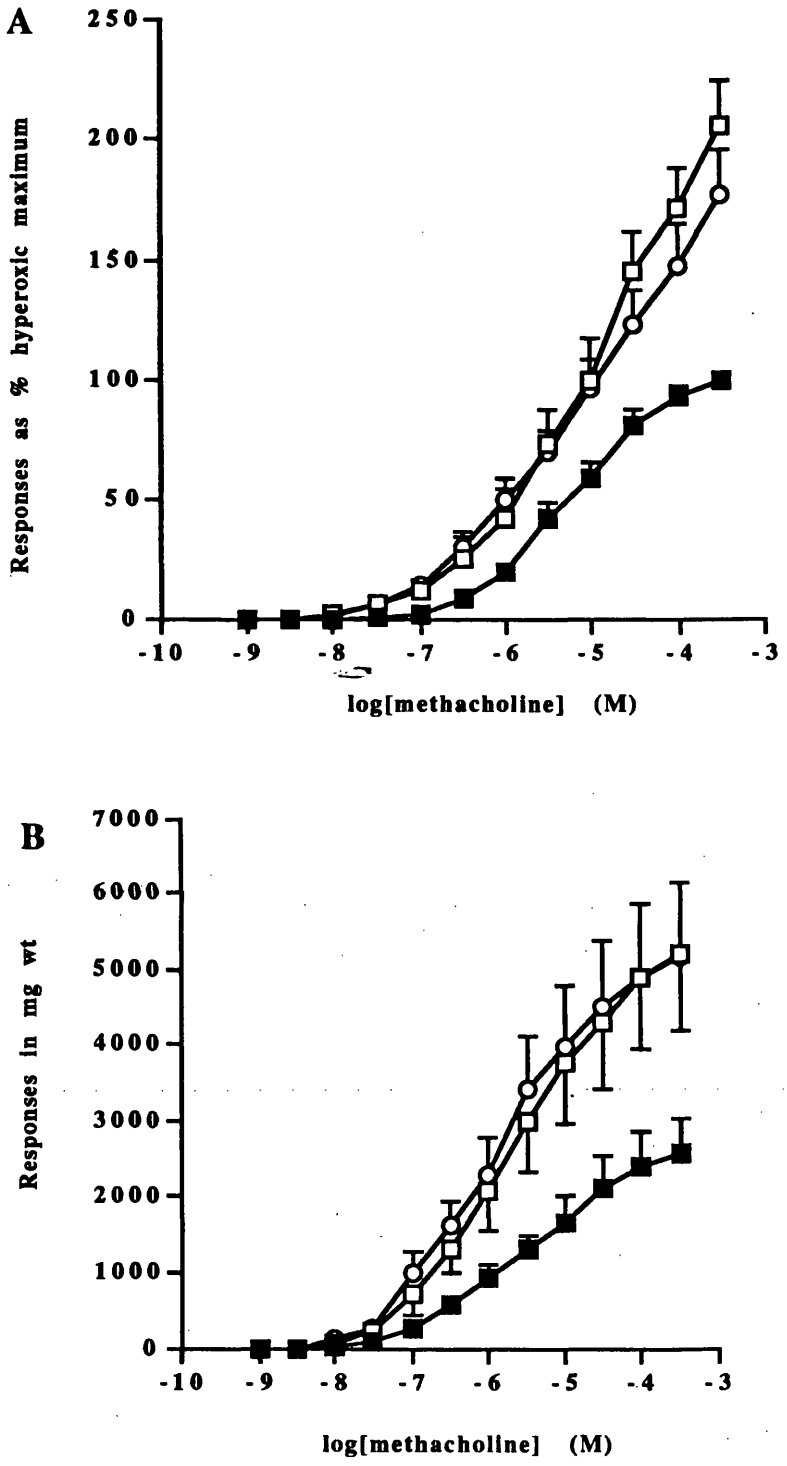


Figure 3.3
Cumulative concentration response curves to methacholine in bovine bronchi in hyperoxia (■), normoxia (□) and hypoxia (○). Responses are expressed as (A) % of the maximum response in hyperoxia and (B) in mg wt. Both normoxia and hypoxia significantly ($P<0.001$) potentiate methacholine responses compared to hyperoxia. There was no significant difference between responses in normoxia and hypoxia. Expressing the results as either % of the maximum response in hyperoxia or in absolute terms did not significantly alter the estimation of EC_{50} values. Number of observations (n) = 8 in each case

3.3.3 Reversal Of Methacholine-Induced Tone

Responses to the single concentration ($3x10^{-6}M$) of methacholine were dependent upon the O_2 tension used (Figure 3.4): in 95% O_2, a contraction of 1.20±0.16g wt was produced, whereas in 20 and 0%, the responses were significantly ($P<0.001$ for data set, n=12) greater (2.37±0.31 and 2.19±0.31g wt, respectively). Again, there was no difference between responses in 20 and 0% O_2.

Figure 3.4

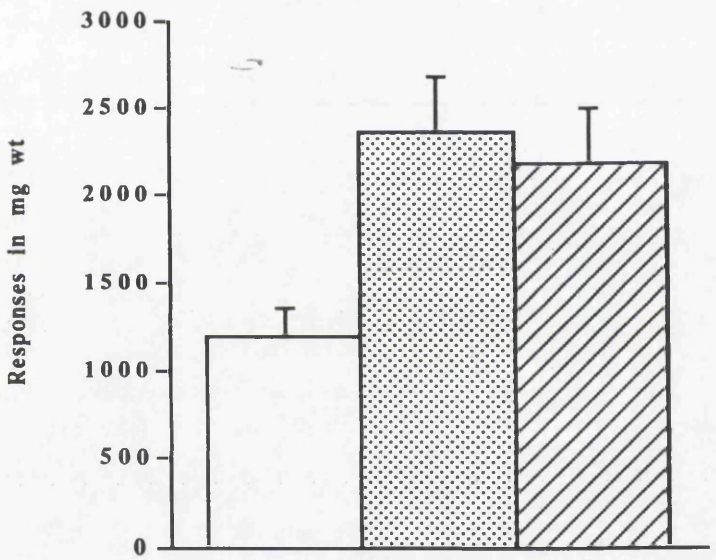


Figure 3.4

Responses evoked by a single concentration ($3x10^{-6}M$) of methacholine in bovine bronchi in hyperoxia (open column), normoxia (stippled column) and hypoxia (striped column). Responses are expressed in milligrams weight (mg wt). Both normoxia and hypoxia significantly ($P<0.001$) potentiate methacholine responses compared to hyperoxia. Number of observations (n) = 12 in each case.

Figure 3.5

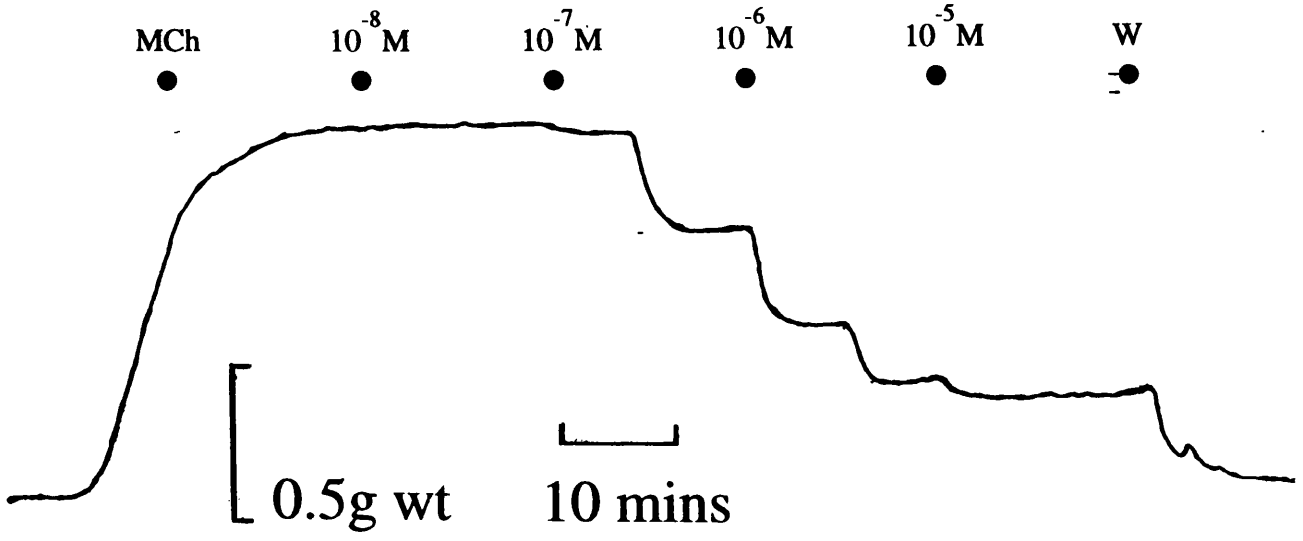


Figure 3.5

Representative trace depicting relaxtory responses evoked by salbutamol in bovine bronchial rings. After a stable contraction to methacholine was achieved, salbutamol was added cumulatively to give final bath concentrations of 10^{-8}M - $3x10^{-5}$M. Consecutive concentrations were added after the previous response had reached a plateau.

The ability of the drugs to relax preconstricted tissue was dependent upon the oxygen tension used (Table 3.1). Salbutamol was more effective in normoxia than hypoxia, whereas the other dilators were either equipotent (isosorbide dinitrate) or more effective (atrial natriuretic peptide) in 0% than 20% O_2. In contrast, sodium nitroprusside was more effective in hyperoxia than normoxia.

For salbutamol (10^{-8}-$3x10^{-6}$M) (Figure 3.4), there was no difference between responses in 95 and 20% O_2 (mean maximal inhibitions, at the $3x10^{-6}$M level; 77.09± 11.47% and 98.01± 7.31%, respectively, n=6), or 95 and 0% O_2 (mean maximal inhibitions, at the $3x10^{-6}$M level; 77.09± 11.47% and 70.87± 7.31%, respectively), however, salbutamol was significantly $P<0.001$ for data set, n=6) more effective at reversing tone in 20% than in 0% O_2 (mean maximal inhibitions, at the $3x10^{-6}$M level; 98.01± 7.31% and 70.87± 7.31%, respectively, $P<0.05$ for the data points).

Atrial natriuretic peptide (10^{-9}-10^{-6}M) showed a different pattern of results from salbutamol (Figure 3.5). There was no difference between responses in 95 and 20% O_2 (mean maximal inhibitions, at the 10^{-6}M level; 36.08± 12.38% and 33.03± 7.59%, respectively, n=6). In this case, however, responses in 0% were significantly ($P<0.05$ for data set, n=6) enhanced compared to 20% O_2, albeit with no change in the mean maximum inhibition (mean maximal inhibition, at the 10^{-6}M level; 50.30± 8.50%).

Figure 3.6

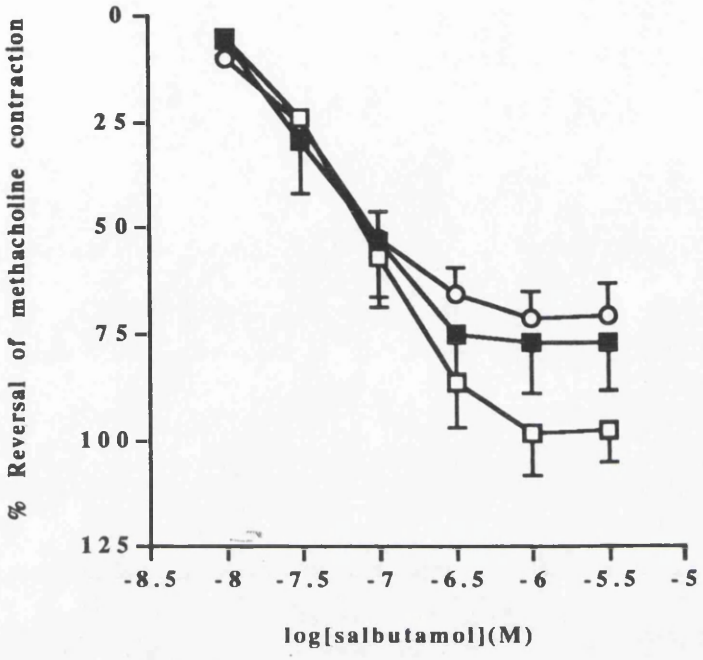


Figure 3.6

The ability of salbutamol to reverse methacholine induced tone in bovine bronchi in 95% (■), 20% (❑) and 0% O_2 (○). Salbutamol was added cumulatively to give final bath concentrations of (10^{-8}-3x10^{-6}M). Responses are expressed as a % reversal of the methacholine contraction. Salbutamol was significantly ($P<0.001$) more effective in 20% O_2 than in 0%, however there was no difference between responses in 95 and 20%, or between 95 and 0%. Number of observations (n) = 6 in each case.

Figure 3.7

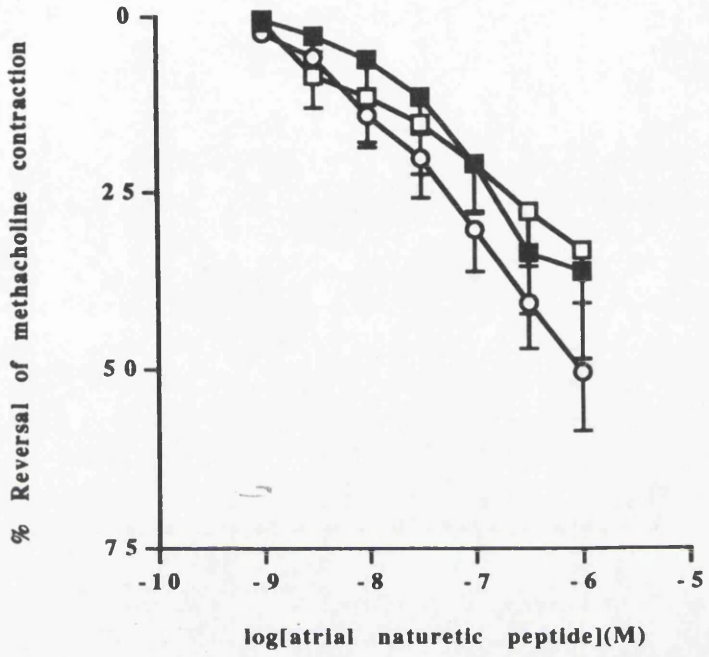


Figure 3.7

The ability of atrial natriuretic peptide (ANP) to reverse methacholine induced tone in bovine bronchi in 95% (■), 20% (❑) and 0% O_2 (○). ANP was added cumulatively to give final bath concentrations of (10^{-9}-10^{-6}M). Responses are expressed as a % reversal of the methacholine contraction. ANP was significantly ($P<0.05$) more effective in 0% O_2 when compared with both 95 and 20%. All experiments involving atrial natriuretic peptide were conducted in the presence of phosphoramidon to prevent rapid hydrolysis by neutral endopeptidases. Number of observations (n) = 6 in each case.

For sodium nitroprusside (Figure 3.8), responses throughout the whole curve (10^{-8}-10^{-5}M) were significantly (P<0.01 for data set) greater in 95% compared to 20% O_2, although there was no significant difference between the maximum responses (mean maximal inhibitions, at the 10^{-5}M level; 83.74±11.21% and 77.77±9.27%, in 95 and 20%, respectively). Responses in hypoxia (mean maximal inhibition; 90.19± 11.51%) were not significantly different from those in either 95 or 20%.

Figure 3.8

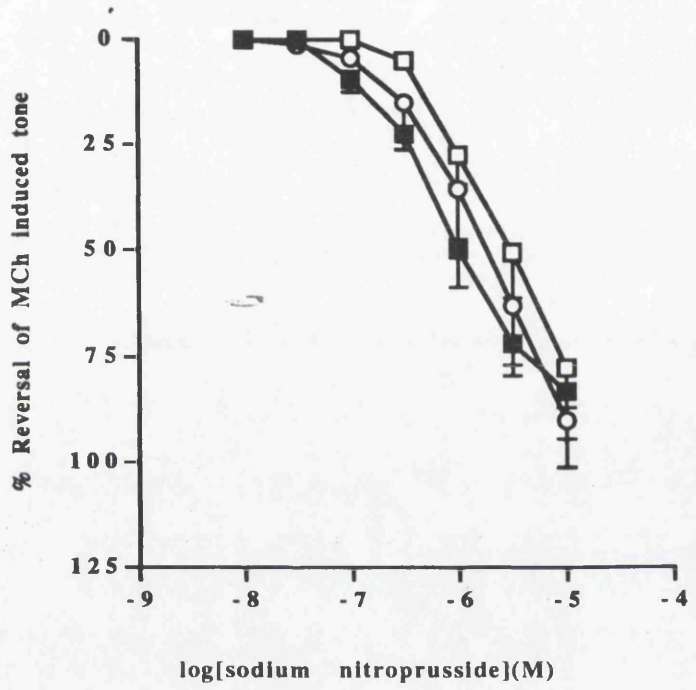


Figure 3.8

The ability of sodium nitroprusside to reverse methacholine induced tone in bovine bronchi in 95% (■), 20% (❑) and 0% O_2 (○). SNP was added cumulatively to give final bath concentrations of (10^{-8}-10^{-5}M). Responses are expressed as a % reversal of the methacholine contraction. Sodium nitroprusside was significantly (P<0.01) more effective in 95% O_2 than 20% O_2. Responses in 0% O_2 were not significantly different than either 95% or 20% O_2. Number of observations (n) = 6 in each case.

Isosorbide dinitrate (10^{-7}-$3x10^{-5}$M) (Figure 3.9), evoked significantly greater relaxations in 20% O_2 than 95% ($P<0.01$ for data set, n=6, mean maximal inhibition at the $3x10^{-5}$M level; 64.51±10.08% and 33.42±5.90%, respectively, $P<0.05$ for data points). Responses to isosorbide in 0% O_2 were also significantly enhanced ($P<0.001$ for data set, n=6, mean maximal inhibition at the $3x10^{-5}$M level; 80.37±5.32%, $P<0.001$ for data points) compared to 95% O_2, although there was no significant difference between responses in 20% and 0% O_2.

Figure 3.9

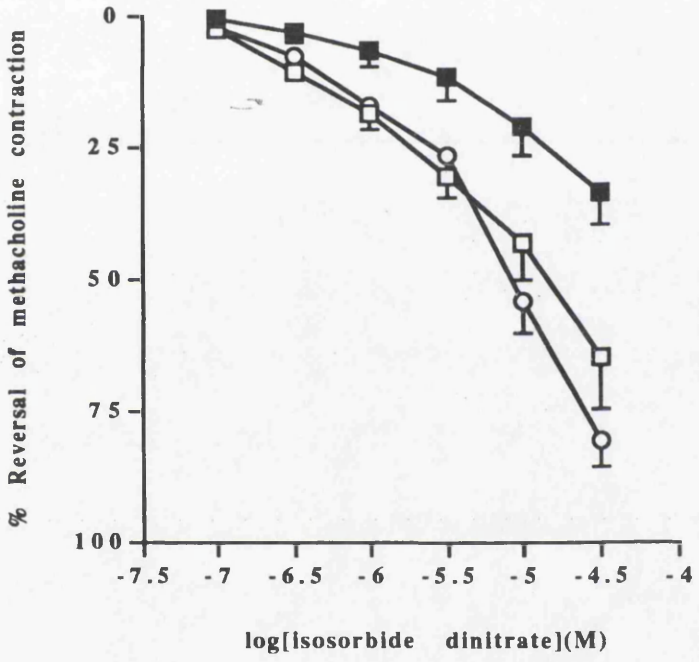


Figure 3.9

The ability of isosorbide dinitrate to reverse methacholine induced tone in bovine bronchi in 95% (■), 20% (❑) and 0% O_2 (○). Isosorbide was added cumulatively to give final bath concentrations of (10^{-7}-$3x10^{-5}$M). Responses are expressed as a % reversal of the methacholine contraction. Isosorbide was significantly more effective in 0% and 20% O_2 ($P<0.001$ and $P<0.01$, respectively) than in 95%. Number of observations (n) = 6 in each case.

Table 3.1: Reversal of methacholine-induced contraction

Bronchodilator	Oxygen tension	Maximum (±s.e.m)	2-way ANOVA
Salbutamol (10^{-8}-3x10^{-6}M)	95%	77.09 (11.47)	
	20%	98.01 (7.31)†	
	0%	70.87 (7.31)	20% > 0% (p<0.001)
ANP (10^{-9}-10^{-6}M)	95%	36.08 (12.38)	
	20%	33.03 (7.59)	
	0%	50.30 (8.50)	0% > 95%, 20% (p<0.05)
SNP (10^{-8}-10^{-5}M)	95%	83.7 (11.2)	95% > 20% (p<0.01)
	20%	77.8 (9.3)	
	0%	90.2 (11.5)	
ISDN (10^{-7}-3x10^{-5}M)	95%	33.42 (5.90)	
	20%	64.51 (10.08)*	20% > 95% (p<0.01)
	0%	80.37 (5.32)***	0% > 95% (p<0.001)

Table 3.1 displays the mean maximum reversal of methacholine-induced tone for each bronchodilator in the three oxygen tensions: 95, 20 and 0%. Values are expressed as the mean (± s.e.m.) % reversal of the induced tone. Statistical significance between mean maximum responses was determined using Student's t-test, while differences between the data sets from each concentration response curve were calculated using 2-way analysis of variance (ANOVA). The concentration of methacholine used was $3x10^{-6}M$. Number of observations (n) = 6 in each case. Abbreviations: ANP, atrial natriuretic peptide, SNP, sodium nitroprusside, ISDN, isosorbide dinitrate. Statistical significance for mean maximal inhibition: † $P<0.05$ for salbutamol in 20% compared with 0% O_2, * $P<0.05$ for isosorbide dinitrate in 20% compared with 95% O_2 and *** $P<0.001$ for isosorbide dinitrate in 0% compared with 95% O_2.

3.3.3 Protection Against Subsequent Challenge With Methacholine

Contractile responses to methacholine

Methacholine evoked concentration-dependent contractions in the bovine isolated bronchial rings, with the threshold concentration for contraction being between $3x10^{-8}M$ and $3x10^{-7}M$. In each of the experiments, there was no difference between two consecutive concentration-response curves to methacholine alone (data not shown), indicating that methacholine-induced responses did not alter with time.

As stated in section 3.3.1, we have shown that lowering the oxygen tension from hyperoxia to normoxia or hypoxia significantly enhances methacholine-induced contractions in bovine bronchial rings. Our results from these present experiments are in agreement with this, and again, changing the oxygen tension did not alter the sensitivity of the tissue to methacholine (methacholine pD_2 values: in 524mm Hg; 5.04±0.09, n=72, in 147mm Hg; 5.15±0.07, n=72 and in 26mm Hg; 5.18±0.09, n=72). There was no difference between two consecutive methacholine concentration-response curves conducted in the same oxygen tension, indicating that any change between the two curves was due to the effect of the bronchodilator.

Atrial natriuretic peptide

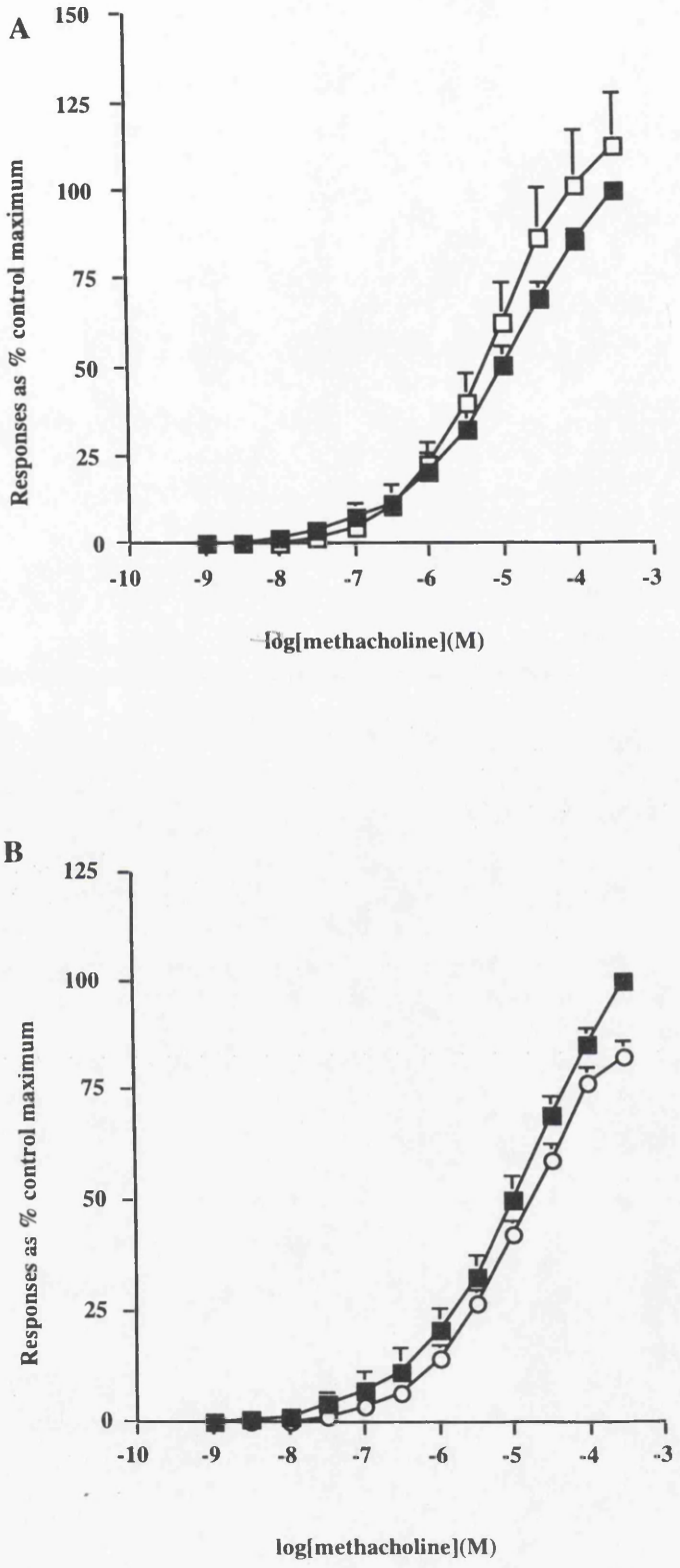
In hyperoxia, pre-incubation of ANP, in the presence of phosphoramidon, attenuated methacholine-induced responses in a concentration-dependent manner. At a concentration of $10^{-7}M$, ANP did not alter the contractions evoked by methacholine (methacholine pD_2 values; control for ANP $10^{-7}M$; 5.01±0.09, n=6, methacholine plus ANP $10^{-7}M$; 5.14±0.12, Figure 3.10A). Pre-incubation of ANP at concentrations of $3x10^{-7}M$ and $10^{-6}M$ (Figure 3.10 B and C, respectively) significantly ($P<0.05$ and $P<0.001$ for data sets, respectively) attenuated the methacholine concentration-response curve compared to control responses. This attenuation was not manifested as a change in the sensitivity of the tissue (methacholine pD_2 values; control for ANP $3x10^{-7}M$; 5.11±0.11, n=6, plus ANP $3x10^{-7}M$; 4.94±0.14. Control for ANP $10^{-6}M$; 5.39±0.12, n=6, plus ANP $10^{-6}M$; 5.20±0.12. In each case $P>0.05$ for data points, compared to control.).

The ability of higher concentrations ($3x10^{-7}M$ and $10^{-6}M$) of ANP to protect against challenge with methacholine in 95% O_2 was lost when the oxygen tension in the gas mixture was reduced to 20%. At concentrations of $10^{-7}M$, $3x10^{-7}M$ and $10^{-6}M$, ANP did not alter subsequent responses to methacholine (methacholine pD_2 values; control for ANP $10^{-7}M$; 5.20±0.18, methacholine plus ANP $10^{-7}M$; 5.25±0.16, control for ANP $3x10^{-7}M$; 5.17±0.12, methacholine plus ANP $3x10^{-7}M$; 5.07±0.12 and control for ANP $10^{-6}M$; 4.87±0.14, methacholine plus ANP $10^{-6}M$; 4.94±0.16 (Figures 3.11 A, B and C, respectively). In each case there was no significant ($P>0.05$) difference between corresponding data points and controls).

Under hypoxic conditions, ANP actually enhanced responses to methacholine in a concentration-dependent manner. At a concentration of 10^{-7}M, pre-incubation of ANP did not alter contractions evoked by methacholine (methacholine pD_2 values; control for ANP 10^{-7}M; 5.08±0.12, n=6, methacholine plus ANP 10^{-7}M; 5.18±0.08, Figure 3.12A). After pre-incubation of either $3x10^{-7}$M or 10^{-6}M ANP, the methacholine concentration-response curve was significantly ($P<0.01$ and $P<0.001$, respectively) enhanced compared to control responses, albeit with no change in the sensitivity of the tissue (methacholine pD_2 values; control for ANP $3x10^{-7}$M; 5.12±0.12, n=6, methacholine plus ANP $3x10^{-7}$M; 5.10±0.11. Control for ANP 10^{-6}M; 5.01±0.13, n=6, methacholine plus ANP 10^{-6}M; 4.85±0.15 (Figure 3.12B and C, respectively). In each case there was no significant ($P>0.05$) difference between corresponding data points and controls).

Phosphoramidon ($3.7x10^{-5}$M) alone did not alter contractile responses to methacholine in any of the oxygen tensions studied (results not shown).

Figure 3.10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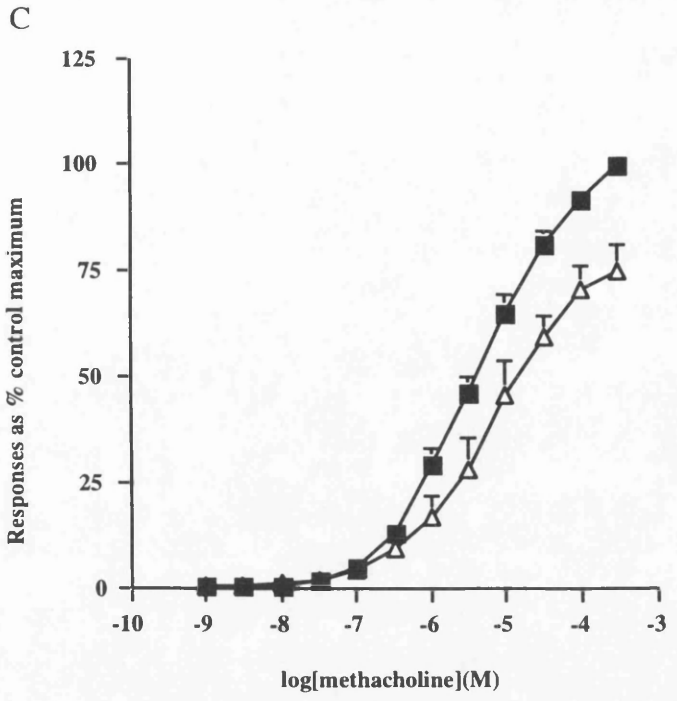
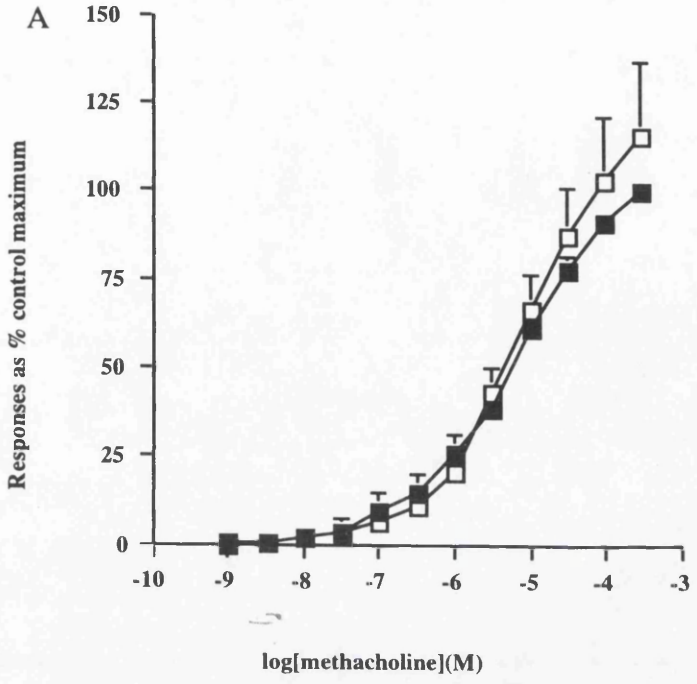


Figure 3.10

In hyperoxia (95% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of atrial natriuretic peptide (ANP) at concentrations of 10^{-7}M (A), $3x10^{-7}$M (B) and 10^{-6}M (C). When ANP was used phosphoramidon was pre-incubated to prevent its degradation. Responses are expressed as a % of the control maximum response. When pre-incubated at concentrations of $3x10^{-7}$M and 10^{-6}M, ANP significantly ($P<0.05$ and $P<0.001$, respectively) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.11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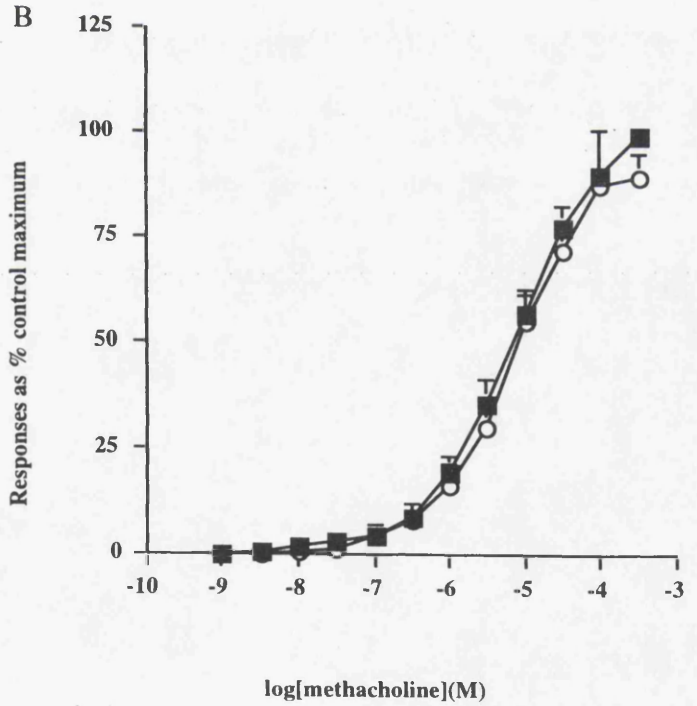
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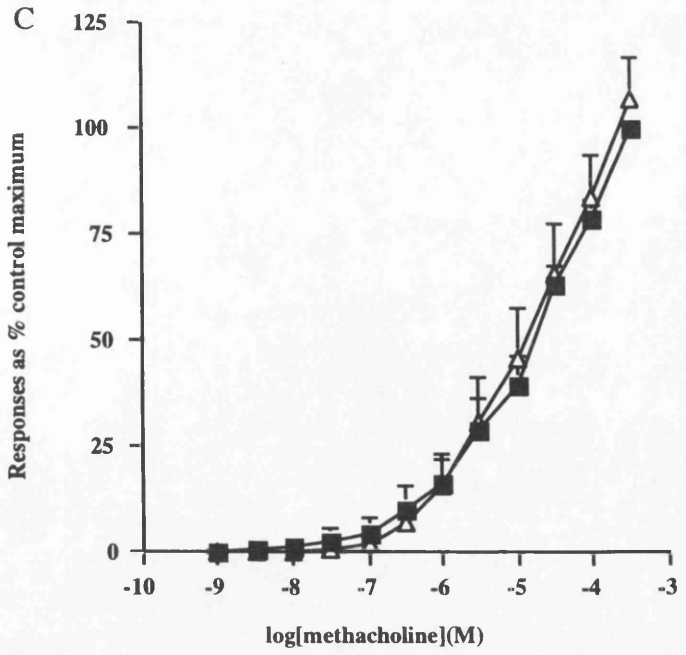
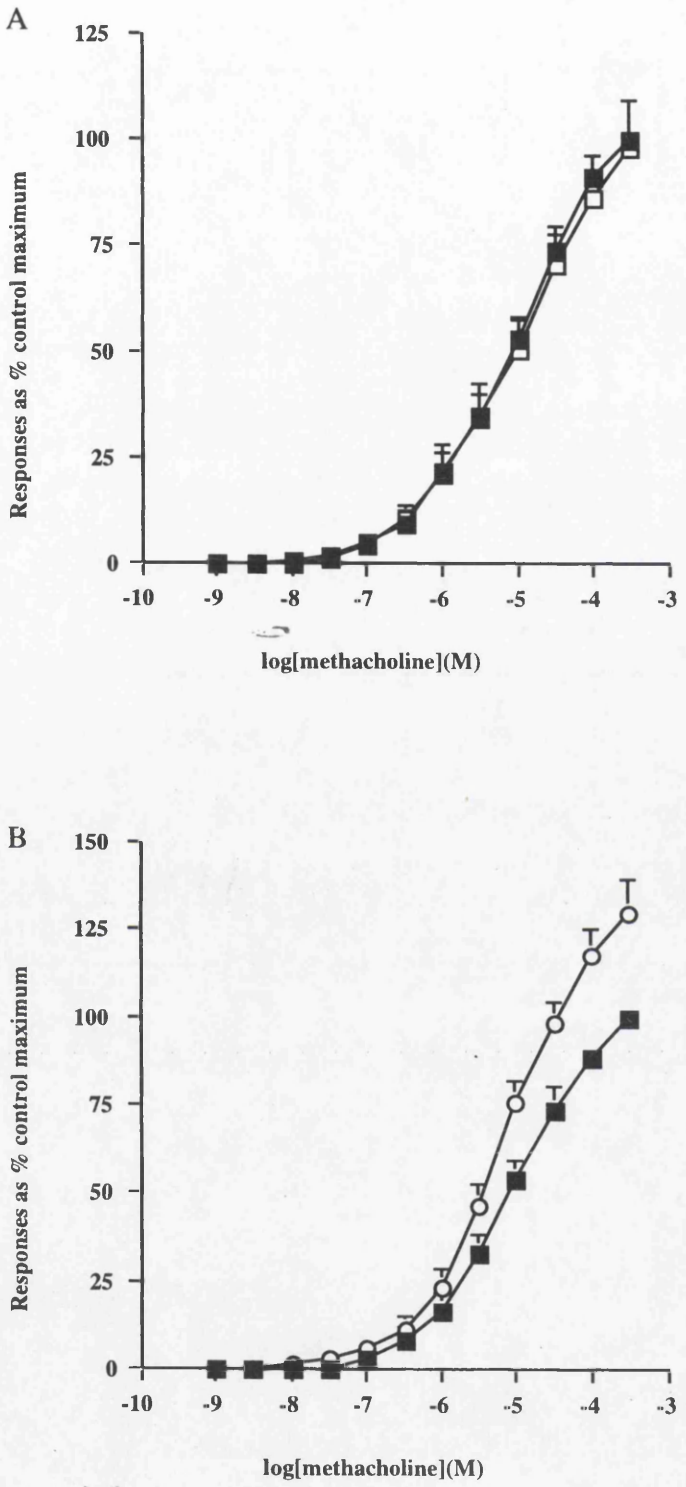


Figure 3.11

In normoxia (20% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of atrial natriuretic peptide (ANP) at concentrations of 10^{-7}M (A), $3x10^{-7}$M (B) and 10^{-6}M (C). When ANP was used phosphoramidon was pre-incubated to prevent its degradation. Responses are expressed as a % of the control maximum response. Pre-incubation of ANP had no effect on methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.12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log[methacholine](M)
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Responses as % control maximum
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log[methacholine](M)

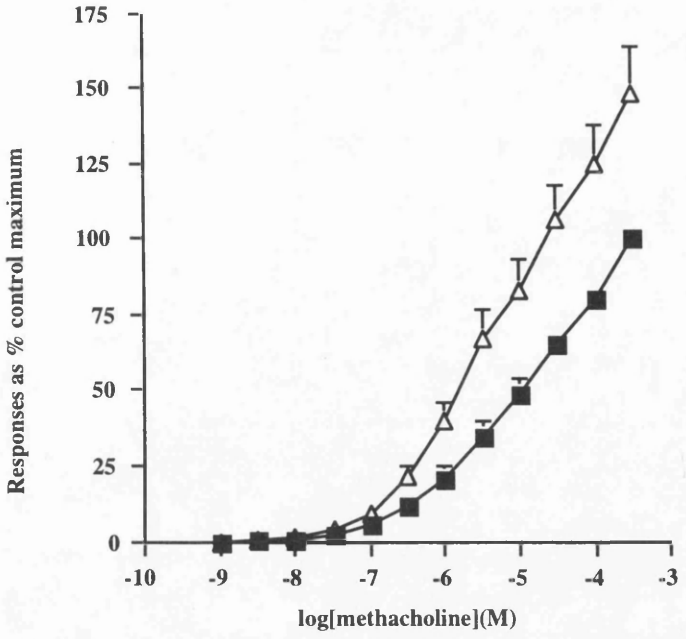


Figure 3.12

In hypoxia (0% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of atrial natriuretic peptide (ANP) at concentrations of 10^{-7}M (A), $3x10^{-7}$M (B) and 10^{-6}M (C). When ANP was used phosphoramidon was pre-incubated to prevent its degradation. Responses are expressed as a % of the control maximum response. When pre-incubated at concentrations of $3x10^{-7}$M and 10^{-6}M, ANP significantly ($P<0.01$ and $P<0.001$, respectively) enhanced methacholine-induced contractions. Number of observations (n) = 6 in each case.

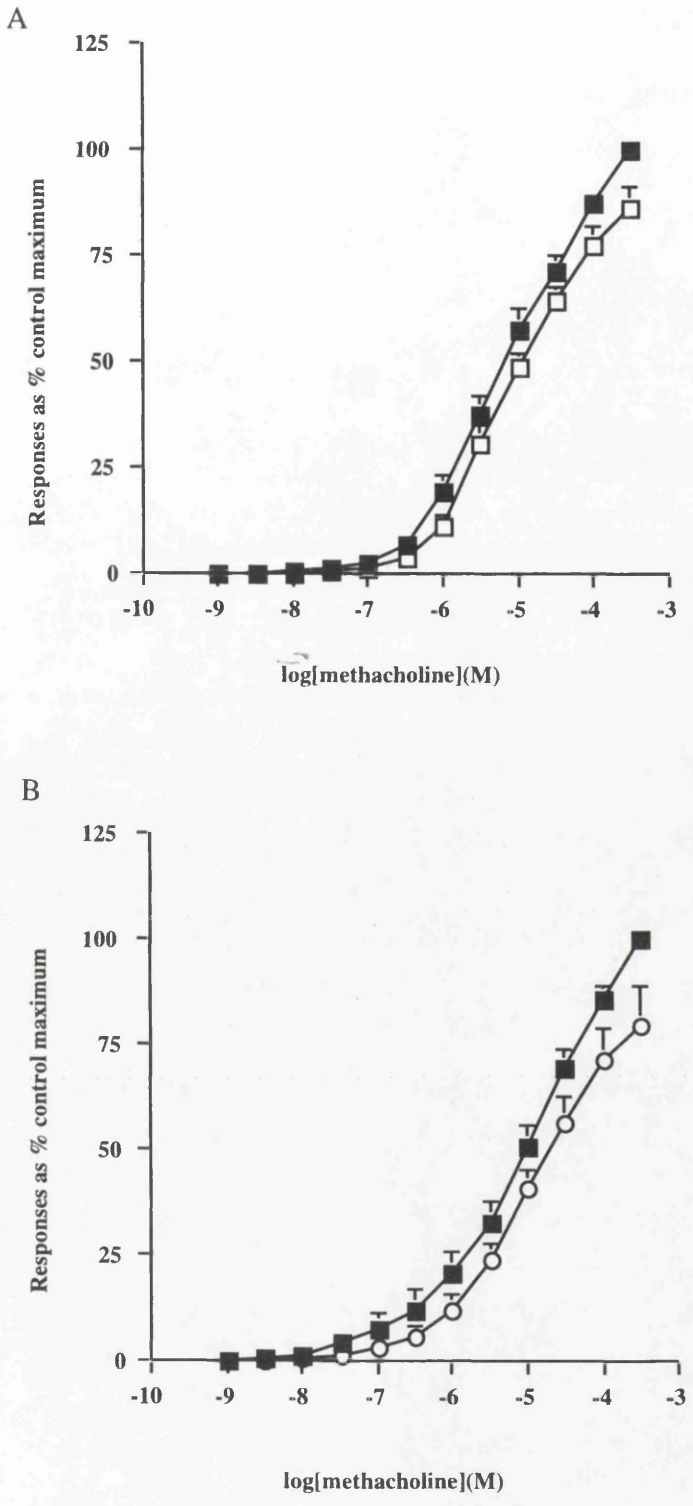
Salbutamol

In hyperoxia, salbutamol produced a similar pattern of responses to ANP, namely a concentration-dependent inhibition of the methacholine response. At a concentration of 10^{-7}M, salbutamol did not alter the contractions evoked by methacholine (methacholine pD_2 values; control for salbutamol 10^{-7}M; 5.21±0.09, n=6, methacholine plus salbutamol 10^{-7}M; 5.18±0.13, Figure 3.13A). After pre-incubation of either $3x10^{-7}$M or 10^{-6}M salbutamol, the methacholine concentration-response curve was significantly ($P<0.05$ and $P<0.001$ for data sets, respectively) attenuated compared to control responses. As was the case with ANP, this attenuation was not manifested as a change in the sensitivity of the tissue (methacholine pD_2 values; control for salbutamol $3x10^{-7}$M; 5.01±0.10, n=6, plus salbutamol $3x10^{-7}$M; 4.81±0.13. Control for salbutamol 10^{-6}M; 5.37±0.15, n=6, plus salbutamol 10^{-6}M; 5.01±0.18 (Figure 3.13B and C, respectively). In each case there was no significant ($P>0.05$) difference between corresponding data points and controls.).

As was the case with ANP, when the oxygen tension was reduced to normoxic levels, salbutamol did not confer protection against subsequent challenge with methacholine (methacholine pD_2 values; control for salbutamol 10^{-7}M; 5.20±0.15, methacholine plus salbutamol 10^{-7}M; 5.02±0.17, control for salbutamol $3x10^{-7}$M; 5.17±0.12, methacholine plus salbutamol $3x10^{-7}$M; 5.02±0.12 and control for salbutamol 10^{-6}M; 4.96±0.11, methacholine plus salbutamol 10^{-6}M; 4.80±0.16 (Figure 3.14). In each case there was no significant ($P>0.05$) difference between corresponding data points and controls).

Therefore, like ANP, salbutamol conferred protection against methacholine challenge in hyperoxia but did not alter responses in normoxia. In hypoxia, pre-incubation of salbutamol at concentrations of 10^{-7}M, $3x10^{-7}$M and 10^{-6}M did not alter contractions evoked by subsequent addition of methacholine (methacholine pD_2 values; control for salbutamol 10^{-7}M; 5.08±0.09, methacholine plus salbutamol 10^{-7}M; 4.98±0.10, control for salbutamol $3x10^{-7}$M; 5.20±0.13, methacholine plus salbutamol $3x10^{-7}$M; 5.12±0.19 and control for salbutamol 10^{-6}M; 5.08±0.09, methacholine plus salbutamol 10^{-6}M; 4.95±0.15 (Figure 3.15 A, B and C, respectively). In each case there was no significant ($P>0.05$) difference between corresponding data points and controls).

Figure 3.13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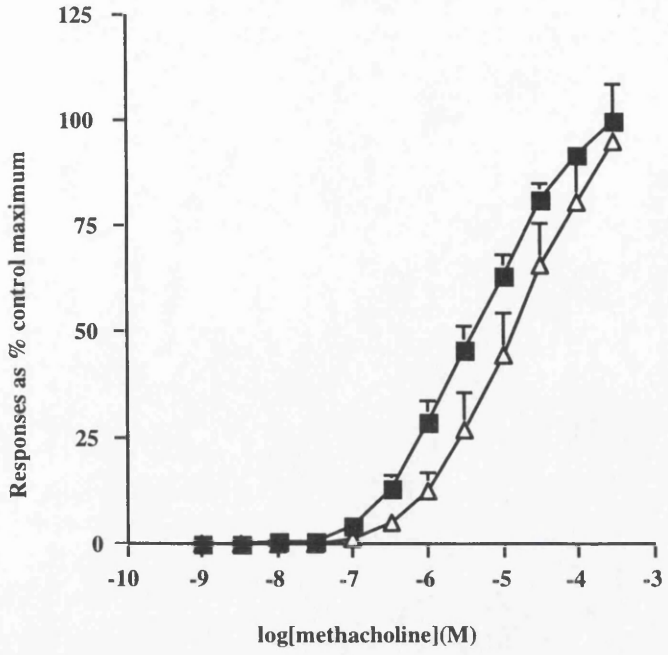
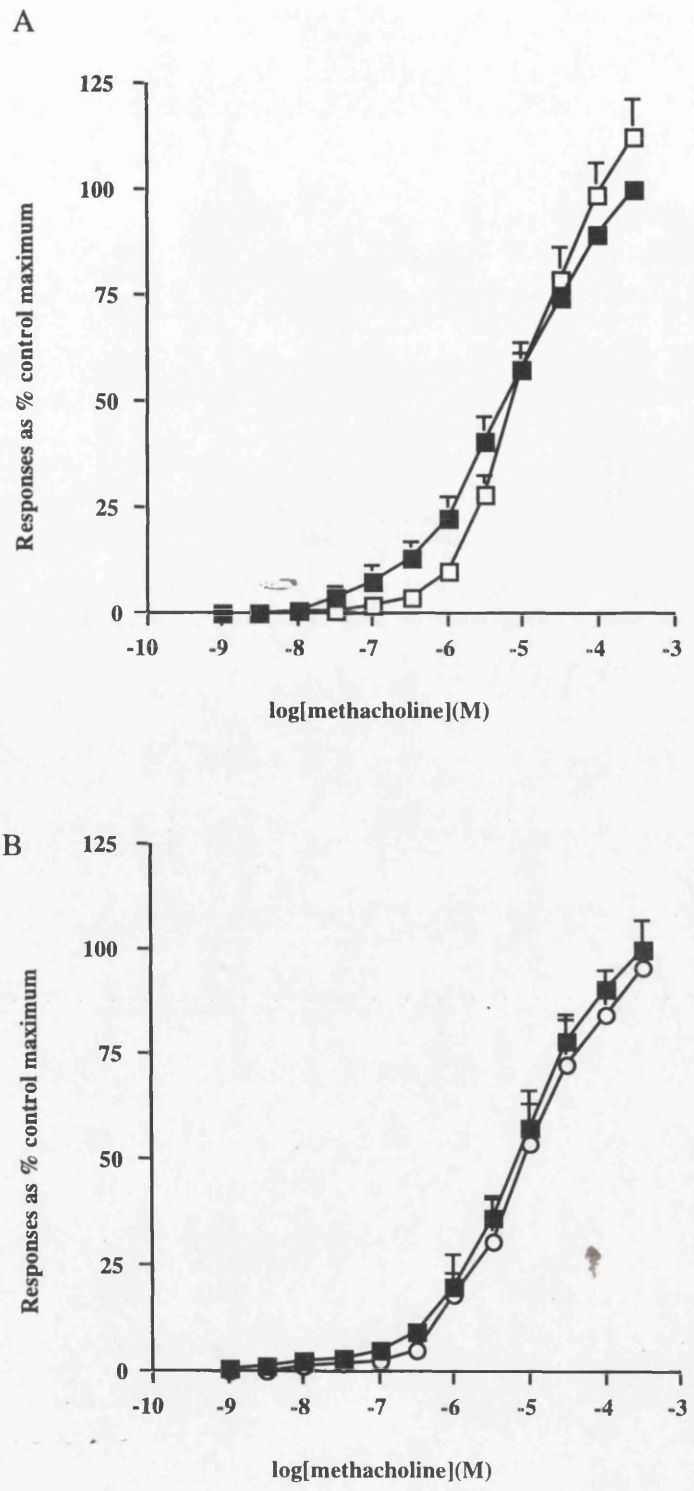


Figure 3.13

In hyperoxia (95% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of salbutamol at concentrations of 10^{-7}M (A), $3x10^{-7}$M (B) and 10^{-6}M (C). Responses are expressed as a % of the control maximum response. When pre-incubated at concentrations of $3x10^{-7}$M and 10^{-6}M, salbutamol significantly ($P<0.05$ and $P<0.001$, respectively) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.14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log[methacholine](M)
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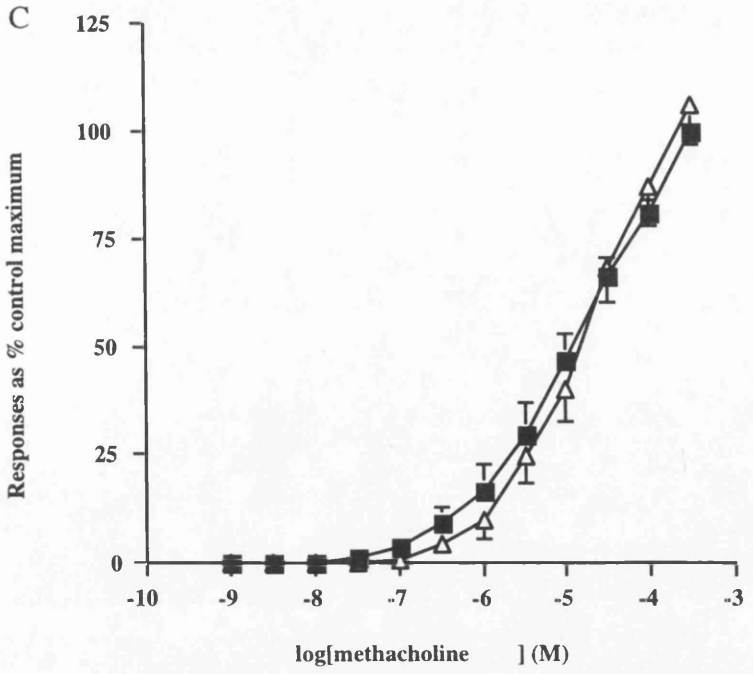
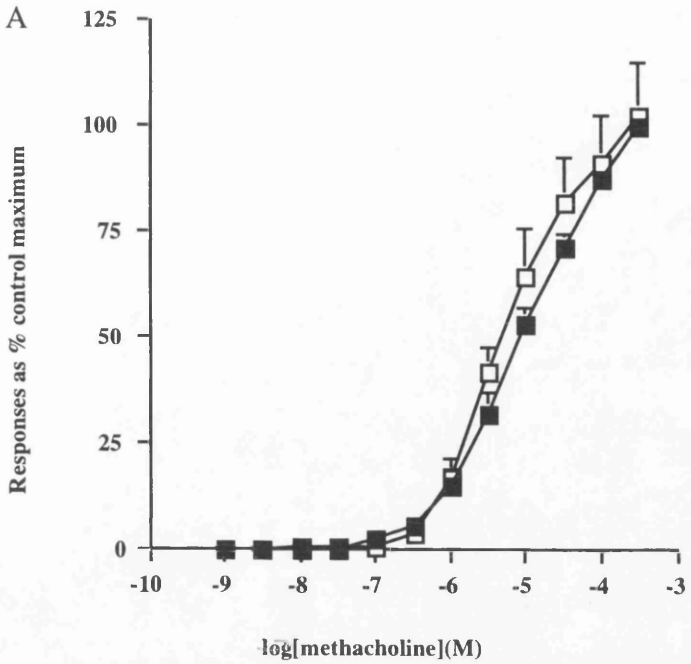


Figure 3.14

In normoxia (20% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of salbutamol at concentrations of 10^{-7}M (A), $3x10^{-7}$M (B) and 10^{-6}M (C). Responses are expressed as a % of the control maximum response. Pre-incubation of salbutamol had no effect on methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.15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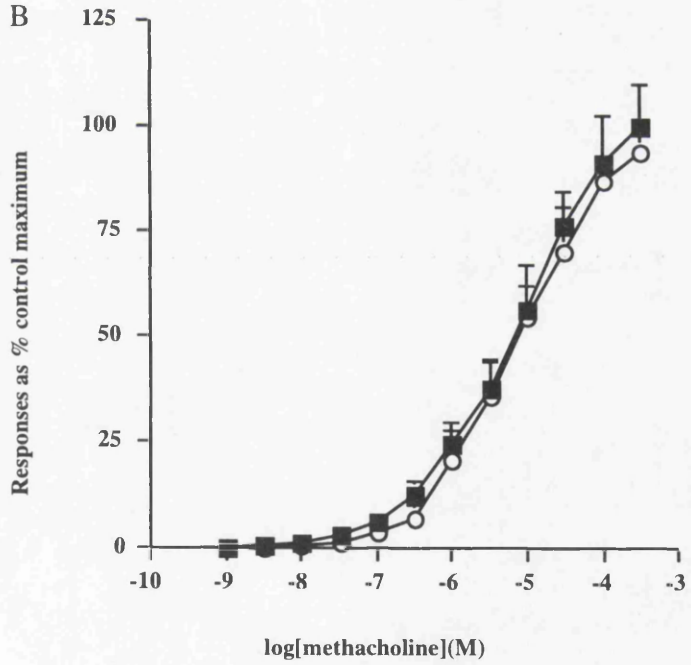
log[methacholine](M)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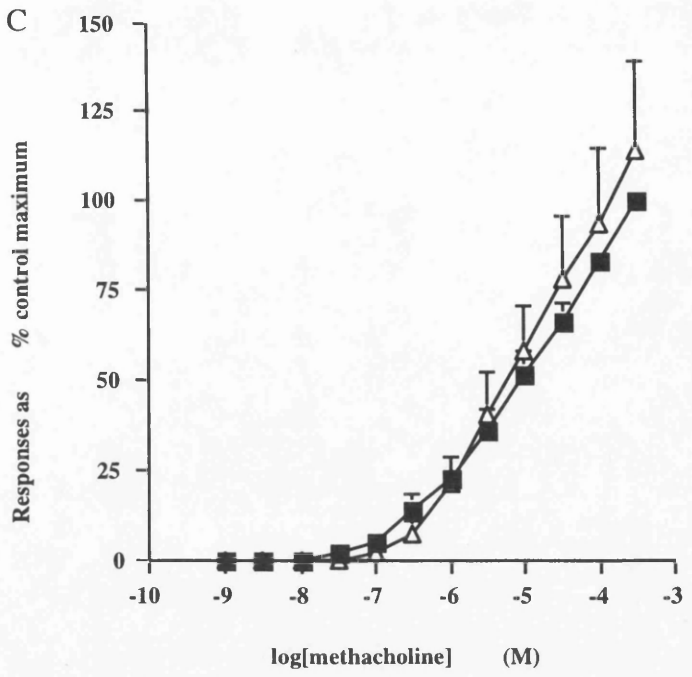


Figure 3.15

In hypoxia (0% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of salbutamol at concentrations of 10^{-7}M (A), $3x10^{-7}$M (B) and 10^{-6}M (C). Responses are expressed as a % of the control maximum response. Pre-incubation of salbutamol had no effect on methacholine-induced contractions. Number of observations (n) = 6 in each case.

Sodium nitroprusside

In hyperoxia pre-incubation of SNP at concentrations of 10^{-6}M and $3x10^{-6}$M did not alter subsequent contractions evoked by methacholine (methacholine pD_2 values; control for SNP 10^{-6}M; 5.37±0.14, n=6, plus SNP 10^{-6}M; 5.23±0.19. Control for sodium nitroprusside $3x10^{-6}$M; 4.95±0.16, n=6, plus SNP $3x10^{-6}$M; 5.10±0.18. In each case there was no significant ($P>0.05$) difference between corresponding data points and controls, Figures 3.16 A and B). When the concentration of SNP was increased to 10^{-5}M however, methacholine-induced responses were significantly ($P<0.01$, for data set) attenuated, albeit with no change in the sensitivity of the tissue (methacholine pD_2 values; control for SNP 10^{-5}M; 5.05±0.12, plus SNP 10^{-5}M; 5.02±0.14 (Figure 3.16C). In each case there was no significant ($P>0.05$) difference between corresponding data points and controls.).

Lowering the oxygen tension to normoxic levels did not alter the ability of 10^{-6}M SNP to protect against subsequent challenge with methacholine. As was the case under hyperoxia, pre-incubation with this concentration of SNP did not protect against methacholine-induced contractions (methacholine pD_2 values; control for SNP 10^{-6}M; 5.04±0.10, n=6, plus SNP 10^{-6}M; 4.89±0.18, in each case there was no significant ($P>0.05$) difference between corresponding data points and controls, Figure 3.17A).

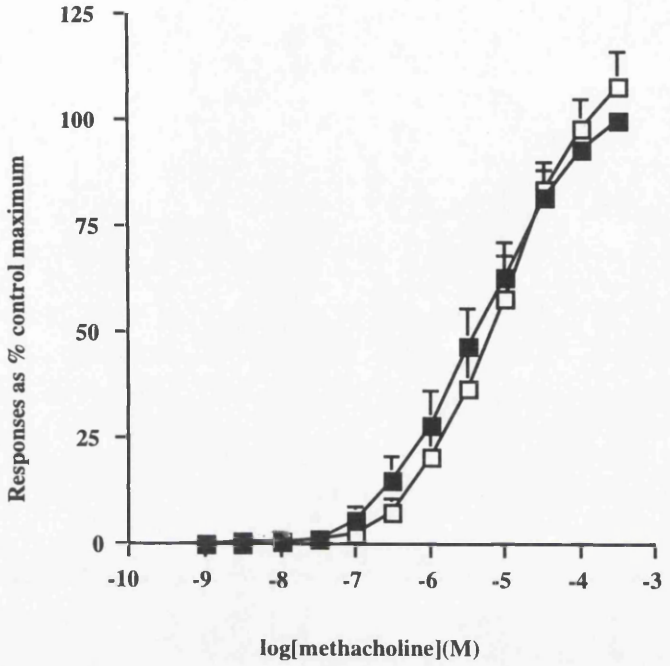
When the concentration of pre-incubated SNP was increased to $3x10^{-6}$M, the methacholine response curve was significantly ($P<0.05$, for data set) attenuated albeit with no change in the sensitivity of the tissue (methacholine pD_2 values; control for SNP $3x10^{-6}$M; 5.17±0.12, plus SNP $3x10^{-6}$M; 4.83±0.09, in this case there was no significant ($P>0.05$) difference between corresponding data points and controls.

(Figure 3.17B). Furthermore, when the concentration of SNP was increased to 10^{-5}M (Figure 3.17C), the methacholine concentration-response curve was also attenuated ($P<0.001$, for data set), in this case with a concomitant decrease in the sensitivity of the tissue (methacholine pD_2 values; control for SNP 10^{-5}M; 5.17±0.12, plus SNP 10^{-5}M; 4.77±0.10, representing a significant ($P<0.05$) difference between the corresponding data points and the controls.).

This pattern of responsiveness was similar in hypoxia, with SNP being ineffective at the 10^{-6}M level (methacholine pD_2 values; control for SNP 10^{-6}M; 5.11±0.15, n=6, plus SNP 10^{-6}M; 4.98±0.16. There was no significant ($P>0.05$) difference between corresponding data points and controls, Figure 3.18A), while significantly ($P<0.01$ and $P<0.001$ for data sets, respectively) attenuating methacholine-induced contractions when pre-incubated at concentrations of $3x10^{-6}$ and 10^{-5}M. This attenuation occurred without any significant change in the sensitivity of the tissue (methacholine pD_2 values; control for SNP $3x10^{-6}$M; 5.22±0.06, plus SNP $3x10^{-6}$M; 4.91±0.12 and control for SNP 10^{-5}M; 5.22±0.06, plus SNP 10^{-5}M; 4.91±0.07 (Figures 3.18B and C). In each case there was no significant ($P>0.05$) difference between corresponding data points and controls.).

Figure 3.16

A



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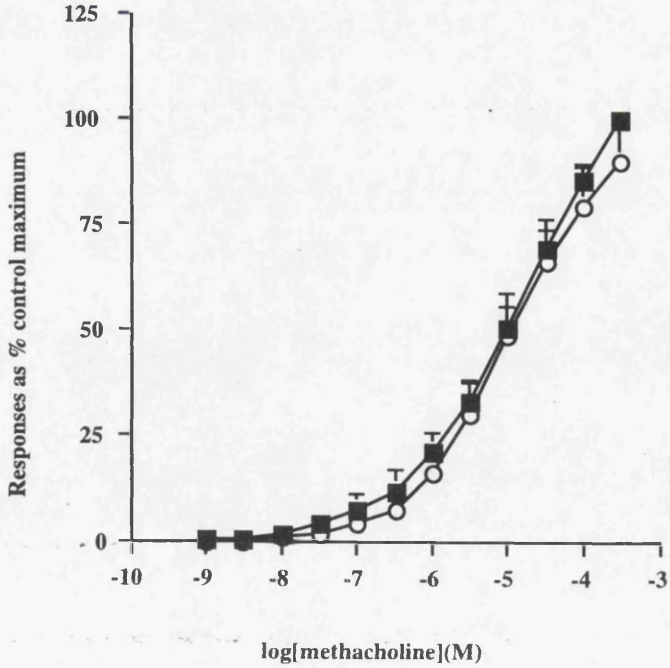

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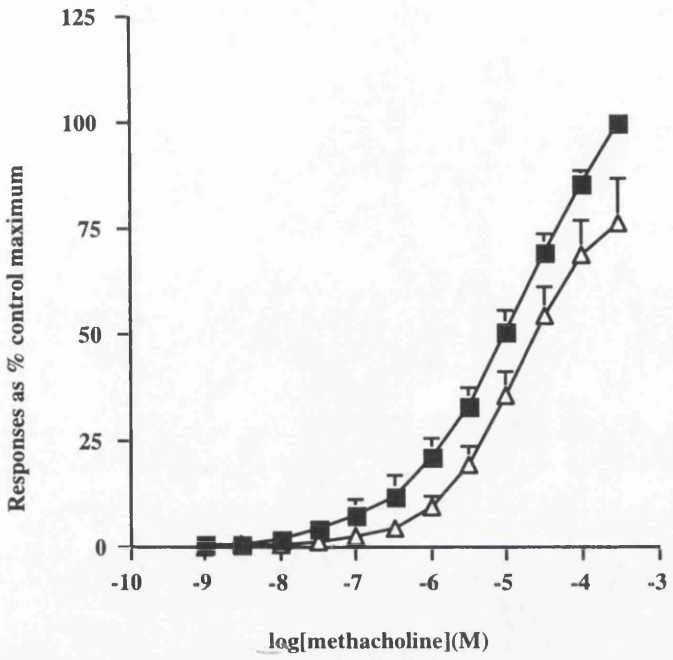
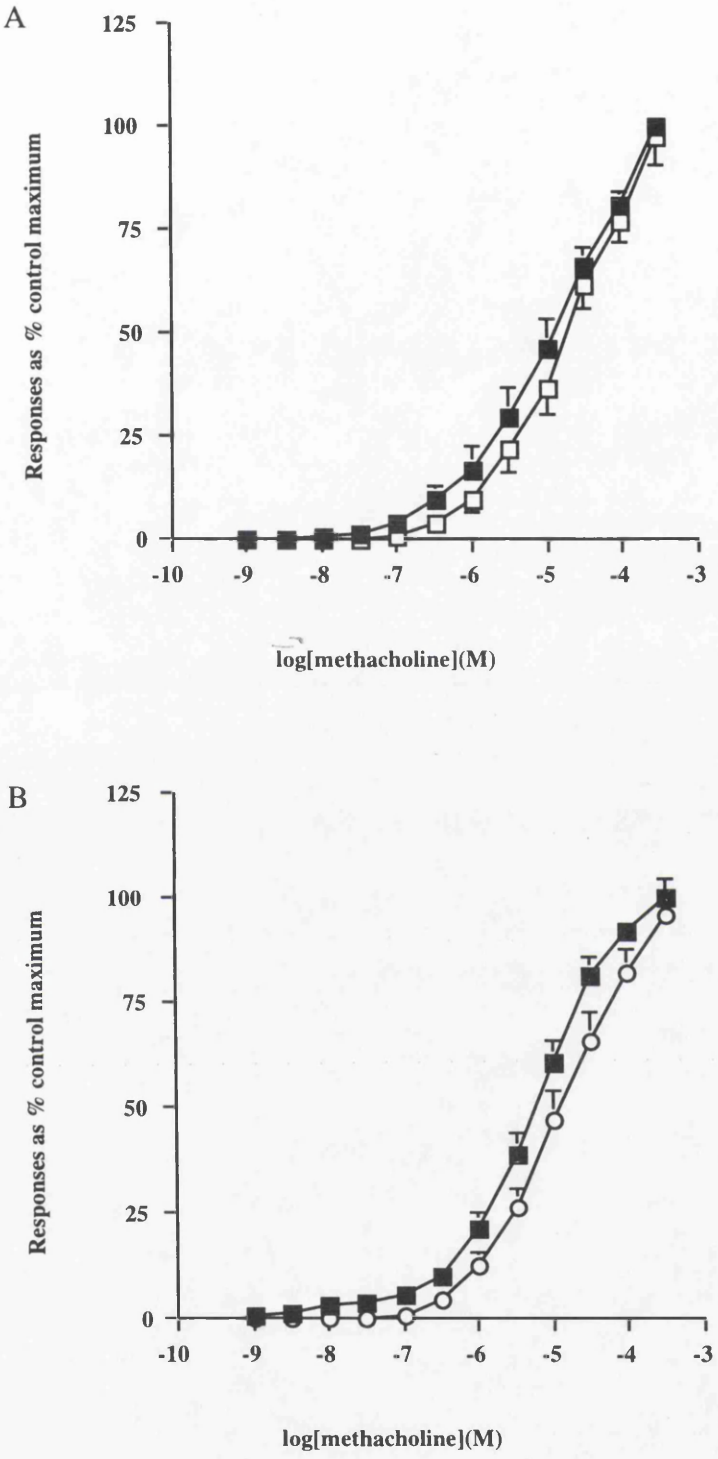


Figure 3.16

In hyperoxia (95% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of sodium nitroprusside (SNP) at concentrations of 10^{-6}M (A), $3x10^{-6}$M (B) and 10^{-5}M (C). Responses are expressed as a % of the control maximum response. When pre-incubated at a concentration of 10^{-5}M, SNP significantly ($P<0.01$) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.17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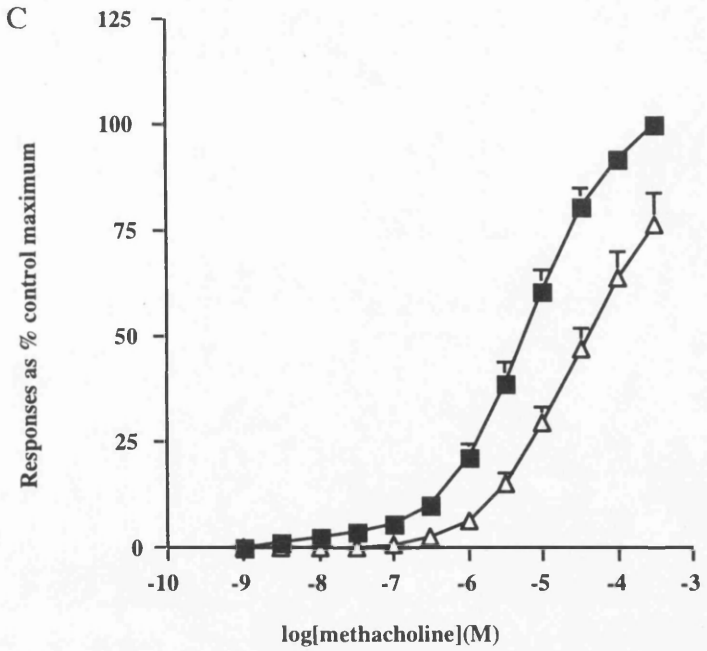
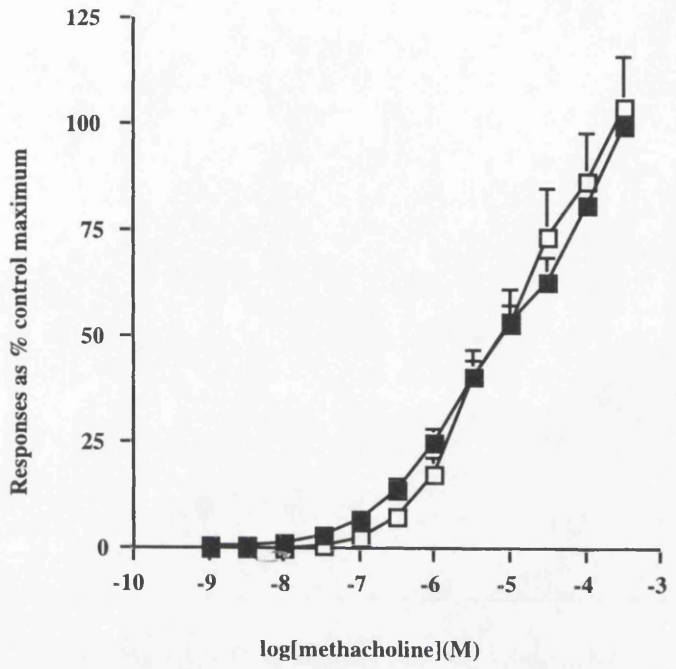


Figure 3.17

In normoxia (20% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of sodium nitroprusside (SNP) at concentrations of 10^{-6}M (A), $3x10^{-6}$M (B and 10^{-5}M (C). Responses are expressed as a % of the control maximum response. When pre-incubated at concentrations of $3x10^{-6}$M and 10^{-5}M, SNP significantly ($P<0.05$ and $P<0.001$, respectively) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.18

A



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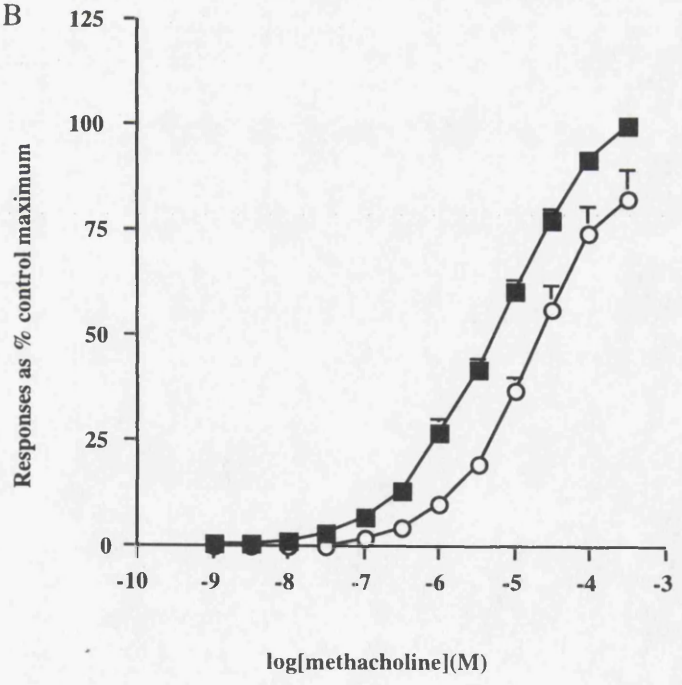


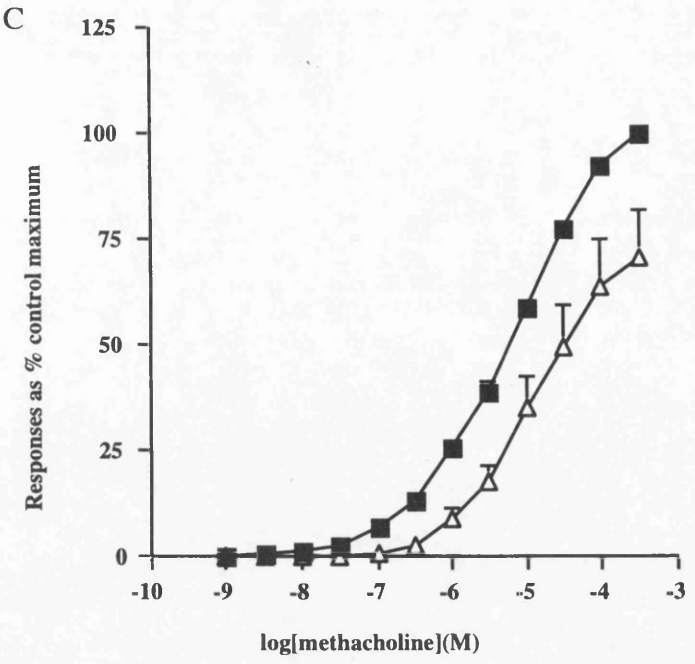


Figure 3.18

In hypoxia (0% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of sodium nitroprusside (SNP) at concentrations of 10^{-6}M (A), $3x10^{-6}$M (B) and 10^{-5}M (C). Responses are expressed as a % of the control maximum response. When pre-incubated at concentrations of $3x10^{-6}$M and 10^{-5}M, SNP significantly ($P<0.01$ and $P<0.001$, respectively) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

Isosorbide Dinitrate

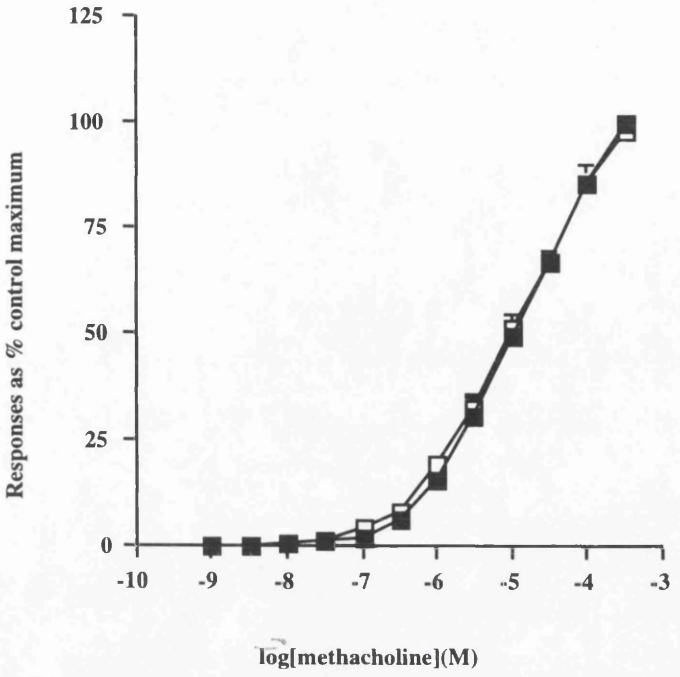
In hyperoxia, pre-incubating ISDN at concentrations of 10^{-5}M and $3x10^{-5}$M did not alter contractions evoked by subsequent challenge with methacholine (methacholine pD_2 values; control for ISDN 10^{-5}M; 4.98±0.09, plus ISDN 10^{-5}M; 5.04±0.12 and control for ISDN $3x10^{-5}$M; 4.95±0.12, plus ISDN $3x10^{-5}$M; 4.89±0.16. In each case there was no significant ($P>0.05$) difference between corresponding data points and controls. Figures 3.19A and B). At the 10^{-4}M level, however, ISDN significantly ($P<0.05$ for data set) attenuated methacholine-induced responses, albeit with no change in the sensitivity of the tissue (methacholine pD_2 values; control for isosorbide dinitrate 10^{-4}M; 5.01±0.24, plus ISDN 10^{-4}M; 4.69±0.13 (Figure 3.19C). There was no significant ($P>0.05$) difference between corresponding data points and controls.).

Lowering the oxygen tension in the gas mixture to 20% did not alter the effect of 10^{-5}M ISDN on methacholine challenge. As was the case in hyperoxia, pre-incubation with this concentration of bronchodilator had no effect on the methacholine-response curve (methacholine pD_2 values; control for ISDN 10^{-5}M; 5.14±0.15, plus ISDN 10^{-5}M; 5.02±0.14. There was no significant ($P>0.05$) difference between corresponding data points and controls. Figure 3.20A). In contrast to hyperoxia, however, pre-incubation of $3x10^{-5}$M ISDN in normoxia significantly ($P<0.01$ for data set) attenuated methacholine-induced contractions albeit with no change in the sensitivity of the tissue (methacholine pD_2 values; control for ISDN $3x10^{-5}$M; 4.95±0.16, plus ISDN $3x10^{-5}$M; 4.78±0.17. There was no significant ($P>0.05$) difference between corresponding data points and controls. Figure 3.20B). Furthermore, increasing the concentration of ISDN to 10^{-4}M, evoked a greater ($P<0.001$) attenuation of the methacholine-response curve, in this case with a corresponding decrease in the sensitivity of the tissue (methacholine pD_2 values; control for ISDN 10^{-4}M; 5.21±0.10, plus ISDN 10^{-4}M; 4.81±0.06 (Figure 3.20C). This reflects a significant ($P<0.01$) difference between the corresponding data points and controls.).

When the oxygen tension was lowered to hypoxic levels, ISDN, at both the 10^{-5}M and $3x10^{-5}$M level, did not alter the methacholine-induced contractions (methacholine pD_2 values; control for ISDN 10^{-5}M; 5.01±0.08, plus ISDN 10^{-5}M; 5.12±0.14 and control for ISDN $3x10^{-5}$M; 4.98±0.10, plus ISDN $3x10^{-5}$M; 4.89±0.11. There was no significant ($P>0.05$) difference between corresponding data points and controls. Figures 3.21 A and B, respectively). As was the case in hyperoxia and normoxia, pre-incubating ISDN at the 10^{-4}M level significantly ($P<0.01$ for data set in 0% O_2) attenuated responses to subsequent challenge with methacholine. In this instance, ISDN also evoked a significant rightward shift of the methacholine response (methacholine pD_2 values; control for ISDN 10^{-4}M; 5.14±0.10, plus ISDN 10^{-4}M; 4.63±0.05 (Figure 3.21C). This reflects a significant ($P<0.001$) difference between the corresponding data points and controls.).

Figure 3.19

A



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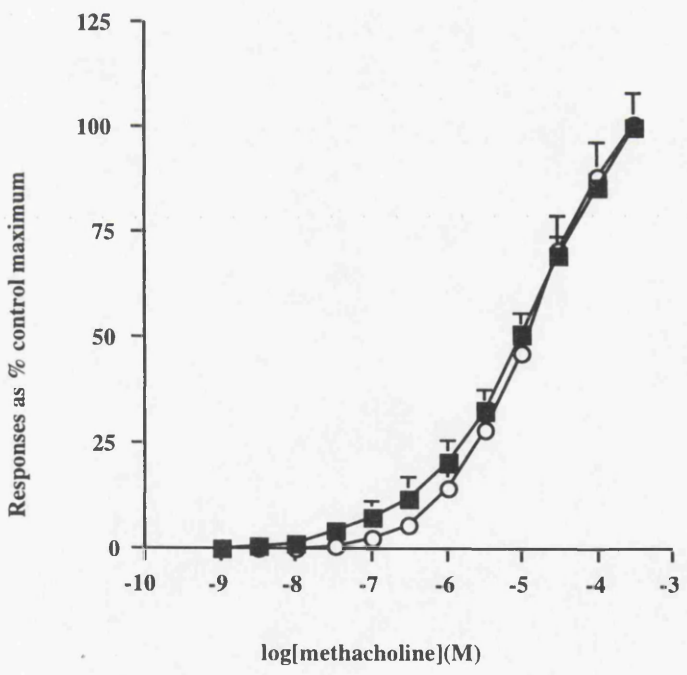


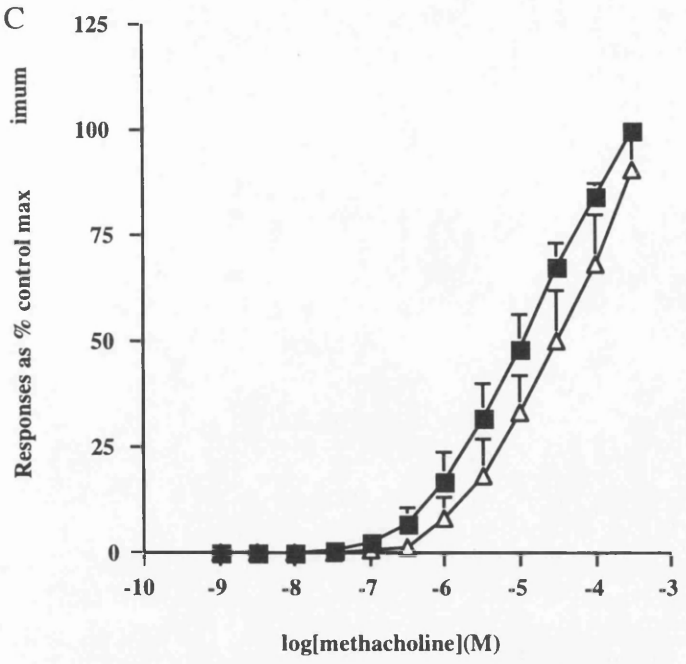
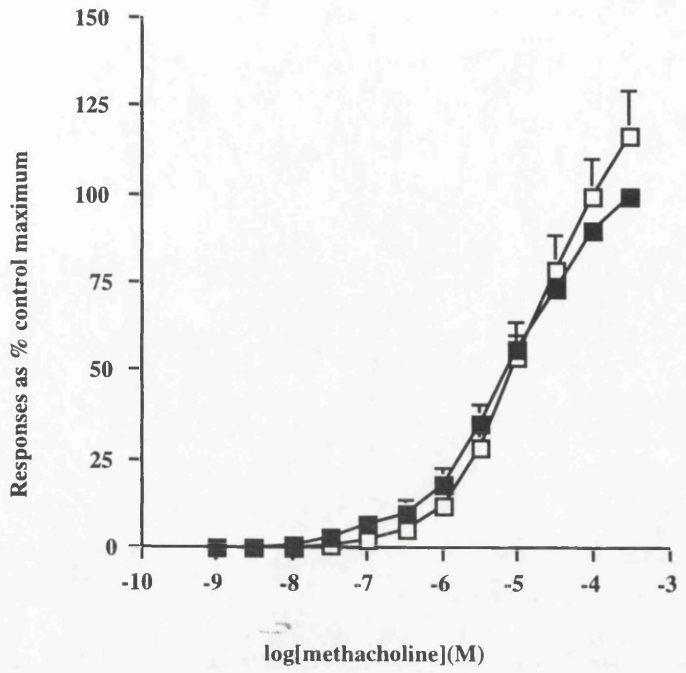


Figure 3.19

In hyperoxia (95% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of isosorbide dinitrate (ISDN) at concentrations of 10^{-5}M (A), $3x10^{-5}$M (B) and 10^{-4}M (C). Responses are expressed as a % of the control maximum response. When pre-incubated at a concentration of 10^{-4}M, ISDN significantly ($P<0.05$) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.20

A



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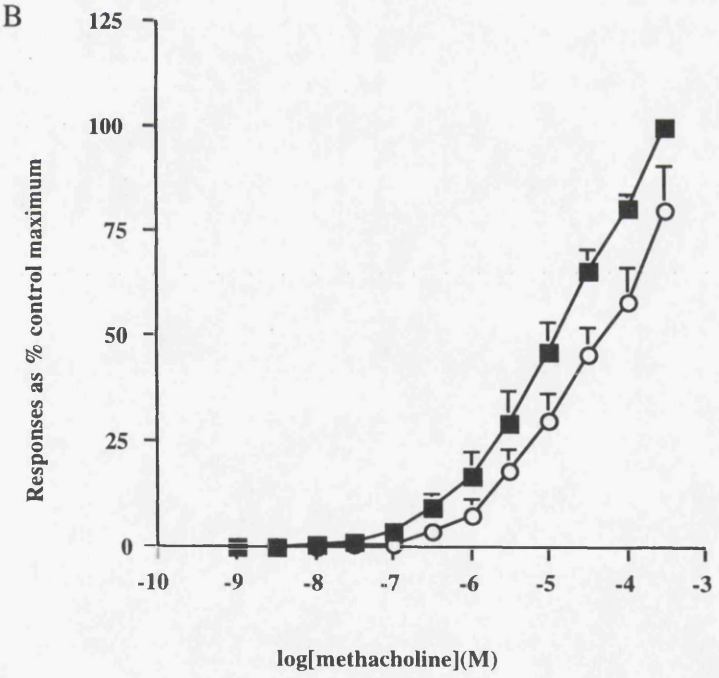


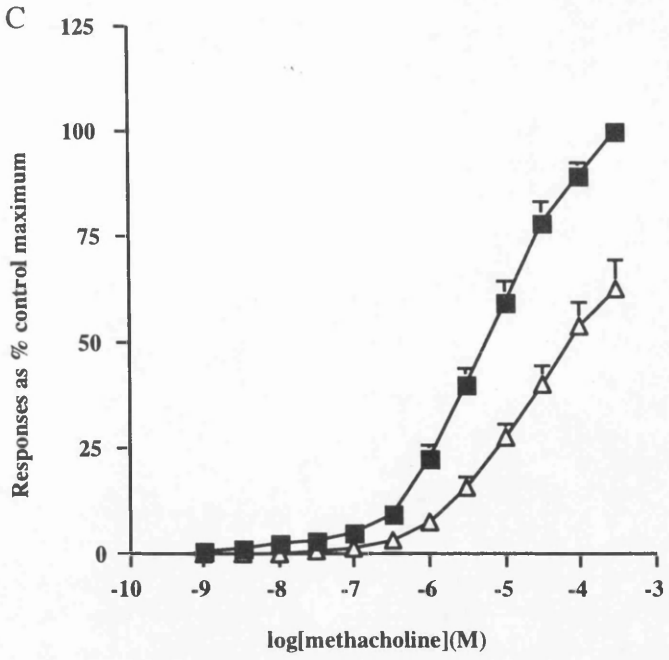
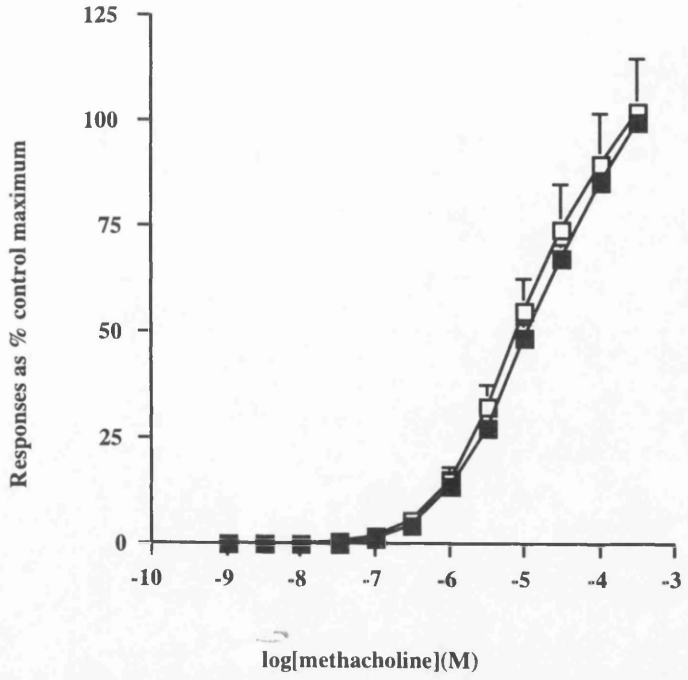


Figure 3.20

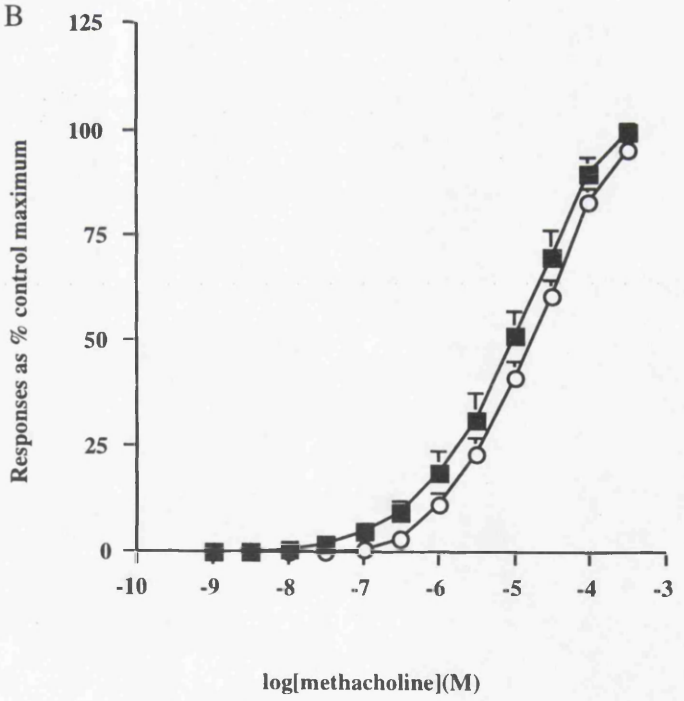
In normoxia (20% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of isosorbide dinitrate (ISDN) at concentrations of 10^{-5}M (A), $3x10^{-5}$M (B) and 10^{-4}M (C). Responses are expressed as a % of the control maximum response. When pre-incubated at concentrations of $3x10^{-5}$M and 10^{-4}M, ISDN significantly ($P<0.01$ and $P<0.001$, respectively) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

Figure 3.21

A



B



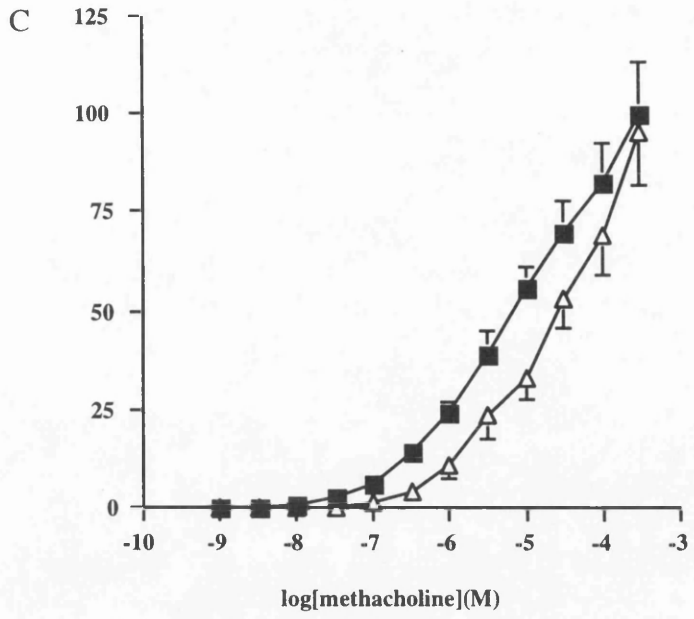


Figure 3.21

In hypoxia (0% O_2), cumulative concentration response curves evoked by methacholine (MCh, 10^{-9}-$3x10^{-4}$M) were constructed in bovine bronchial rings in the absence (■) and in the presence of isosorbide dinitrate (ISDN) at concentrations of 10^{-5}M (A), $3x10^{-5}$M (B) and 10^{-4}M (C). Responses are expressed as a % of the control maximum response. When pre-incubated at a concentration of 10^{-4}M, ISDN significantly ($P<0.01$) attenuated methacholine-induced contractions. Number of observations (n) = 6 in each case.

3.4 DISCUSSION

Changing the oxygen tension had a marked effect on the responses evoked by both methacholine and the bronchodilators presently studied.

Contractile responses to methacholine

It was found that responses evoked by methacholine were significantly enhanced when the oxygen tension was reduced from 95%. This was true for either the addition of a single concentration of methacholine or for responses throughout the whole concentration-response curve. It has previously been suggested that the hypoxia-induced enhancement of airway reactivity to spasmogens may be mediated by a vagal reflex (Vidruk & Sorkness, 1985), however, the fact that we found a similar enhancement of responses using isolated tissue suggests that reflex pathways are not the only mechanisms involved. The hypothesis put forward by D'Brot and Ahmed (1988) that leukotrienes (or some other mediator) may cause the enhancement of spasmogen responses remains more likely, given that a number of contractile substances are released in response to hypoxia. While the studies conducted by D'Brot and Ahmed involved whole animals, it remains possible that the epithelial layer or even the smooth muscle of an isolated bronchial ring could be a site for release of mediators causing enhancement of the smooth muscle reactivity. In fact, the potent bronchoconstricting peptide endothelin-1 is released from isolated bovine endothelial cells in response to hypoxia (Hieda & Gomez-Sanchez, 1990). Several other possibilities exist regarding this enhancement of methacholine responses; our group has previously shown that the vasoactive peptide hormone angiotensin II (AII), in concentrations below those which evoke significant contractions *per se*, can potentiate methacholine responses in human and bovine bronchi *in vitro* (Nally *et al.*, 1994c) and that angiotensin II is released during acute asthma (Millar *et al.*, 1994). It is therefore possible that angiotensin II or another spasmogenic substance is released locally from isolated tissue in concentrations which potentiate responses to methacholine, however this remains to be established.

Reversal of methacholine-induced tone

In the experiments involving reversal of methacholine-induced tone, we found salbutamol to be extremely effective in all three oxygen tensions, however it was most potent in normoxia, producing almost 100% reversal of the induced contraction. Since hypoxia can result in a loss of ß-adrenoceptors in cardiac muscle (Voelkel *et al.*, 1981) and since we found salbutamol to be significantly less effective in low oxygen, it would be tempting to speculate that hypoxia may also cause a reduction in ß-adrenoceptors in the bronchi.

The bronchodilating abilities of atrial natriuretic peptide have been studied in airways both *in vivo* and *in vitro* (Angus *et al.*, 1994a and b) Its mechanism of action is

proposed to be via activation of particulate guanylyl cyclase leading to elevation of cGMP (Ishii & Murad, 1989). We found atrial natriuretic peptide to be most effective in 0% O_2, reversing approximately 50% of the induced tone, suggesting that this drug may be more potent in hypoxic conditions. This enhancement in hypoxia was not seen with either salbutamol or isosorbide dinitrate.

The nitrate drug isosorbide dinitrate was also effective at reversing methacholine-induced tone. Given that both isosorbide dinitrate and atrial natriuretic peptide are thought to evoke their responses by stimulation of guanylyl cyclase (Ishii & Murad, 1989; Ignarro & Kadowitz, 1985), one would perhaps expect similarities in the responses evoked by these drugs in different oxygen tensions. Indeed, both of these dilators were significantly more effective in hypoxia than hyperoxia. There were some differences, however, in the pattern of responses evoked by each drug. For example, isosorbide dinitrate was more effective in normoxia than hyperoxia, whereas atrial natriuretic peptide was equipotent in these two oxygen tensions. These differences may be due to the fact that atrial natriuretic peptide stimulates particulate guanylyl cyclase (Ishii & Murad, 1989) whereas isosorbide dinitrate stimulates soluble guanylyl cyclase (Ignarro & Kadowitz, 1985). Activation of soluble guanylyl cyclase is also the mechanism of action of sodium nitroprusside. The results from this study showed that responses to sodium nitroprusside were greater in hyperoxia than normoxia, therefore the pattern of responses differed between sodium nitroprusside and isosorbide. This was unexpected given that both agents are purported to act via the same mechanism. These differences may be due to the fact that sodium nitroprusside can stimulate soluble guanylyl cyclase directly (Ignarro *et al.*, 1981), whereas isosorbide must first form an intermediate complex (Ignarro and Gruetter, 1980; Ignarro *et al.*, 1981).

A cautionary note must be added regarding the use of sodium nitroprusside, since it is possible that prolonged exposure to high concentrations of this agent might have a non-specific toxic effect due to release of cyanide from its ferricyanide complex. Indeed sodium nitroprusside has been shown to be cytotoxic in cultured endothelial cells under hypoxic conditions (Ioannidis *et al.*, 1996). In the study in question, however, the endothelial cells were exposed to extremely high (20mM) concentrations of sodium nitroprusside over a prolonged (8 hours) period. Furthermore, the chemically unrelated nitric oxide donor, S-nitroso-N-acetylpenicillamine (SNAP), was also cytotoxic under these conditions, despite the fact it does not possess a cyanide moiety (Ioannidis *et al.*, 1996).

It is now generally well accepted that the smooth muscle relaxant effects of SNP and SNAP are mediated via increases in cellular cGMP (see Warner *et al.*, 1994 for review) and that this effect is due to release of nitric oxide either spontaneously or upon enzymatic degradation of the original compound. Nitric oxide, not the parent compound, is believed to be responsible for the activation of soluble guanylyl cyclase and the resulting elevation of cGMP (Schmidt *et al.*, 1993). Indeed both SNP and SNAP evoke a dose-dependent increase in cGMP in cultured human airway smooth

muscle cells (Hamad *et al.*, 1997). In certain smooth muscle preparations, for example vascular smooth muscle, SNAP has been shown to be a more potent nitric oxide donor than SNP (Marks *et al.*, 1995), however, SNP remains widely used as a pharmacological means of activating soluble guanylate cyclase (for example, Hennan & Diamond, 1998).

Salbutamol was the only bronchodilator used in this study which was more effective in normoxia than hypoxia. In every other case, the dilators were either equally (isosorbide dinitrate, sodium nitroprusside) or more (atrial natriuretic peptide) effective in hypoxia. This may be of importance given the potential for hypoxia during an asthmatic attack. It could therefore be postulated that isosorbide dinitrate would retain its bronchodilating abilities in a hypoxic situation, indeed this drug has been shown to be a useful bronchodilator in asthmatic patients (Okayama *et al.*, 1984). Our results show that the potency of atrial natriuretic peptide is actually enhanced under hypoxic conditions and more importantly, salbutamol becomes less effective. This finding perhaps suggests a role for combining O_2 therapy with salbutamol in patients already in a hypoxic state.

Protection against subsequent challenge with methacholine

Changing the oxygen tension had a marked effect on the ability of the bronchodilators used in this study to protect against methacholine-induced contractions. The pattern varied however, between the bronchodilators used which may reflect the different second-messenger pathways utilised by each of these agents. For example, atrial natriuretic peptide, which acts via opening of potassium channels (Winquist *et al.*, 1984; Ohlstein & Berkowitz, 1985) and stimulation of particulate guanylyl cyclase (Ishii & Murad, 1989), significantly attenuated methacholine-induced contractions in hyperoxia whereas in normoxia this effect was lost. Furthermore, when the oxygen tension was lowered to hypoxic levels, ANP actually enhanced methacholine responses. The attenuation of methacholine responses in hyperoxia is in keeping with results from a previous study where pre-incubating ANP at a concentration of 10^{-6}M significantly reduced contractions evoked by methacholine in isolated human and bovine bronchi (Angus *et al.*, 1994a). Under normoxic conditions, inhaled ANP protects against methacholine challenge in asthmatic patients (Angus *et al.*, 1994b), however, in the present study, ANP failed to produce a similar effect in isolated bovine bronchi. It would be interesting to determine if, in isolated human bronchi, ANP protects against methacholine challenge under normoxia.

The enhancement of methacholine responses in hypoxia remains to be explained, however it cannot be attributed to phosphoramidon since when pre-incubated alone, this neutral endopeptidase inhibitor had no effect on methacholine responses. In addition to hydrolysis by neutral endopeptidases, the principal mechanism by which ANP is removed from the circulation is via binding to a non-guanylyl cyclase clearance

receptor (Maak *et al.*, 1987). These "C"-receptors have been shown to internalise ANP, thus performing a major role in the clearance of ANP from plasma (Chiu *et al.*, 1991). While the presence of these C-receptors in bovine bronchi remains to be established, they are expressed in rat airway smooth muscle cultures (James & Burnstock, 1991). It may be that changing the oxygen tension alters the ratio between the biologically active, G-protein coupled ANP receptors and the uncoupled clearance receptors. For example, lowering the oxygen tension from hyperoxia may increase the relative number of C-receptors, thus explaining the loss of ANP's effectiveness in normoxia. This would not, however, explain the enhancement of methacholine-induced contractions in hypoxia.

Salbutamol, a β_2 agonist acting via potassium channel opening (Miura *et al.*, 1992) as well as stimulation of adenylyl cyclase (Rinard *et al.*, 1983), was effective only in hyperoxia. In normoxia and hypoxia, pre-incubation with salbutamol had no effect on the methacholine concentration-response curve. The ability of salbutamol to protect against bronchoconstrictors remains controversial; studies conducted upon isolated human bronchi have shown little (Advenier *et al.*, 1988) or no (Gustaffson & Persson, 1991; Advenier *et al.*, 1991) pre-protectant effect of this drug. In contrast, clinical studies (e.g. Tattersfield, 1987; Britton *et al.*, 1988) have shown an ability of salbutamol to inhibit bronchial reactivity to spasmogens. In addition, a previous study by this group showed that salbutamol, in hyperoxia, significantly attenuated methacholine-induced contractions in human isolated bronchi (Nally *et al.*, 1994b) and the results from this present study appear to indicate a similar effect in bovine bronchi at this oxygen tension. When the oxygen tension was lowered to normoxic or hypoxic levels however, this pre-protectant effect of salbutamol was lost. While the reasons for this remain unclear, hypoxia has been shown to cause, in cardiac muscle, uncoupling of β_2-adrenoceptors from their regulatory G proteins (Richalet, 1990). It is possible, therefore, that lowering the oxygen tension reduces the number of functionally coupled β_2-adrenoceptors in the bronchi. This may explain the loss of salbutamol's pre-protectant effect in hypoxia, however, one would expect salbutamol to retain its effectiveness in normoxia, especially as β_2-agonists confer protection *in vivo* at this oxygen tension. It has been suggested however (Persson *et al.*, 1982), that β_2-agonists have effects on cells other than airway smooth muscle (for example, inhibition of mediator release, Gorenne *et al.*, 1995) and that this may contribute to their protective effects *in vivo*.

Since both sodium nitroprusside and isosorbide dinitrate are purported to act via similar second-messenger systems (nitric oxide donation resulting in stimulation of soluble guanylyl cyclase, Feelisch & Novak, 1987) one would expect similarities in their pattern of responses. In hyperoxia, SNP, at the 10^{-5}M level, evoked a slight attenuation of the methacholine response whereas this attenuation was significantly greater in both normoxia and hypoxia. Pre-incubation of isosorbide dinitrate at a concentration of 10^{-}

more marked shift in hypoxia, however, isosorbide dinitrate was most effective in normoxia. Thus the pattern of responses evoked by these two dilators are similar but not identical. This may be due to the fact that sodium nitroprusside stimulates soluble guanylyl cyclase directly (Ignarro *et al.*, 1981) whereas isosorbide dinitrate interacts with a cellular constituent, such as a sulfhydryl, to form an intermediate which then activates guanylyl cyclase (Ignarro *et al.*, 1981; Ignarro & Gruetter, 1980).
It is interesting to note that atrial natriuretic peptide and salbutamol only protected against methacholine challenge in hyperoxia, whereas both sodium nitroprusside and isosorbide dinitrate were least effective in this oxygen tension. This may suggest that agonists acting via stimulation of soluble guanylyl cyclase would be more effective in oxygen tensions less than hyperoxia.

Comparing the ability of these dilators to reverse methacholine induced tone with the ability to protect against subsequent challenge, there are some interesting differences. For example, the ability of ANP to reverse methacholine-induced contraction was enhanced under hypoxic conditions, whereas in the present study, the ability of ANP to protect against subsequent challenge was lost when the oxygen tension was reduced from hyperoxia to either normoxia or hypoxia. Preincubation of salbutamol (at concentrations of $3x10^{-7}M$ and $10^{-6}M$) attenuated the methacholine concentration-response curve, although only under hyperoxic conditions. In terms of reversing tone however, salbutamol was most effective in normoxia. Sodium nitroprusside was more effective at reversing tone in hyperoxia than normoxia, whereas in terms of protecting against subsequent challenge, its effectiveness increased as the oxygen tension was lowered from hyperoxia to either normoxia or hypoxia. In both cases, however, isosorbide dinitrate was shown to be more effective in normoxia and hypoxia than in hyperoxia. Therefore, the effect of changes in oxygen tension on the ability of isosorbide dinitrate to, firstly, reverse induced tone and secondly, protect against subsequent challenge was consistent between the two studies. In contrast, the effects of oxygen tension on ANP and salbutamol responses varied between the two studies, indicating that the pharmacology of relaxation may be dissimilar to that of protection.

In summary, changes in oxygen tension can alter: i)contractile responses to methacholine ii)the ability of bronchodilators to reverse methacholine-induced tone and iii)the ability of bronchodilators to protect against methacholine-induced contraction. Contractile responses to methacholine were enhanced when the oxygen tension of the gas mixture was lowered from 95% to 20 or 0%, although the direction of the changes varied among the dilators. This suggests that the responses evoked by bronchodilators in hyperoxic conditions may not necessarily predict those in the physiological range of oxygen tensions and that the relative effectiveness of bronchodilators may vary between normoxic and hypoxic conditions.

CHAPTER 4

THE EFFECT OF CHRONIC HYPOXIA ON THE RESPONSIVENESS OF RAT ISOLATED AIRWAYS TO CONTRACTILE AND RELAXATORY AGONISTS

4.1 INTRODUCTION

As stated in section 1.6.4.2, the effect of chronic hypoxia on airway responsiveness has not been extensively studied. In contrast, the effect of chronic hyperoxia on airway responsiveness has been more clearly defined. Chronic exposure to hyperoxia induces airway hyperresponsiveness, both *in vivo* and *in vitro*, to contractile agents such as 5-hydroxytryptamine and methacholine (Szarek, 1989; Hershenson *et al.*, 1992a; Hershenson *et al.*, 1994).

In this present study, we compared responses evoked by the spasmogens methacholine, endothelin-1 (ET-1) and potassium chloride (KCl) in isolated trachea from control and chronically hypoxic rats. Our initial results indicated that the contractile responses evoked by each of these agonists were significantly reduced in trachea from chronically hypoxic rats, therefore further experiments were carried out in an attempt to elucidate the mechanisms underlying this attenuation. Several studies indicate that chronic hyperoxia increases airway epithelial and smooth muscle layer thickness (Szarek, 1989; Hershenson *et al.*, 1992a and b; Hershenson *et al.*, 1994) and that this increase in airway wall thickness correlates with the increase in airway reactivity (Hershenson *et al.*, 1992). More recently, however, Szarek and co-workers (1995) showed that in hyperoxic rats, the increase in airway reactivity occurs before the remodelling process develops, indicating that other mechanisms are also involved in the development of hyperoxia-induced airway hyperresponsiveness. Interestingly, Hershenson *et al* (1992) showed that pretreatment with the cyclooxygenase inhibitor indomethacin or removal of the epithelium reversed hyperoxia-induced airway hyperresponsiveness in immature rats *in vitro*. This indicates that epithelium-derived prostanoids contribute to the development of hyperoxia-induced enhancement of airway responsiveness. In this present study, we sought to examine if the release of (inhibitory) cyclooxygenase metabolites could also be responsible for the attenuation of contractile responses caused by exposure to chronic hypoxia.

Alternatively, several studies indicate that nitric oxide synthase expression is upregulated in the lungs of chronically hypoxic rats (Shaul *et al.*, 1995; Xue *et al.*, 1994). To test the hypothesis that release of nitric oxide was responsible for the attenuation of contractile responses in chronically hypoxic rat airways, we also studied the effect of the nitric oxide synthase inhibitor, l-nitroarginine methyl ester (L-NAME), on responses to methacholine and ET-1 in trachea from control and chronically hypoxic rats.

In the previous chapter, I demonstrated that acute exposure to hypoxia can significantly enhance contractions evoked by methacholine in isolated bovine bronchi (section 3). It remained to be seen, however, if this is also the case in rat bronchi or indeed if chronic exposure to hypoxia alters the sensitivity of the airways to acute changes in oxygen tension. In this study, we measured, *in vitro,* responses evoked by methacholine and

ET-1 in the three oxygen tensions used in the previous study; 95% O_2 (hyperoxia), 20% (normoxia) and 0% (hypoxia).

4.2 METHODS

4.2.1 Development Of Chronic Hypoxia

Rats were made chronically hypoxic as described in section 2.1.7.4. Age matched control rats were reared alongside the hypobaric chamber but were maintained in room air throughout.

4.2.2 Tissue Preparation And Measurement Of Contractile Responses

Tracheal ring preparations were obtained from control and chronically hypoxic rats as described in section 2.1.2.1 and mounted in 5ml organ baths as described in section 2.1.1. Contractile responses were measured in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution at 37 ± 0.5°C. Pilot experiments were conducted to determine the optimum level of applied tension for trachea from both control and hypoxic animals (see Figure 4.1). Under hyperoxic conditions, an initial resting tension of 0.25g wt was applied to each tracheal ring. Tissues were allowed to equilibrate for 15 minutes before addition of a single concentration of methacholine (10^{-4}M). This concentration was chosen as it evoked a near maximal contractile response. When the contraction had reached a plateau, the tissues were washed three times with fresh Krebs-Henseleit solution over a 15 min period, or until the tension returned to baseline. Tension was then removed before increasing the degree of initial tension to the next increment (0.5g wt).

For subsequent measurement of agonist responses, the optimum tension (1.0g wt) was applied and tissues were allowed to equilibrate for 45 minutes, during which time tension was reapplied where necessary. Cumulative concentration-response curves were then constructed to either methacholine (10^{-10}-$3x10^{-4}$M), endothelin-1 (ET-1, 10^{-11}-$3x10^{-7}$M) or potassium chloride (1-70mM). Results are expressed in mg wt.

To test whether exposure to chronic hypoxia might induce the release of cyclooxygenase metabolites or nitric oxide from the tracheal epithelium, we examined responses evoked by both methacholine and ET-1 in intact and epithelium-denuded tracheal rings in the presence and absence of (1) the cyclooxygenase inhibitor indomethacin ($3x10^{-6}$M) and (2) the nitric oxide synthase inhibitor LNAME (10^{-4}M). Responses to methacholine are expressed as a % of the first curve (methacholine alone) maximum. On each experimental day, one tissue was subjected to two consecutive concentration-response curves to methacholine alone to ensure that responses did not alter with time.

Due to tachyphylaxis, it is not possible to perform consecutive concentration-response curves to ET-1 (see section 2.1.6), therefore tissues were initially stimulated with a maximal concentration (10^{-4}M) of methacholine and responses to ET-1 expressed as a % of this response. Removal of the epithelial layer from tracheal preparations was verified by light microscopy.

In a separate series of experiments, tracheal rings were preconstricted with a single concentration of methacholine ($3x10^{-7}M$ for control rat trachea and $10^{-6}M$ for hypoxic rat trachea). The concentrations used were approximately the EC_{30} for methacholine in trachea from control and chronically hypoxic rats. Once contractions had plateaued, cumulative concentration-response curves were constructed to salbutamol (10^{-9}-$10^{-4}M$) and sodium nitroprusside (10^{-9}-$10^{-4}M$). Results are expressed as % reversal of the initial methacholine contraction. On each experimental day, one tissue acted as a time control to ensure that the methacholine contraction was sustained.

To examine the effect of acute changes in oxygen tension on responses to methacholine and ET-1, the oxygen tension in the organ bath was reduced from hyperoxic levels as previously described (3.2.2).

For methacholine, four consecutive cumulative concentration-response curves (10^{-10}-$3x10^{-4}M$), were constructed in each of the three oxygen tensions, firstly in hyperoxia and then in either normoxia or hypoxia followed by a final curve in hyperoxia again. Results are expressed as a % of the maximum response of the initial curve under hyperoxia. On each experimental day, one tissue acted as a time control whereby three consecutive concentration-response curves to methacholine were conducted in hyperoxia alone.

As stated above, it is not possible to conduct three consecutive concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}M$) in the same tracheal ring, therefore each oxygen tension was imposed upon a different tracheal ring. As before, tissues were initially stimulated with a maximal concentration ($10^{-4}M$) of methacholine and responses to ET-1 expressed as a % of this response.

4.2.3 Materials

The following chemicals were used; endothelin-1, indomethacin, L-NAME, methacholine, potassium chloride, salbutamol and sodium nitroprusside (for a list of suppliers and full chemical names, see section 2.1.8). Concentrations in the text refer to the salts, with the exception of salbutamol which is expressed as the base. Stock solutions of drugs were prepared in distilled water and subsequent dilutions made in Krebs-Henseleit solution, with the exception of indomethacin which was dissolved in ethanol. In experiments where indomethacin was used, one tissue acted as a vehicle control whereby an appropriate volume of ethanol was added to the bath.

4.2.4 Analysis Of Results

As stated in section 2.1.9, results are expressed as mean ± s.e.mean. Statistical significance between data sets was tested by two-way analysis of variance. Significance between maximum responses and pD_2 values (the negative log of the concentration evoking 50% of the maximum response) was calculated using Student's t-test. Due to

financial restraints, a maximum contractile response to ET-1 could not be obtained and hence a pD_2 value could not be calculated. In this study, therefore, results for ET-1 have been expressed in terms of the negative log concentration of the EC_{400mg} (see section 2.1.9.2). This system of expressing results is similar to that used by MacLean *et al.* (1992) and Nally *et al.* (1994b). Significance between EC_{400mg} values was calculated using Student's t-test. A probability level of $P<0.05$ was considered significant. Number of observations (n) refers to the number of animals used.

4.3 RESULTS

4.3.1 Optimisation Of Initial Resting Tension

For both chronically hypoxic rat trachea and control rat trachea, changing the degree of applied (passive) tension significantly altered the contractile (active) response evoked by a near maximal concentration of methacholine (10^{-4}M). The measured contractile response increased as the applied tension was increased from 0.25g wt to 0.5g wt, reaching a maximum contraction at 1.0g wt (Figure 4.1). When the applied tension was increased beyond 1.0g wt, there was no significant change in the contractile response to 10^{-4}M methacholine. For control rat trachea, the maximum response to methacholine, at a resting tension of 1.0g wt, 1156.1±181.3mg wt, was not significantly different than the maximum response in chronically hypoxic rat trachea, 1121.4±193.0 mg wt. Number of observations (n) =6 in each case.

Figure 4.1

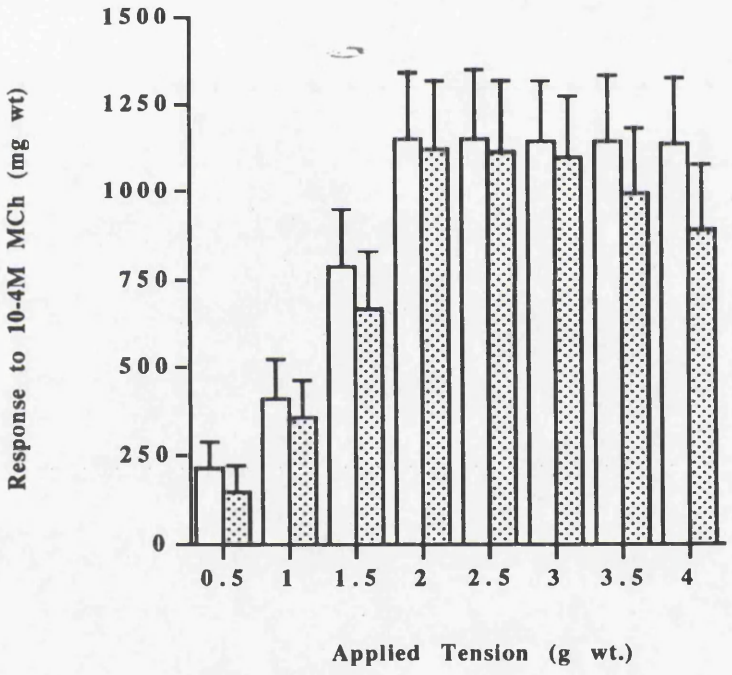


Figure 4.1

The effect of changing the degree of initial resting tension on contractile responses. Under hyperoxic conditions, contractions evoked by a single concentration (10^{-4}M) of methacholine were measured in tracheal rings from control (open columns) and chronically hypoxic rats (stipled columns). Responses are expressed in milligrams weight (mg wt). For both control and chronically hypoxic rat trachea, the optimum resting tension was 1.0g wt. At this tension, the contractile response to methacholine in control rat trachea was not significantly different from the response in trachea from chronically hypoxic rats. Number of observations (n) = 6 in each case.

4.3.1 The Effect Of Chronic Hypoxia On Agonist Responses

Methacholine, ET-1 and KCl each evoked concentration-dependent contractions of tracheal rings isolated from control rats (see Figures 4.2 and 4.3). The threshold concentration for contraction in each case was between $3x10^{-10}$ and $3x10^{-9}$M for methacholine, between 10^{-10} and $3x10^{-10}$M for ET-1 and between 1 and 10mM for KCl. Exposure to chronic hypoxia significantly attenuated contractile responses to methacholine ($P<0.05$ for data sets, n=32), ET-1 ($P<0.01$ for data sets, n=16) and KCl ($P<0.01$ for data sets, n=24) (Figure 4.3). Chronic hypoxia attenuated the sensitivity of the tissue to methacholine (pD_2 values for methacholine in control rat trachea; 5.99±0.08 and for methacholine in hypoxic rat trachea; 5.68±0.10, $P<0.05$ for data points, n=32), but not to ET-1 (-log EC_{400mg} values for ET-1 in control rat trachea, 8.29±0.12 and ET-1 in hypoxic rat trachea, 8.04±0.18, n=8). Maximum responses to methacholine were not altered by chronic hypoxia (maximum response to methacholine in control rat trachea; 1224.5±113.6mg wt and maximum response to methacholine in hypoxic rat trachea; 1067.4±121.3mg wt), however, the maximum responses to both ET-1 and KCl were significantly attenuated by chronic hypoxia (maximum response to ET-1 in control rat trachea; 1539.0±157.4mg wt and maximum response to ET-1 in hypoxic rat trachea; 984.1±118.9, $P<0.05$ for data points, n=8. Maximum response to KCl in control rat trachea; 789.3±49.3mg wt and maximum response to KCl in hypoxic rat trachea; 600.1±56.1mg wt, $P<0.05$ for data points, n=24.).

Figure 4.2

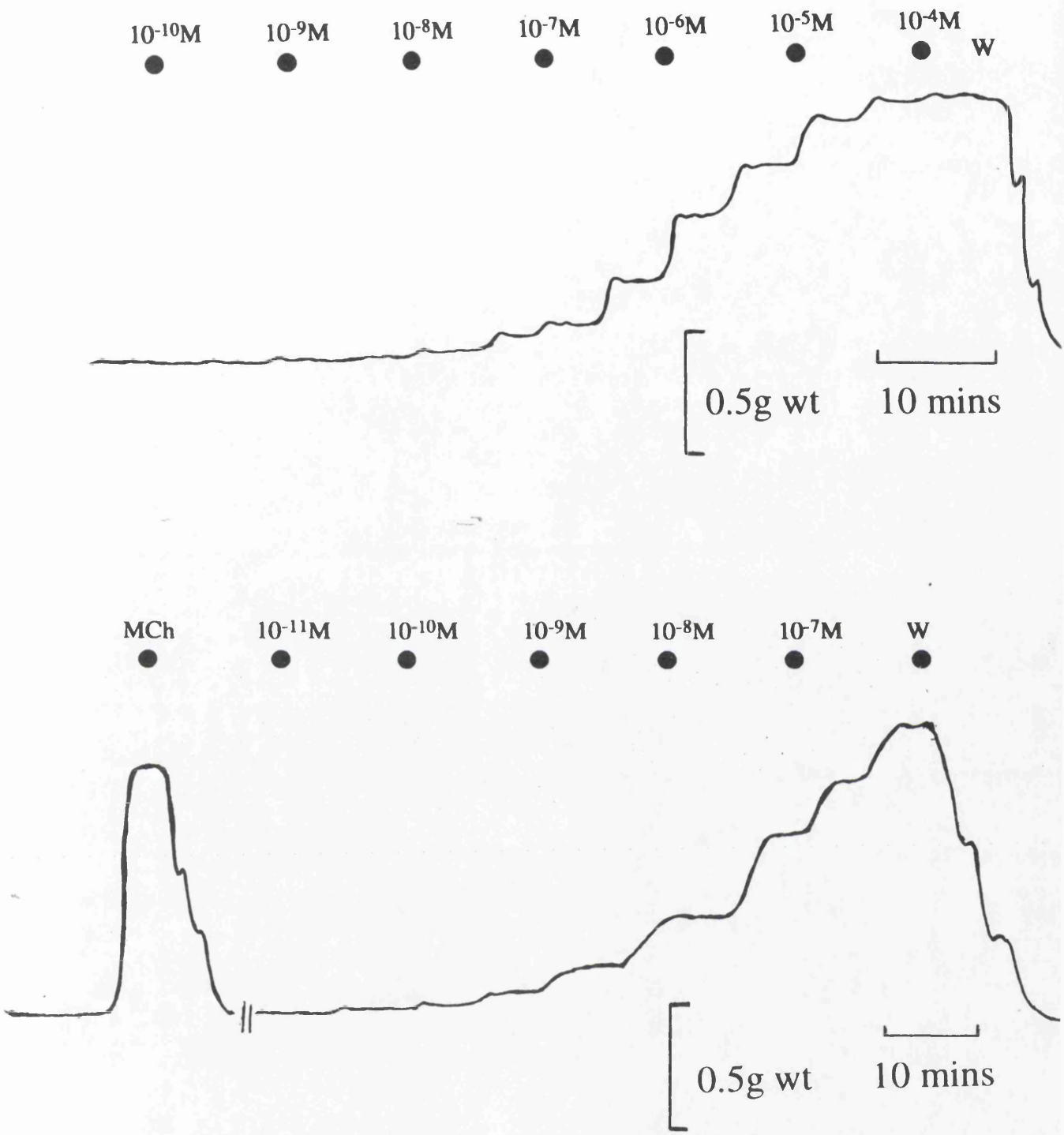
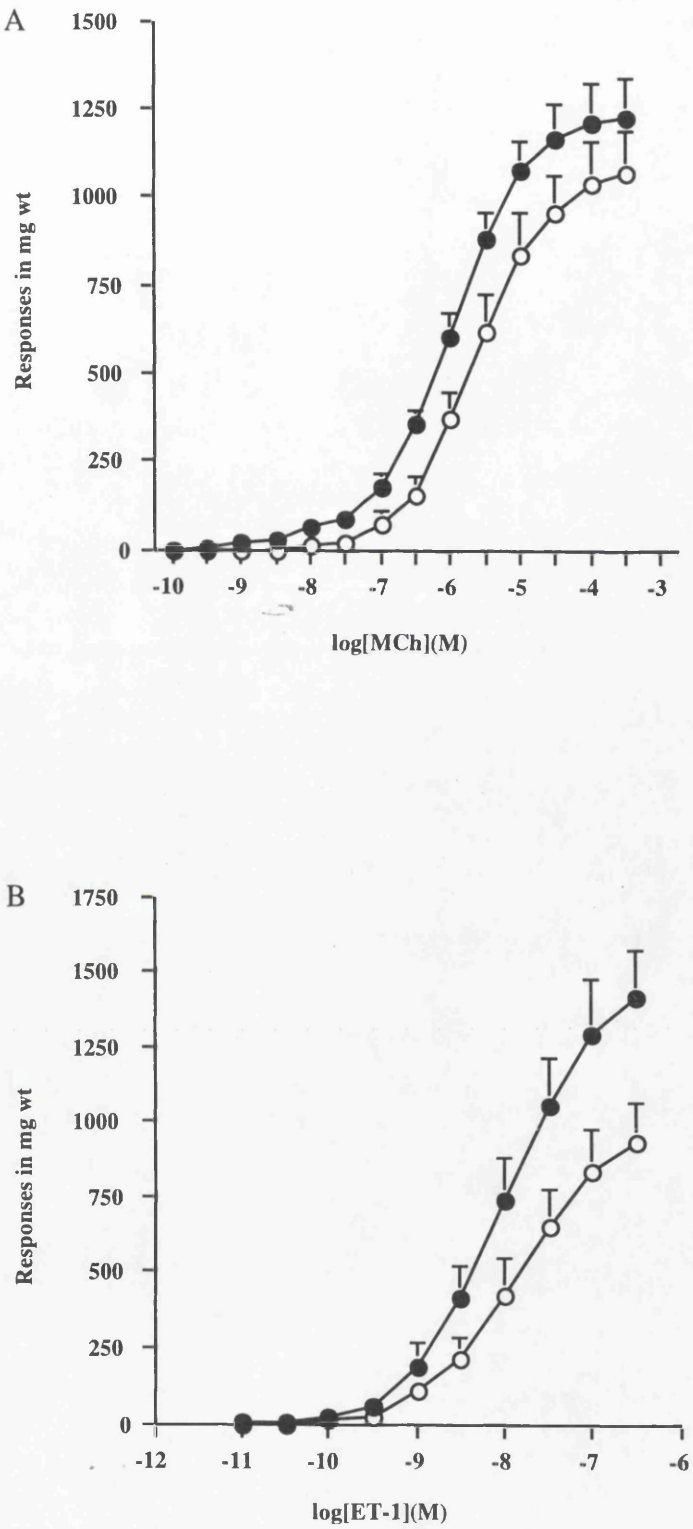


Figure 4.2

Representative traces depicting contractile responses evoked by (A) methacholine and (B) endothelin-1 in rat tracheal rings. Methacholine was added cumulatively to give final bath concentrations of 10^{-10}M - $3x10^{-4}$M. Endothelin-1 was added cumulatively to give final bath concentrations of 10^{-11}M - $3x10^{-7}$M. Before addition of endothelin-1, a single concentration of methacholine (10^{-4}M) was added to produce a reference contraction. Consecutive concentrations were added after the previous response had reached a plateau.

Figure 4.3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1000
750
500
250
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Responses in mg wt
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-7
-6
-5
-4
-3
log[MCh](M)
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1250
1000
750
500
250
0
Responses in mg wt
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-11
-10
-9
-8
-7
-6
log[ET-1](M)

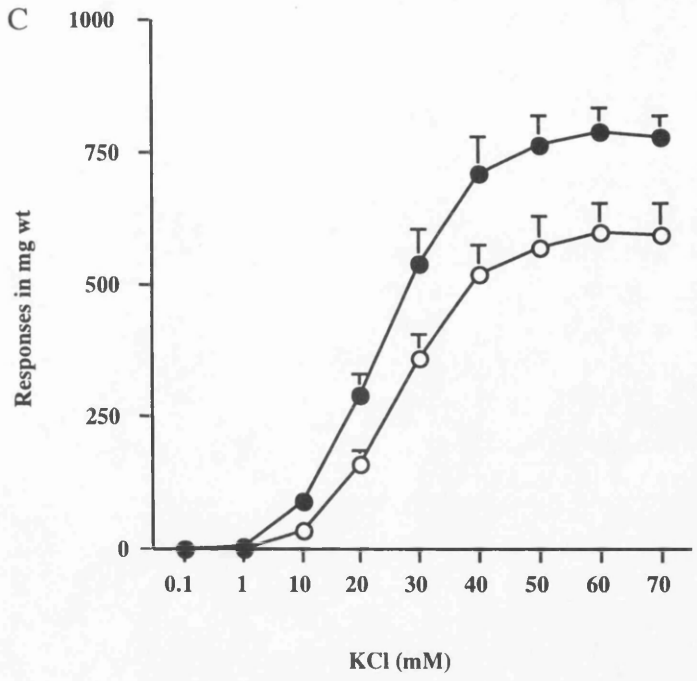


Figure 4.3

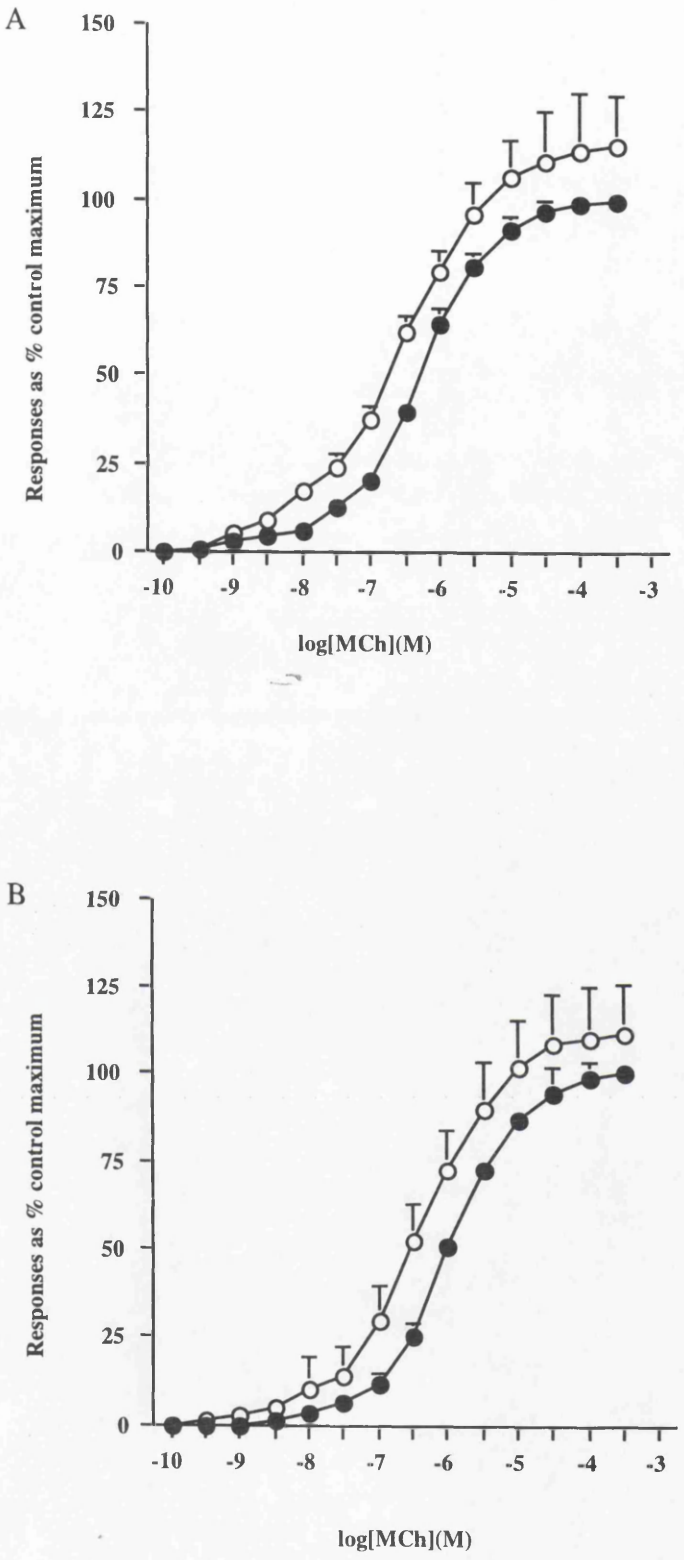
Cumulative concentration-response curves to (A) methacholine, (B) ET-1 and (C) KCl in tracheal rings from control (●) and chronically hypoxic (○) rats. Chronic hypoxia significantly attenuated responses to methacholine ($P<0.05$ for data sets, n=32), ET-1 ($P<0.01$ for data sets, n=16) and KCl ($P<0.01$ for data sets, n=24).

4.3.2 The Effect Of Epithelial Removal On Agonist Responses

Consecutive concentration-response curves to methacholine alone were not significantly different from each other, indicating that methacholine responses did not alter with time (data not shown). In trachea from control rats, removing the epithelial layer significantly ($P<0.05$ for data sets, n=8) enhanced responses to methacholine (Figure 4.4A). Removing the epithelium enhanced the sensitivity of the tissue to methacholine (pD_2 values for methacholine in intact control rat trachea; 6.32±0.08 and pD_2 values for methacholine in denuded control rat trachea; 6.61±0.15, $P<0.05$ for data points, n=8) but did not alter the maximum contractile response (maximum response to methacholine in intact control rat trachea; 99.9±0.2% and maximum response to methacholine in denuded control rat trachea; 115.2±14.7%, n=8). Removing the epithelium had a similar effect on methacholine responses in trachea from chronically hypoxic rats. Responses to methacholine were significantly ($P<0.05$ for data sets, n=8) enhanced in epithelial-denuded tracheal rings from hypoxic rats (Figure 4.4B). Again, removing the epithelium increased the sensitivity of the tissue to methacholine (pD_2 values for methacholine in intact hypoxic rat trachea; 6.04±0.06 and pD_2 values for methacholine in denuded hypoxic rat trachea; 6.26±0.08, $P<0.05$, n=8) without altering the maximum response to methacholine (maximum response to methacholine in intact hypoxic rat trachea; 99.9±1.1% and maximum response to methacholine in denuded hypoxic rat trachea; 111.6±13.9%, n=8).

In epithelium-denuded tracheal rings from control rats, contractile responses to ET-1 were also significantly ($P<0.05$ for data sets, n=8) enhanced (Figure 4.5A). Removing the epithelium evoked a significant increase in the sensitivity of the tissue to ET-1 (-log EC_{400mg} values for ET-1 in intact control rat trachea; 8.29±0.09 and -log EC_{400mg} values for ET-1 in denuded control rat trachea; 8.69±0.12, $P<0.05$ for data points, n=8), albeit without altering the maximum response (maximum response to ET-1 in intact control rat trachea; 74.8±8.5% and maximum response to ET-1 in denuded control rat trachea; 84.1±9.9%, n=8). In chronically hypoxic rats, removing the epithelium again significantly ($P<0.01$, for data sets, n=8) enhanced responses to ET-1 (Figure 4.5B). Removing the epithelium increased the sensitivity of the tissue to ET-1 (-log EC_{400mg} values for ET-1 in intact hypoxic rat trachea; 7.92±0.11 and -log EC_{400mg} values for ET-1 in denuded hypoxic rat trachea; 8.42±0.09, $P<0.01$ for data points, n=8), but did not alter the maximum response (maximum response to ET-1 in intact hypoxic rat trachea; 84.7±10.6% and maximum response to ET-1 in denuded hypoxic rat trachea; 92.4±13.7%, n=8).

Figure 4.4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125
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Responses as % control maximum
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-8
-7
-6
-5
-4
-3
log[MCh](M)
B
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125
100
75
50
25
0
Responses as % control maximum
-10
-9
-8
-7
-6
-5
-4
-3
log[MCh](M)

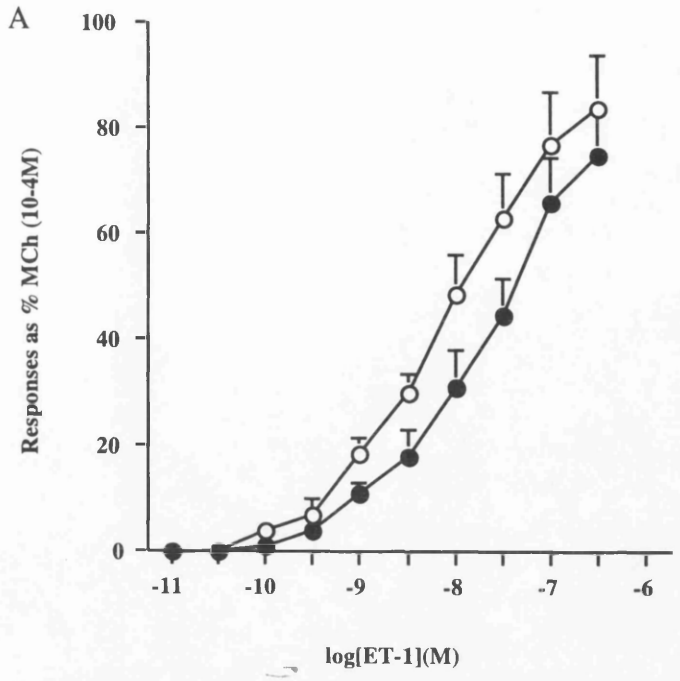
Figure 4.4

Cumulative concentration-response curves to methacholine (10^{-10}-$3x10^{-4}$M) in intact (●) and epithelial-denuded (○) tracheal rings from (A) control rats and (B) chronically hypoxic rats. Removing the epithelium significantly ($P<0.05$ for data sets, n=8) enhanced responses to methacholine in both control and chronically hypoxic rats.

Figure 4.5

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) in intact (●) and epithelial-denuded (○) tracheal rings from (A) control rats and (B) chronically hypoxic rats. Removing the epithelium significantly ($P<0.05$ and $P<0.01$, respectively for data sets, n=8) enhanced responses to ET-1 in both control and chronically hypoxic rats.

Figure 4.5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Responses as % MCh (10-4M)
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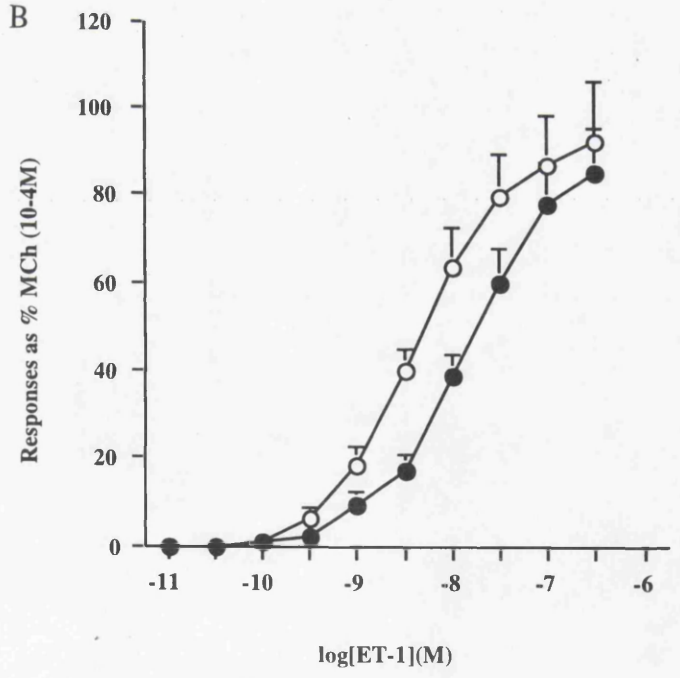
-6
log[ET-1](M)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Responses as % MCh (10-4M)
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-8
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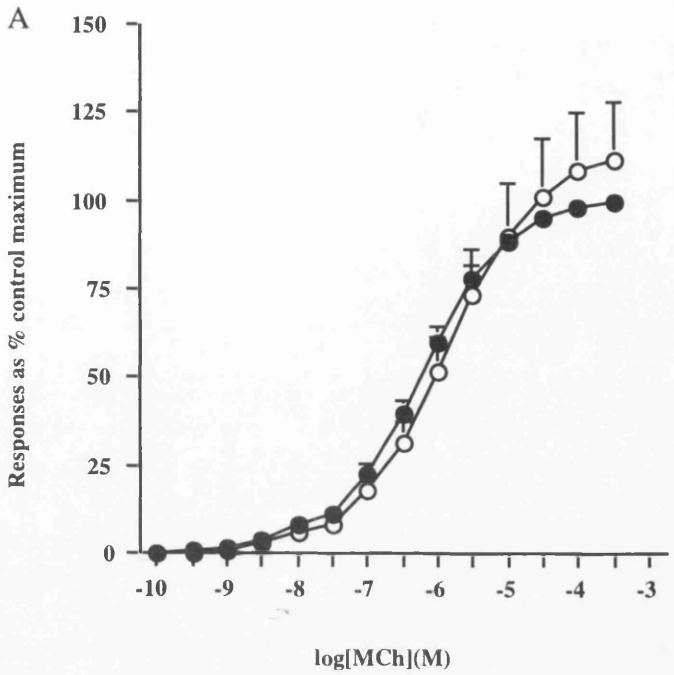
-6
log[ET-1](M)

4.3.3 Effect Of Cyclooxygenase Blockade

Indomethacin ($3x10^{-6}M$) did not alter responses to methacholine in tracheal rings from either control (Figure 4.6A) or chronically hypoxic rats (Figure 4.6B). For control rats, the maximum response to methacholine alone was 99.6±0.3% and the maximum response to methacholine in control rat trachea pretreated with indomethacin was 111.6±16.6%, n=8. The pD_2 values for methacholine alone in control rat trachea were 6.23±0.08 and pD_2 values for methacholine in control rat trachea pretreated with indomethacin were 6.07±0.12, n=8. For chronically hypoxic rats the maximum response to methacholine alone was 99.1±4.7% and the maximum response to methacholine in hypoxic rat trachea pretreated with indomethacin was 95.3±14.4%, n=8. The pD_2 values for methacholine alone in hypoxic rat trachea were 6.21±0.06 and the pD_2 values for methacholine in control rat trachea pretreated with indomethacin were 6.15±0.13, n=8.

In addition, indomethacin did not alter responses evoked by ET-1 in tracheal rings from control rats (Figure 4.7A, maximum response to ET-1 in control rat trachea; 71.5±8.2% and maximum response to ET-1 in control rat trachea pretreated with indomethacin; 77.3±10.2%, n=8. -log EC_{400mg} values for ET-1 in control rat trachea; 8.19±0.07 and pD_2 values for ET-1 in control rat trachea pretreated with indomethacin; 8.08±0.12, n=8) or chronically hypoxic rats (Figure 4.7B, maximum response to ET-1 in hypoxic rat trachea; 87.8±8.2% and maximum response to ET-1 in hypoxic rat trachea pretreated with indomethacin; 87.9±7.6%, n=8. -log EC_{400mg} values for ET-1 in hypoxic rat trachea; 7.86±0.08 and -log EC_{400mg} values for ET-1 in hypoxic rat trachea pretreated with indomethacin; 7.72±0.10, n=8).

Figure 4.6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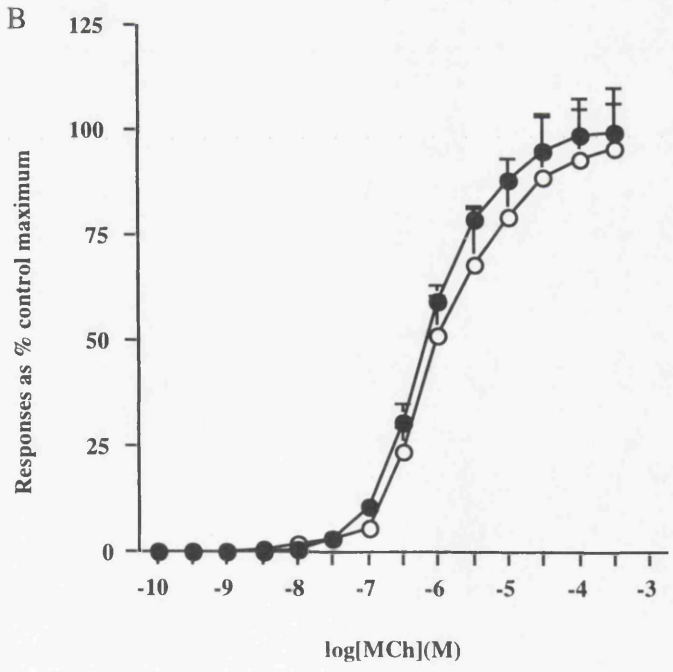
log[MCh](M)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Responses as % control maximum
-10
-9
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-3
log[MCh](M)

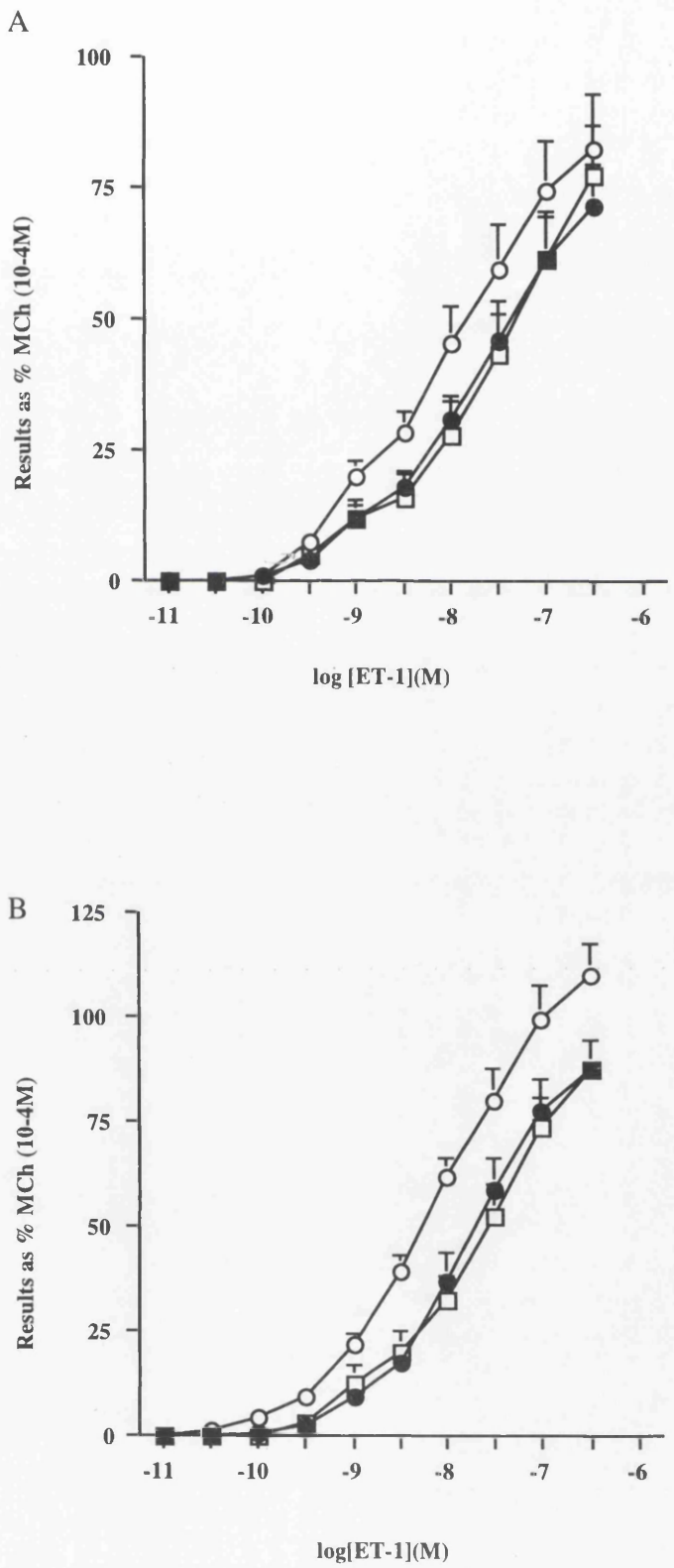
Figure 4.6

Cumulative concentration-response curves to methacholine (10^{-10}-$3x10^{-4}$M) alone (●), or in the presence of $3x10^{-6}$M indomethacin (○) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. Pretreatment with indomethacin did not alter responses to methacholine in either control or chronically hypoxic rats.

Figure 4.7

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) alone (●), or in the presence of either 10^{-4}M L-NAME (○) or $3x10^{-6}$M indomethacin (❑) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. Pretreatment with indomethacin did not alter responses to ET-1 in either control or chronically hypoxic rats. In contrast, pretreatment with L-NAME significantly ($P<0.05$ and $P<0.01$, respectively for data sets) enhanced responses to ET-1 in trachea from both control and hypoxic rats.

Figure 4.7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Results as % MCh (10-4M)
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-7
-6
log [ET-1](M)
B
125
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75
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25
0
Results as % MCh (10-4M)
-11
-10
-9
-8
-7
-6
log[ET-1](M)

4.3.4 Effect Of Nitric Oxide Synthase Blockade

In tracheal rings from control rats, addition of the nitric oxide synthase blocker L-NAME to the organ bath resulted in a small (<200mg wt) but sustained contractile response in 3 from 16 tissue preparations. In tissue from chronically hypoxic rats, the contractile response to L-NAME was of a similar magnitude, but was present in 5 from 16 preparations.

L-NAME (10^{-4}M) significantly ($P<0.05$ for data sets, n=8) potentiated methacholine responses in tracheal rings from control rats (Figure 4.8A, pD_2 values for methacholine in control rat trachea; 6.29±0.10 and pD_2 values for methacholine in control rat trachea pretreated with L-NAME; 6.62±0.14, $P<0.05$ for data points, n=8), albeit without altering the maximum response (maximum response to methacholine in control rat trachea; 99.9±0.1% and maximum response to methacholine in control rat trachea pretreated with L-NAME; 116.2±14.6%, n=8).

In trachea from chronically hypoxic rats, L-NAME significantly ($P<0.01$ for data sets) enhanced responses to methacholine, in this case with a concomitant increase in the maximum response (Figure 4.8B, maximum response to methacholine in hypoxic rat trachea; 99.9±1.1% and maximum response to methacholine in hypoxic rat trachea; 126.2±11.9%, $P<0.05$ for data points, n=8) as well as an increase in the sensitivity of the tissue to methacholine (pD_2 values for methacholine in hypoxic rat trachea; 5.98±0.09 and pD_2 values for methacholine in hypoxic rat trachea pretreated with L-NAME; 6.49±0.11, $P<0.01$ for data points, n=8).

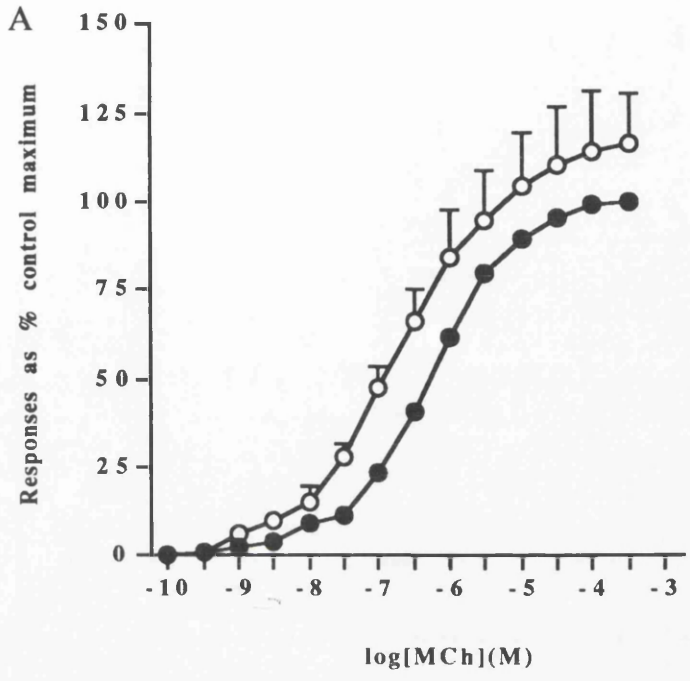
In addition, L-NAME (10^{-4}M) significantly ($P<0.05$ for data sets, n=8) potentiated responses to ET-1 in tracheal rings from control rats (Figure 4.7A, -log EC_{400mg} values for ET-1 in control rat trachea; 8.20±0.11 and -log EC_{400mg} values for ET-1 in control rat trachea pretreated with L-NAME; 8.51±0.07, $P<0.05$ for data points, n=8), albeit without altering the maximum response (maximum response to ET-1 in control rat trachea; 71.5±8.2% and maximum response to ET-1 in control rat trachea pretreated with L-NAME; 82.3±10.5%, n=8).

In trachea from chronically hypoxic rats, L-NAME significantly ($P<0.01$ for data sets) enhanced responses to ET-1, in this case with a concomitant increase in the maximum response (Figure 4.7B, maximum response to ET-1 in hypoxic rat trachea; 87.8±8.2% and maximum response to ET-1 in hypoxic rat trachea; 110.2±7.2%, $P<0.05$ for data points, n=8) as well as an increase in the sensitivity of the tissue to ET-1 (-log EC_{400mg} values for ET-1 in hypoxic rat trachea; 7.86±0.08 and -log EC_{400mg} values for ET-1 in hypoxic rat trachea pretreated with L-NAME; 8.16±0.09, $P<0.05$ for data points, n=8).

Figure 4.8

Cumulative concentration-response curves to methacholine (10^{-10}-$3x10^{-4}$M) alone (●), or in the presence of 10^{-4}M L-NAME (○) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. Pretreatment with L-NAME significantly ($P<0.05$ and $P<0.01$, respectively for data sets, n=8) enhanced responses to methacholine in both control and chronically hypoxic rats.

Figure 4.8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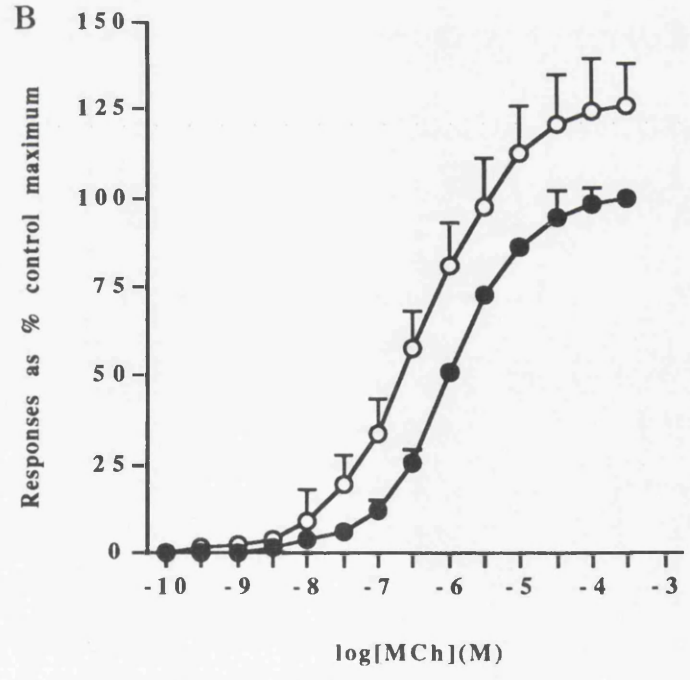
log[MCh](M)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Responses as % control maximum
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-6
-5
-4
-3
log[MCh](M)

4.3.5 Effect Of Chronic Hypoxia On Relaxatory Responses To Salbutamol And Sodium Nitroprusside

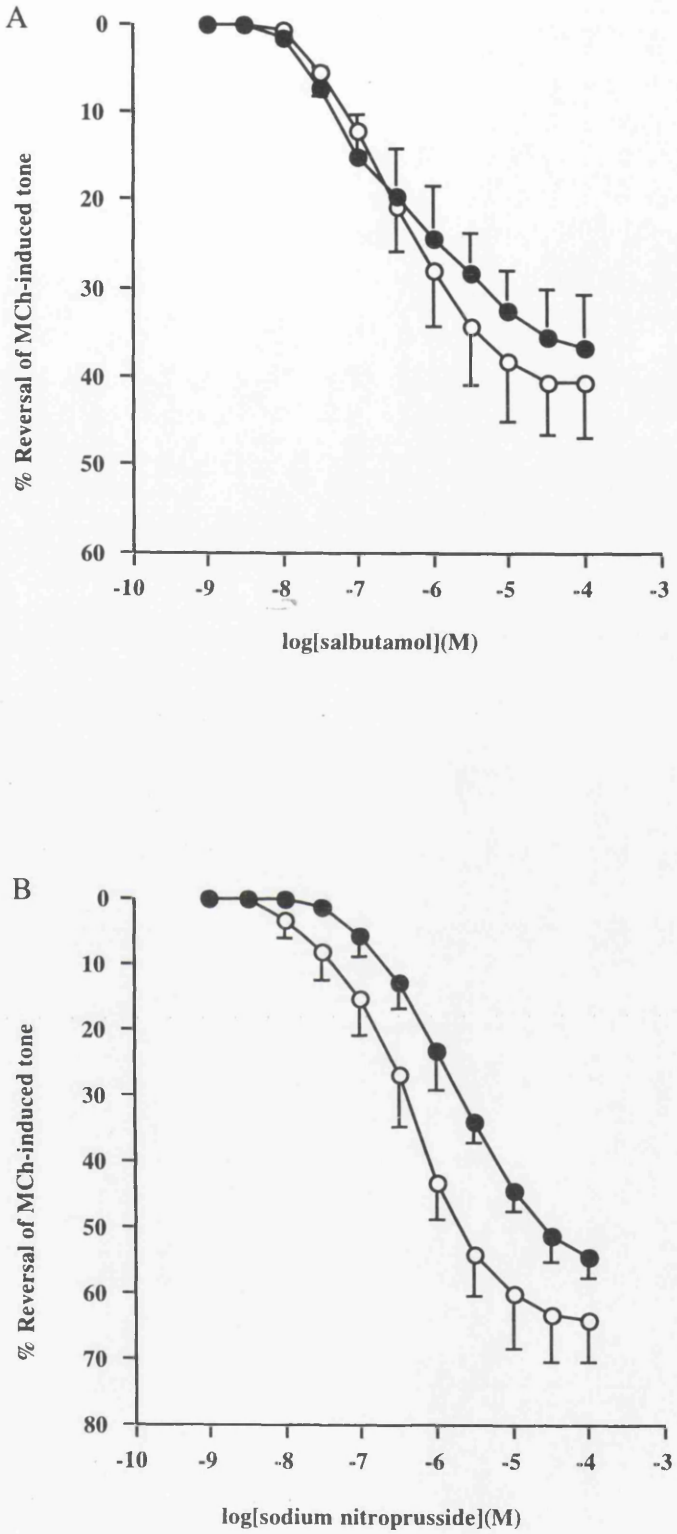
Salbutamol and sodium nitroprusside both reversed methacholine-induced contractions in a concentration dependent manner. Salbutamol initiated responses at concentrations of 10^{-8}M in control rat bronchi and between 10^{-8}M and $3x10^{-8}$M in hypoxic rat bronchi (Figure 4.9A). Chronic hypoxia did not alter the ability of salbutamol to reverse methacholine-induced contraction (mean maximal inhibitions, at the 10^{-4}M level; 36.6±6.0% in control rat bronchi and 40.7±6.3% in bronchi from chronically hypoxic rats).

Sodium nitroprusside was of similar potency to salbutamol, initiating responses at concentrations of $3x10^{-8}$M in control rat bronchi and 10^{-8}M in bronchi from hypoxic rats (Figure 4.9B). In contrast, chronic hypoxia significantly ($P<0.001$ for data sets, n=8) enhanced responses evoked by sodium nitroprusside, albeit without altering the maximum responses (mean maximal inhibitions, at the 10^{-4}M level; 54.6±3.1% in control rat bronchi and 64.1±6.4% in bronchi from chronically hypoxic rats).

Figure 4.9

Reversal of methacholine-induced tone in tracheal rings from control (●) and chronically hypoxic rats (○) by cumulative addition of (A) salbutamol (10^{-9}-10^{-4}M) and (B) sodium nitroprusside (10^{-9}-10^{-4}M). Responses to sodium nitroprusside, but not salbutamol, were significantly ($P<0.001$ for data sets, n=8) greater in trachea from chronically hypoxic rats.

Figure 4.9


A
% Reversal of MCh-induced tone
0
10
20
30
40
50
60
-10
-9
-8
-7
-6
-5
-4
-3
log[salbutamol](M)
B
% Reversal of MCh-induced tone
0
10
20
30
40
50
60
70
80
-10
-9
-8
-7
-6
-5
-4
-3
log[sodium nitroprusside](M)

4.3.6 Effect Of Acute Changes In Oxygen Tension On Methacholine And ET-1 Responses

In trachea from control rats, changing the oxygen tension from 95% to 20% did not alter the responses evoked by methacholine (pD_2 in 20% O_2; 6.10±0.14 compared with the pD_2 value in 95%; 6.28±0.11. Maximum response in normoxia; 96.8±10.9% compared with the maximum response in hyperoxia; 99.7±0.3%). When the oxygen tension was lowered to hypoxic levels, responses to methacholine were significantly ($P<0.01$ for data sets, n=6) attenuated, albeit with no change in the sensitivity of the tissue (pD_2 in hypoxia; 6.27±0.19 compared with pD_2 in 95%; 6.28±0.11). The maximum response to methacholine, however, was significantly ($P<0.05$ for data points, n=6) attenuated in hypoxia (maximum response in hypoxia; 70.1±9.8% compared with the maximum response in hyperoxia; 99.7±0.3%, see Figure 4.10A).
When the oxygen tension was returned to hyperoxic levels and a fourth concentration response curve constructed to methacholine, responses were not significantly different from the initial responses in hyperoxia (pD_2 for second hyperoxic curve; 6.17±0.15 compared with the pD_2 value for the initial hyperoxic curve; 6.28±0.11. Maximum response for the second hyperoxic curve; 92.8±9.4% compared with the maximum response for the initial hyperoxic curve; 99.7±0.3%).

Trachea from hypoxic rats showed a similar pattern in that responses to methacholine were not altered by lowering the oxygen tension from 95% to 20% (pD_2 in 20% O_2; 6.30±0.12 compared with pD_2 in 95%; 6.08±0.16. Maximum response in 20% O_2; 107.8±13.4% compared with the maximum response in hyperoxia; 99.9±0.03%, whereas in hypoxia responses to methacholine were significantly ($P<0.05$ for data set, n=6) attenuated. Again there was no change in the sensitivity of the tissue to methacholine (pD_2 in hypoxia O_2; 6.14±0.10 compared with pD_2 in hyperoxia; 6.08±0.16), while the maximum response in hypoxia was significantly ($P<0.05$ for data points, n=6) attenuated. (Maximum response in hypoxia; 75.1±10.3% compared with the maximum response in hyperoxia; 99.9±0.03%, see Figure 4.10B).
In addition, when the oxygen tension was returned to hyperoxic levels and a fourth concentration response curve constructed to methacholine, responses were not significantly different from the initial responses in hyperoxia (pD_2 for second hyperoxic curve; 6.19±0.18 compared with the pD_2 value for the initial hyperoxic curve; 6.08±0.16. Maximum response for the second hyperoxic curve; 90.8±10.4% compared with the maximum response for the initial hyperoxic curve; 99.8±0.2%).

There was no significant difference between the three consecutive methacholine-concentration response curves conducted in 95% O_2 (data not shown), thus indicating that responses to methacholine are not altered by time.

Figure 4.10

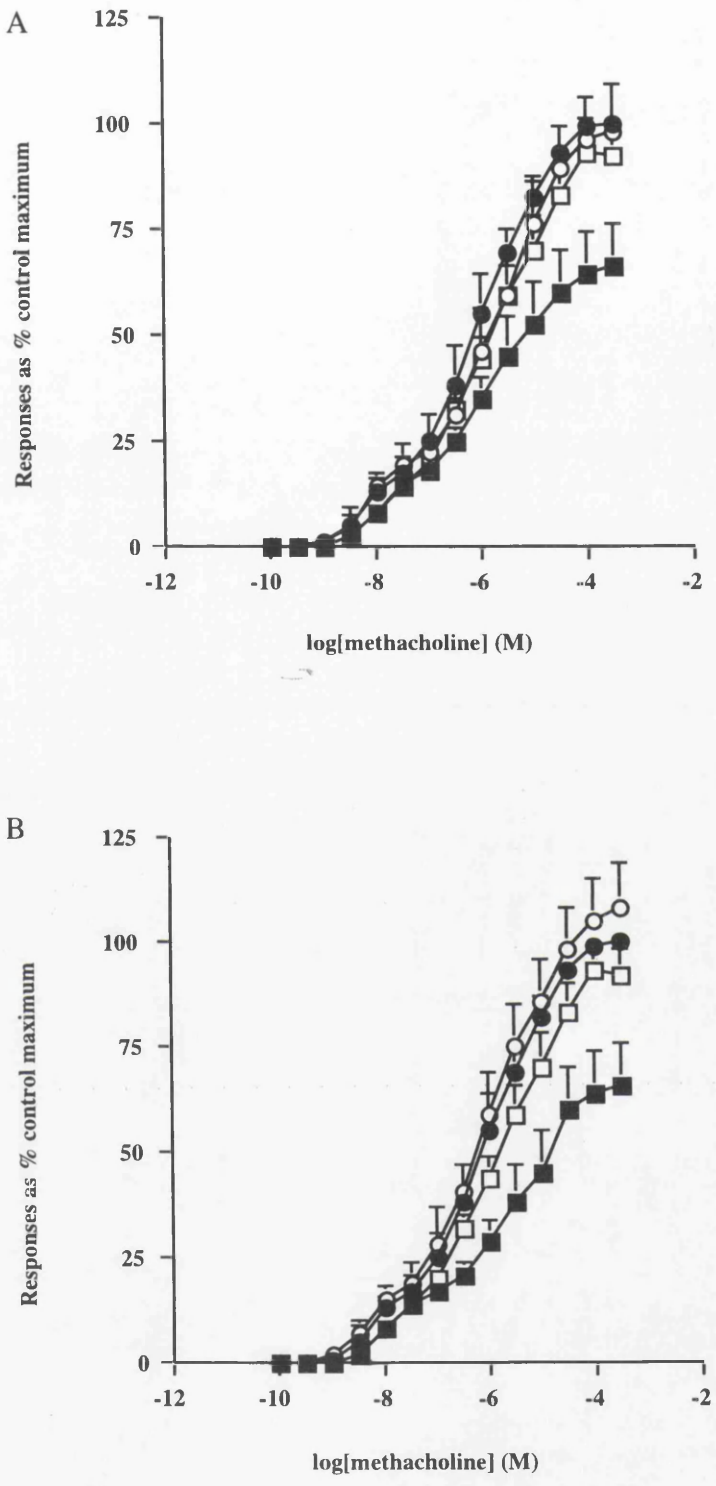


Figure 4.10

Cumulative concentration-response curves to methacholine (10^{-10}-$3x10^{-4}$M) in oxygen tensions of hyperoxia (initial curve, ●), normoxia (○), hypoxia (■) and hyperoxia again (final curve, ❑) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. Acute hypoxia significantly impaired responses to methacholine in both control ($P<0.01$, respectively for data sets, n=6) and chronically hypoxic ($P<0.05$, respectively for data sets, n=6) rats.

There were close similarities in the pattern of responses evoked by methacholine and ET-1. In control rat trachea, changing the oxygen tension from 95% to 20% did not alter the responses evoked by ET-1 (-log EC_{400mg} values for ET-1 in 20% O_2; 8.28±0.13 compared with -log EC_{400mg} values in 95%; 8.34±0.12. Maximum response in normoxia; 76.1±10.6% compared with the maximum response in hyperoxia; 70.7±9.9%). When the oxygen tension was lowered to hypoxic levels, however, responses evoked by ET-1 were significantly ($P<0.01$ for data sets, n=6) attenuated, with a significant ($P<0.05$ for data points) decrease in the maximum response (maximum response in hypoxia; 48.5±7.9% compared with the maximum response in hyperoxia; 70.7±9.9%). There was no change, however, in the sensitivity of the tissue to ET-1 (-log EC_{400mg} values for ET-1 in hypoxia; 8.08±0.19 compared with -log EC_{400mg} values in hyperoxia; 8.34±0.12, see Figure 4.11A).

To illustrate the comparability of expressing the data in different ways, results are expressed both in absolute terms (mg wt) and as a % of the reference contraction to methacholine. Expressing the results either as a % of the initial contractile response to methacholine (Figure 4.11A) or in mg wt (Figure 4.11B) did not alter the overall pattern of responses. In both cases, contractile responses to ET-1 were not significantly different in hyperoxia or normoxia, but were significantly ($P<0.01$ for data sets, n=6) attenuated.

In trachea from hypoxic rats, there was no difference between responses to ET-1 in 95 and 20% O_2 (-log EC_{400mg} values for ET-1 in 20% O_2; 7.93±0.13 compared with -log EC_{400mg} values in 95%; 7.89±0.12. Maximum response in normoxia; 85.6±11.2% compared with the maximum response in hyperoxia; 81.9±9.8%). In hypoxia, however, responses were significantly ($P<0.001$ for data set, n=6) attenuated, with a significant ($P<0.01$ for data points) decrease in the maximum response (maximum response in hypoxia; 42.2±7.9% compared with the maximum response in hyperoxia; 81.9±9.8%). There was no change, however, in the sensitivity of the tissue to ET-1 (-log EC_{400mg} values for ET-1 in hypoxia; 7.60±0.21 compared with -log EC_{400mg} values in hyperoxia; 7.89±0.12, see Figure 4.12).

Expressing the results either as a % of the initial contractile response to methacholine (Figure 4.12A) or in mg wt (Figure 4.12B) did not alter the overall pattern of responses. In both cases, contractile responses to ET-1 were not significantly different in hyperoxia or normoxia, but were significantly ($P<0.01$ for data sets, n=6) attenuated.

Figure 4.11

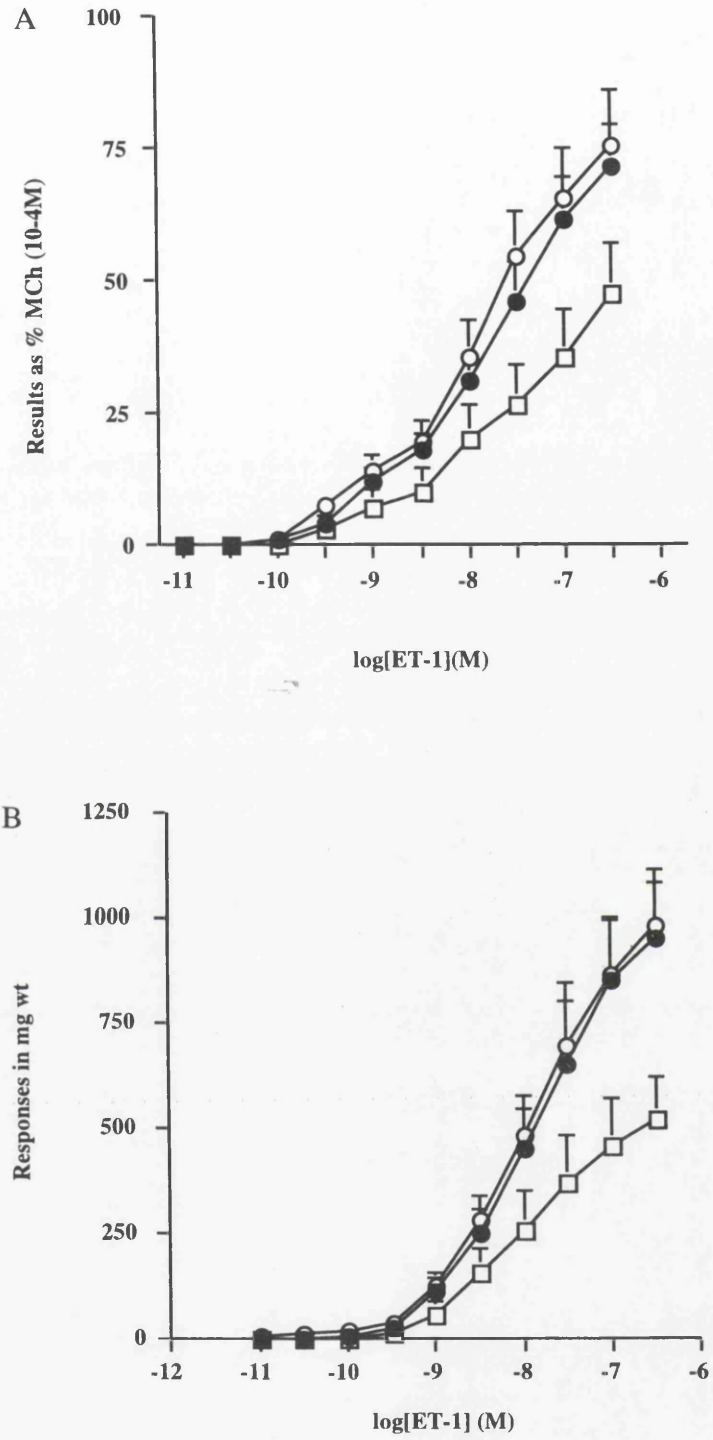


Figure 4.11

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) in oxygen tensions of hyperoxia (●), normoxia (○) or hypoxia (❑) in tracheal rings from control rats. Responses are expressed as (A) a % of the initial reference contraction to methacholine and (B) in mg wt. In both cases, acute hypoxia significantly (in each case, $P<0.01$, respectively for data sets, n=6) impaired responses to ET-1 in control rats.

Figure 4.12

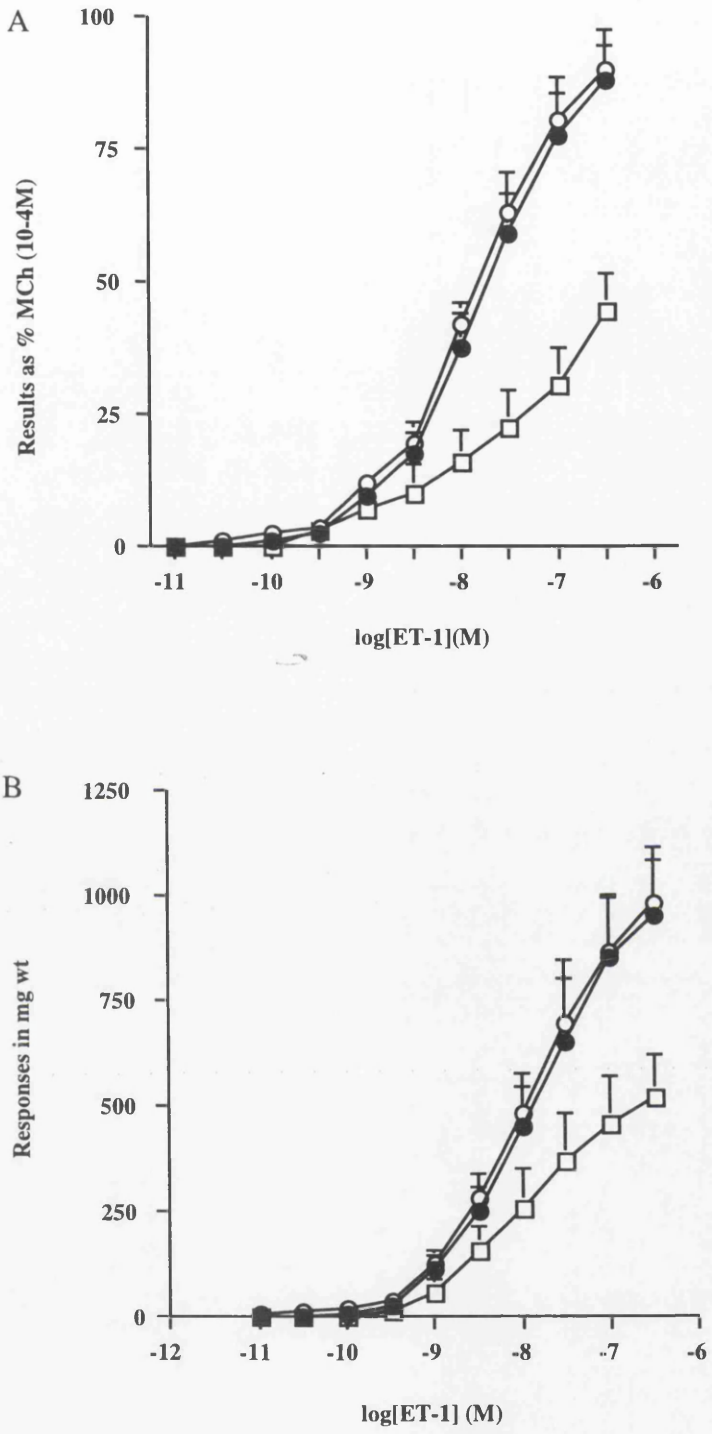


Figure 4.12

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) in oxygen tensions of hyperoxia (●), normoxia (○) or hypoxia (❑) in tracheal rings from chronically hypoxic rats. Responses are expressed as (A) a % of the initial reference contraction to methacholine and (B) in mg wt. In both cases, acute hypoxia significantly (in each case, $P<0.01$, respectively for data sets, n=6) impaired responses to ET-1 in chronically hypoxic rats.

4.4 DISCUSSION

Our initial results showed that responses evoked by the bronchoconstrictors methacholine and ET-1 were significantly attenuated in tracheal rings from chronically hypoxic rats compared to controls. These results are perhaps in keeping with the finding that chronic hyperoxia potentiates contractile responses in rat airways both *in vivo* and *in vitro* (Szarek, 1989; Hershenson *et al.*, 1992a and b; Hershenson *et al.*, 1994; Szarek *et al.*, 1995). A number of possible mechanisms exist which could explain the attenuation which we found in this present study. Both methacholine and ET-1 bind to and activate membrane-bound receptors which are coupled to G proteins and both agonists are thought to initiate contraction of airway smooth muscle via generation of the second messengers IP_3 and DAG (Grandordy *et al.*, 1986; Mattoli *et al.*, 1991). This led us to speculate that chronic hypoxia may inhibit some part of the signalling process which occurs between receptor activation and initiation of contraction. For example, in cardiac tissue, chronic hypoxia has been shown to cause down-regulation of β-adrenoceptors (Voelkel *et al.*, 1981) as well as uncoupling of these receptors from their regulatory G proteins (Richalet, 1990). Whether this occurs with airway muscarinic and ET-1 receptors is unknown, however, chronic hypoxia has been shown to cause a down-regulation in cholinergic responsiveness in rat pulmonary arteries (Orton *et al.*, 1988). Furthermore, it has also been demonstrated that lung ET-1 levels are elevated by chronic hypoxia (Elton & Oparil, 1992), which would be expected to result in down-regulation of endothelin receptors.

Our results with KCl, however, would suggest that the attenuation of contractile responses is due to some reason other than alterations in receptor number or impairment of signal transduction processes. KCl evokes contraction of smooth muscle by causing depolarization of the smooth muscle membrane, resulting in the stimulation of 'L'-type voltage operated Ca^{2+} channels (see section 1.3.2.4). Contractions evoked by KCl therefore represent a direct stimulation of airway smooth muscle which does not involve receptor activation and generation of second messengers. The results for KCl followed a similar pattern to methacholine and ET-1, namely a significant impairment of contractile responses in trachea from chronically hypoxic rats. This indicates that some other mechanism(s) must be responsible for the attenuation of agonist-induced contractions.

A previous study by Hershenson *et al* (1994) demonstrated that hyperoxia-induced airway hyperresponsiveness was substantially reduced by either epithelial removal or treatment with the cyclooxygenase inhibitor indomethacin. This strongly suggests that epithelium-derived cyclooxygenase metabolites contribute to the increased airway reactivity in rats exposed to chronic hyperoxia. This led us to postulate that release of cyclooxygenase metabolites (perhaps inhibitory prostaglandins or prostacyclin) could be responsible for the attenuation of contractile responses in chronically hypoxic rat

airways. In this present study, removing the epithelium enhanced responses to methacholine and ET-1 in tracheal rings from both control and chronically hypoxic rats, suggesting that the epithelium is indeed releasing an inhibitory factor in both of these preparations. Indomethacin, however, did not alter responses evoked by these agonists in either control or hypoxic rats, indicating that this inhibitory factor is not a cyclooxygenase metabolite. Our results suggest that this inhibitory factor may be nitric oxide or a nitric oxide-like substance, since the nitric oxide synthase inhibitor L-NAME enhanced contractions evoked by methacholine and ET-1. Chronic hypoxia increases nitric oxide synthase expression in the rat lung and induces *de novo* nitric oxide synthase expression in the rat pulmonary artery endothelium (Xue *et al.*, 1994), supporting the suggestion of an increased basal release of nitric oxide in the chronically hypoxic rat lung. More recently Shaul *et al* (1995) demonstrated that expression of nitric oxide synthase I, the isoform of nitric oxide synthase localized to the airway epithelium in rats (Schmidt *et al.*, 1992), is increased in rats exposed to chronic hypoxia. This suggests that an increased release of nitric oxide may be responsible for the attenuation in contractile responses in airways from chronically hypoxic rats. Perhaps in keeping with the finding that nitric oxide synthase is expressed in the airway epithelium of both control and chronically hypoxic rats (Xue *et al.*, 1994), we found that L-NAME not only potentiated contractions in chronically hypoxic trachea, it also enhanced responses in control tissue. It would appear, therefore, that the hyporesponsiveness of chronically hypoxic rat trachea is not simply due to the release of nitric oxide, since nitric oxide is also released from control rat trachea. This led us to speculate that the responsiveness of the airway smooth muscle to nitric oxide may be altered by chronic hypoxia. To this end, we conducted experiments whereby the ability of the nitric oxide donor SNP to reverse methacholine-induced tone was compared in trachea from control and chronically hypoxic rats. As shown in Figure 4.7, responses to SNP were significantly greater in trachea from chronically hypoxic rats. This does not appear to represent a non-specific enhancement of bronchodilator responses, since responses to the β_2-adrenoceptor agonist salbutamol, which acts via stimulation of adenylyl cyclase (Rinard *et al.*, 1983) as well as opening of membrane potassium channels (Miura *et al.*, 1992), were not altered by chronic hypoxia. Mindful of the reduced contractile response to methacholine in hypoxic rat trachea, we used concentrations of methacholine which produced approximately 30% of the maximum contractile response in trachea from both control and hypoxic rats. This, together with the finding that salbutamol responses were unaltered by chronic hypoxia, indicates that the enhancement of SNP responses is unlikely to be due to the inverse relationship between the level of airway tone and the potency of relaxant agonists (Van den Brink, 1973).

Interestingly, a recent study in Fisher rats, a strain of rats which exhibit airway hyperrresponsiveness, found results which are in direct contrast to our findings. Jia *et al* (1995) demonstrated that contractile responses to carbachol were potentiated in the

Fisher rats while relaxant responses to SNP were attenuated. The authors speculated that the increased responsiveness to spasmogens in these rats is due to a decreased guanylyl cyclase response to nitric oxide. In favour of this hypothesis is the finding that SNP produces significantly less cGMP in airway smooth muscle from Fisher rats than controls (Jia *et al.*, 1995). Our results with L-NAME indicate that airways of chronically hypoxic and control rats release nitric oxide or a nitric oxide-like substance which opposes contractile responses. It may be possible, therefore, that the decreased response to spasmogens and increased response to SNP which we found in chronically hypoxic rats in our study is due to an increased guanylyl cyclase response produced by chronic exposure to hypoxia, although further studies must be undertaken to elucidate this.

Nitric oxide may be released from a number of potential sources within the airways, including airway smooth muscle (Xue *et al.*, 1994), non-adrenergic non-cholinergic nerve terminals (Belvisi *et al.*, 1991; Li & Rand, 1991), alveolar macrophages (Stuehr & Marletta, 1987) and inflammatory cells (McCall *et al.*, 1989). Our results showed that removing the epithelium mimics the effect of L-NAME on contractile responses in both control and hypoxic rats, suggesting that nitric oxide or a nitric oxide-like substance is being released from the epithelial layer in our preparations. In dogs (Gao & Vanhoutte, 1993) and rabbits (Spina & Page, 1991) it has been proposed that the airway epithelium is not a major source for endogenous nitric oxide-like substances, however, previous studies have indeed confirmed nitric oxide synthase expression in the airway epithelium of both control and chronically hypoxic rats (Xue *et al.*, 1994). Soluble guanylyl cyclase, however, appears to be localized to the airway smooth muscle and not the epithelium (Rengasamy *et al.*, 1994), indicating that nitric oxide performs a paracrine role in the respiratory system, being produced by the airway epithelium and activating soluble guanylyl cyclase in airway smooth muscle. The functional role of the nitric oxide synthase signalling pathway in the control of airway function remains controversial (Munakata *et al.*, 1990; Nijkamp *et al.*, 1993; Spina & Page, 1991; Stuart-Smith, 1990), but our results support physiological studies which suggest that endogenous nitric oxide may act as a bronchodilator (Alving *et al.*, 1993). In our present study, adding L-NAME to the organ baths resulted in a small contraction in 3 from 16 tracheal rings from control rats and 5 from 16 from hypoxic rats. This indicates that there was basal release of nitric oxide in only a few tissue preparations, whereas L-NAME significantly potentiated contractions in almost all cases. This suggests that nitric oxide may be released in response to contraction of the airway smooth muscle and would then act to oppose the contractile response.

In addition, I chose to study the effect of acute changes in oxygen tension on contractile responses in both control and chronically hypoxic rats. Our results showed that lowering the oxygen tension from hyperoxic to normoxic levels did not alter responses to methacholine or ET-1, in either the control or the hypoxic rats. When the oxygen tension was lowered to hypoxic levels, however, responses to both methacholine and

ET-1 were significantly attenuated. The pattern of responses was similar between the control and the chronically hypoxic rats, indicating that prior exposure to chronic hypoxia does not alter the sensitivity of the airways to acute changes in oxygen tension. Furthermore, returning the oxygen tension from hypoxic to hyperoxic levels returned the contractile responses to levels similar to those of the first hyperoxic response. This indicates that the attenuation of contractile responses caused by acute hypoxia are reversible and therefore not due to hypoxia-mediated apoptosis of the airway smooth muscle cells.

In summary, contractile responses to methacholine, ET-1 and KCl were attenuated in isolated trachea from chronically hypoxic rats. Indomethacin did not alter responses to methacholine or ET-1, however, addition of L-NAME or removal of the epithelium significantly enhanced contractile responses in both control and chronically hypoxic rats. The ability of SNP to reverse methacholine-induced tone was enhanced in trachea from chronically hypoxic rats. These results show that, in both chronically hypoxic and control rats, contractile responses to methacholine and ET-1 are attenuated by nitric oxide which appears to be released from the airway epithelium. The attenuation of contractile responses in airways from chronically hypoxic rats may be due to an enhanced guanylyl cyclase activity and hence, an increased response to nitric oxide.

CHAPTER 5

THE EFFECT OF CHRONIC HYPOXIA ON ENDOTHELIN RECEPTOR SUBTYPE-MEDIATED RESPONSES IN RAT ISOLATED AIRWAYS

5.1 INTRODUCTION

Endothelin-1 (ET-1) is one of the most potent bronchoconstrictors yet isolated, producing prolonged contractions of airway smooth muscle both *in vivo* and *in vitro* (McKay *et al.*, 1992). ET-1 is produced and released from airway epithelial cells (Black *et al.*, 1989; Mattoli *et al.*, 1990) as well as alveolar macrophages (Ehenreich *et al.*, 1990). Elevated levels of ET-1 have been reported in bronchoalveolar lavage from asthmatic patients (Nomura, 1989) and expression of ET-1 is increased in bronchial epithelial cells cultured from asthmatics (Springall *et al.*, 1991; Vittori *et al.*, 1992) suggesting a possible role for ET-1 as a mediator in asthma.

At least two distinct endothelin receptor subtypes, ET_A and ET_B, exist in mammalian tissues (Arai *et al.*, 1990; Sakurai *et al.*, 1990). The ET_A receptor has a higher affinity for ET-1 or ET-2 compared with ET-3, whereas the ET_B receptor has equal affinity for the three members of the ET family (Masaki, 1991). It is now established that both ET_A and ET_B receptors can mediate ET-1-induced contraction of airway smooth muscle (Hay, 1992; Hay *et al.*, 1993; Henry, 1993), however, there appears to be considerable variation between species in terms of which receptor subtype predominates. For example, in guinea-pig tracheal smooth muscle, ET-1 evokes contraction mainly via activation of ET_B receptors (Hay, 1992), whereas in sheep airways, ET-1-induced bronchoconstriction is mediated by stimulation of ET_A receptors (Abraham *et al.*, 1993; Goldie *et al.*, 1994). In rat trachea and also in human bronchus, ET-1-induced contractions appear to be mediated by activation of both ET_A and ET_B receptors (Henry, 1993; O' Donnell & Kay, 1995; Fukuroda *et al.*, 1996).

Our preliminary experiments indicated that contractile responses to ET-1 were significantly attenuated in isolated airways from rats exposed to chronic hypoxia (see section 4). This may be due to an alteration in the ET receptor population in the airways since hypoxia has been reported to alter the expression of ET_A-receptor mRNA in rat lungs (Li *et al.*, 1994). Given that ET-1 appears to play a pathophysiological role in airway diseases such as asthma (Barnes, 1994), ET receptor antagonists may have a role in the clinical management of this disorder. It may be of importance, therefore, to discover if chronic hypoxia, which can often be a feature in severe asthma, alters the ET receptor subtypes which mediate contraction of airway smooth muscle. In this study, the ET_B selective agonist sarafotoxin S6c (Williams *et al.*, 1991), the ET_A selective antagonist FR 139317 (Sogabe *et al.*, 1993), the ET_B selective antagonist BQ 788 (Ishikawa *et al.*, 1994) and the non-selective ET receptor antagonist SB 209670 (Ohlstein *et al.*, 1994) were used to characterize the ET receptor subtypes which mediate contraction of isolated trachea from chronically hypoxic and control rats.

Furthermore experiments were undertaken to ascertain if ET receptor activation could induce reversal of agonist-induced tone in control and chronically hypoxic rats.

5.2 METHODS

5.2.1 Development Of Chronic Hypoxia

Rats were made chronically hypoxic as described in section 2.1.7.4. Age matched control rats were reared alongside the hypobaric chamber but were maintained in room air throughout.

5.2.2 Tissue Preparation

Tracheal ring preparations were obtained from control and chronically hypoxic rats as described in section 2.1.2.1 and mounted in 5ml organ baths as described in section 2.1.1. Contractile responses were measured in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution at 37 ± 0.5°C. Initial experiments indicated that, for both control and hypoxic animals, the optimum level of applied tension was 1.0 g wt. Tissues were allowed to equilibrate for 45 minutes, during which time tension was reapplied where necessary.

5.2.3 Measurement Of Contractile Responses

A reference response to methacholine (10^{-4}M) was obtained for all preparations and tissues were allowed a further 45 minutes to equilibrate. Concentration-response curves were then constructed to either ET-1 (10^{-11}-$3x10^{-7}$M) or sarafotoxin S6c (10^{-11}-$3x10^{-7}$M) by cumulative addition to the organ baths. The ET receptor antagonists were added 30 minutes before, and L-NAME 5 minutes before, addition of ET-1.

5.2.4 Measurement Of Relaxatory Responses

Rings of trachea from control and chronic hypoxic rats were prepared as above. Tracheal rings were preconstricted with a single concentration of methacholine ($3x10^{-7}$M for control rat trachea and 10^{-6}M for hypoxic rat trachea). The concentrations used were approximately the EC_{30} for methacholine in trachea from control and chronically hypoxic rats. Endothelin receptor antagonists were then preincubated for 30 minutes prior to cumulative addition of either ET-1 (10^{-12}-10^{-8}M) or sarafotoxin S6c (10^{-12}-10^{-8}M). In a separate series of experiments, cumulative concentration-response curves were constructed to sodium nitroprusside (10^{-9}-10^{-4}M) in tracheal rings preconstricted with methacholine. On each experimental day, one tissue acted as a time control to ensure that the methacholine contraction was sustained.

5.2.5 Data Analysis

Due to financial restraints, a maximum contractile response to ET-1 could not be obtained and hence a pEC_{50} value could not be calculated. In this study, therefore, results for ET-1 have been expressed in terms of the negative log concentration of the EC_{200mg} and the EC_{400mg} (see section 2.1.9.2). This system of expressing results is

similar to that used by MacLean *et al.* (1992) and Nally *et al.* (1994b). Relaxatory responses are expressed as % reversal of the methacholine-induced tone.

As stated in section 2.1.9, results are expressed as mean ± s.e.mean. Statistical significance between data sets was tested by two-way analysis of variance. Significance between EC_{200mg} and EC_{400mg} values was calculated using Student's t-test. A probability level of $P<0.05$ was considered significant. Number of observations (n) refers to the number of animals used.

5.2.6 Materials

The following chemicals were used; endothelin-1, BQ 788, FR 139317, L-NAME, methacholine, sarafotoxin S6c, sodium nitroprusside and SB 209670. For a list of suppliers and full chemical names, see section 2.1.8. Stock solutions of drugs were prepared in distilled water and subsequent dilutions made in Krebs-Henseleit solution.

5.3 RESULTS

5.3.1 Responses To ET-1 And Sarafotoxin S6c In Control And Chronically Hypoxic Rats

Exposure to chronic hypoxia significantly ($P<0.01$ for data sets, n=8, Figure 5.1A) attenuated contractile responses to ET-1 in rat isolated tracheal rings, with a significant attenuation of the maximum response to ET-1 (maximum response to ET-1 in control rat trachea; 1412.9±161.3mg wt and maximum response to ET-1 in hypoxic rat trachea; 929.3±129.7, $P<0.05$ for data points, n=8). The sensitivity of the tissue to ET-1 was not, however, altered by chronic hypoxia (see Table 5.1 for EC_{200mg} and EC_{400mg} values).

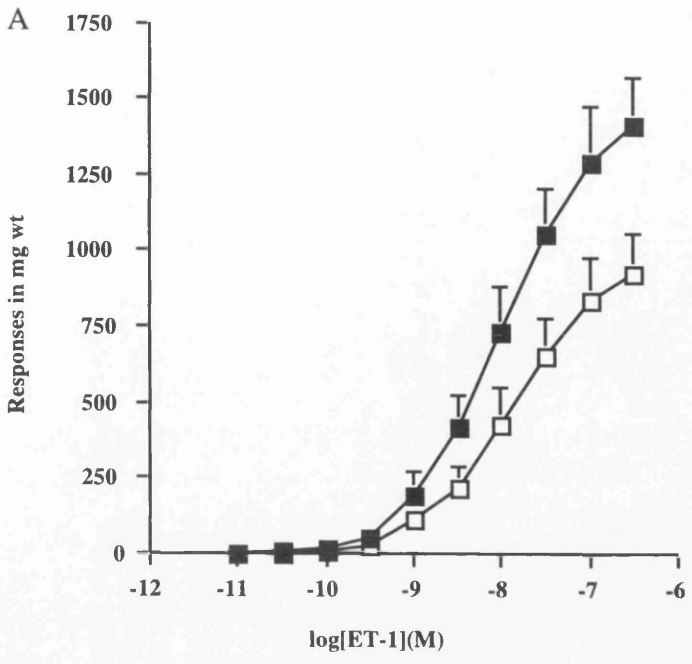
The ET_B receptor-selective agonist, sarafotoxin S6c, induced concentration-dependent contractions of isolated trachea from both control (Figure 5.1B) and chronically hypoxic rats (Figure 5.1C). Sarafotoxin was significantly more potent than ET-1 in control rat trachea, however, in trachea from chronically hypoxic rats, both sarafotoxin and ET-1 were equipotent (see Table 5.1 for EC_{200mg} and EC_{400mg} values).

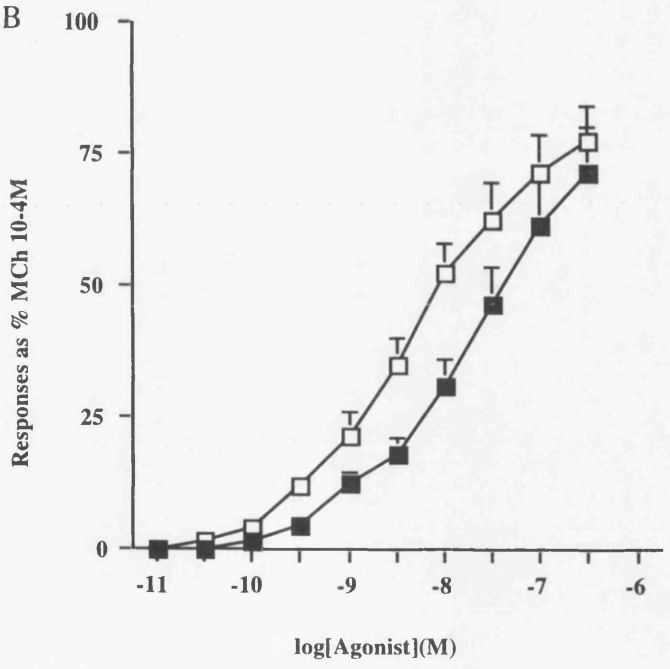
Table 5.1

Group	Control and Chronically Hypoxic rat trachea		
	EC_{200mg} (-log M)	EC_{400mg} (-log M)	(n)
ET-1 control rat trachea	8.83 ± 0.14	8.31 ± 0.11	8
ET-1 hypoxic rat trachea	8.73 ± 0.12	7.98 ± 0.16	8
Sx6c control rat trachea	*9.33 ± 0.18	*8.70 ± 0.15	8
Sx6c hypoxic rat trachea	8.82 ± 0.16	8.12 ± 0.17	8

Table 5.1: Responses to ET-1 and sarafotoxin S6c (Sx6c) in tracheal rings from control and chronically hypoxic rats. Sarafotoxin S6c was significantly more potent than ET-1 in control rat trachea (*$P<0.05$ control S6c more sensitive than control ET-1). In chronically hypoxic rat trachea, ET-1 and sarafotoxin S6c were equipotent.

Figure 5.1


A
1750
1500
1250
1000
750
500
250
0
Responses in mg wt
-12
-11
-10
-9
-8
-7
-6
log[ET-1](M)


B
100
75
50
25
0
Responses as % MCh 10-4M
-11
-10
-9
-8
-7
-6
log[Agonist](M)

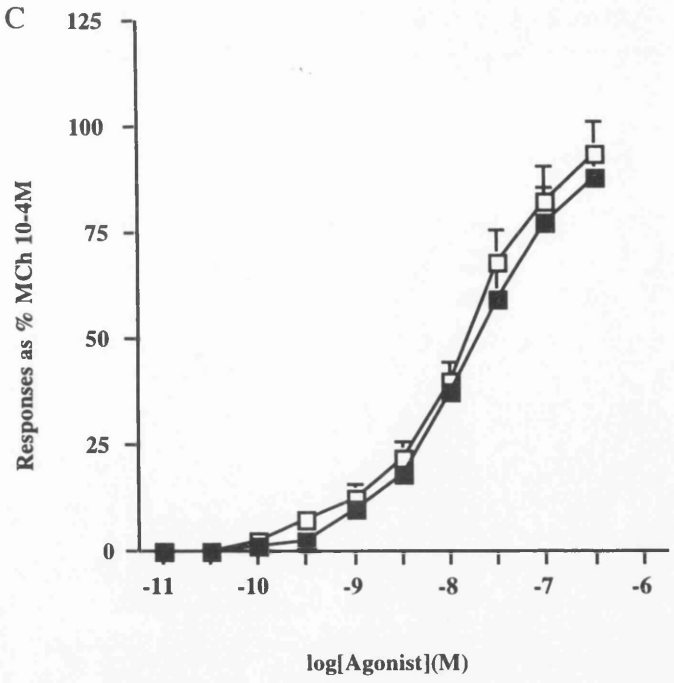


Figure 5.1A

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) in tracheal rings from control (■) and chronically hypoxic (❑) rats. Responses to ET-1 were significantly ($P<0.01$ for data sets) attenuated in tracheal rings from chronically hypoxic rats (n=8).

Figure 5.1B and 5.1C

Cumulative concentration-response curves to ET-1 (■, 10^{-11}-$3x10^{-7}$M) and sarafotoxin S6C (❑, 10^{-11}-$3x10^{-7}$M) in tracheal rings from (B) control rats and (C) chronically hypoxic rats. Sarafotoxin was significantly ($P<0.05$ for data sets) more potent than ET-1 in tracheal rings from control rat trachea, whereas in chronically hypoxic rat trachea, both peptides were of similar potency.

5.3.2 Antagonism Of ET_A Receptors

In trachea from control rats, pre-incubating the ET_A receptor antagonist, FR 139317, at a concentration of 10^{-8}M significantly ($P<0.05$ for data sets) enhanced responses to ET-1 (Figure 5.2A), albeit without altering the sensitivity of the tissue (see Table 5.2A for a summary of EC_{200mg} and EC_{400mg} values). When the concentration of FR 139317 was increased to 10^{-7} or 10^{-6}M however, this enhancement of ET-1 contractions was lost (Figure 5.2A). At these concentrations, FR 139317 had no effect on responses to ET-1 in this tissue.

In trachea from chronically hypoxic rats, pre-incubation of FR 139317 at concentrations of 10^{-8}, 10^{-7} or 10^{-6}M had no significant effect on ET-1-mediated contractions (see Table 5.2B for a summary of EC_{200mg} and EC_{400mg} values). Thus chronic hypoxia appears to have "removed" the ability of low (10^{-8}M) concentrations of FR 139317 to enhance ET-1 contractions (Figure 5.2B).

Figure 5.2

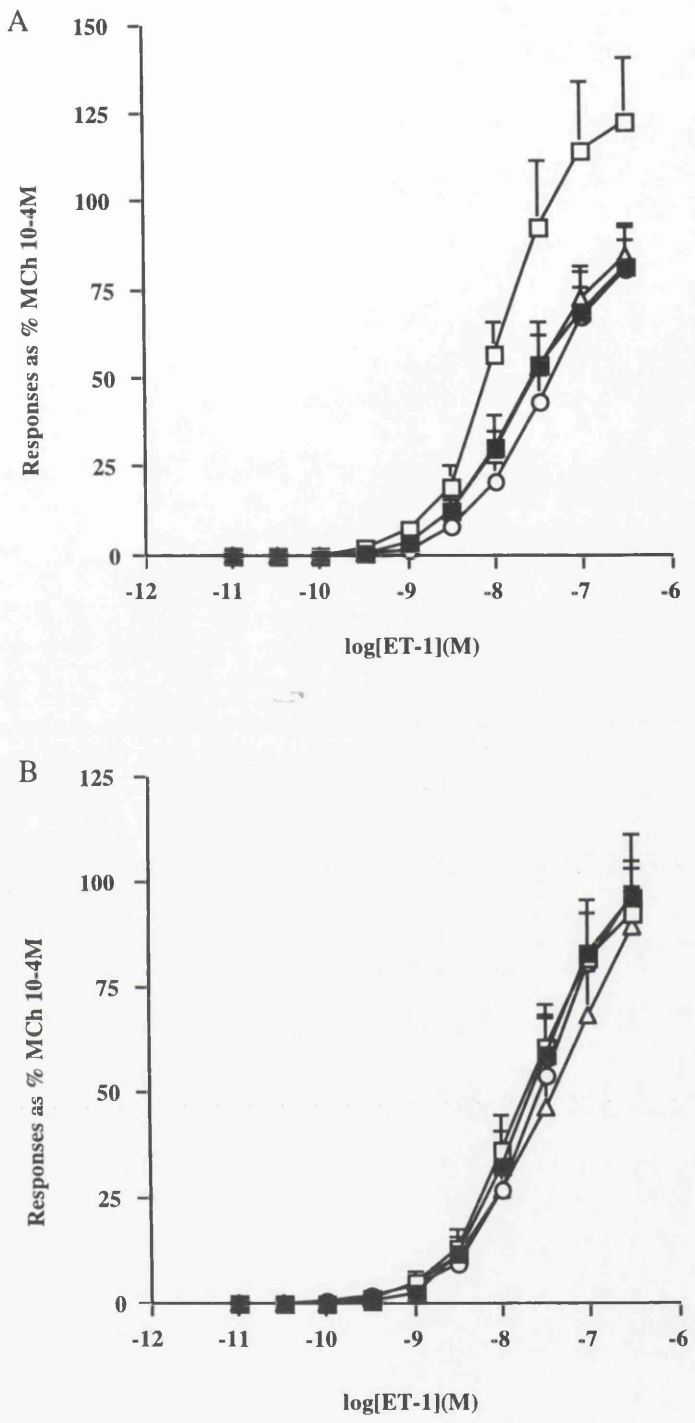


Figure 5.2

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) alone (■), or in the presence of the ET_A receptor antagonist FR 139317 at concentrations of (❑, 10^{-8}M), (○, 10^{-7}M) or (Δ, 10^{-6}M) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. At the 10^{-8}M level, FR 139317 significantly potentiated responses to ET-1 in control rat trachea. At all other concentrations and in chronically hypoxic rat trachea, FR 139317 had no effect on ET-1-mediated contractions.

Table 5.2

A

Group	Control rat trachea EC_{200mg} (-log M)	Control rat trachea EC_{400mg} (-log M)	(n)
ET-1 control	9.03 ± 0.16	8.68 ± 0.18	8
ET-1 plus FR 139317 10^{-8}M	9.17 ± 0.28	8.96 ± 0.19	8
ET-1 plus FR 139317 10^{-7}M	8.93 ± 0.18	8.63 ± 0.17	8
ET-1 plus FR 139317 10^{-6}M	7.96 ± 0.29	8.88 ± 0.15	8

B

Group	Chronically Hypoxic rat trachea EC_{200mg} (-log M)	Chronically Hypoxic rat trachea EC_{400mg} (-log M)	(n)
ET-1 control	8.75 ± 0.07	7.94 ± 0.11	8
ET-1 plus FR 139317 10^{-8}M	8.62 ± 0.16	7.89 ± 0.21	8
ET-1 plus FR 139317 10^{-7}M	8.95 ± 0.25	7.82 ± 0.20	7
ET-1 plus FR 139317 10^{-6}M	8.78 ± 0.24	7.79 ± 0.23	8

Table 5.2: EC_{200mg} and EC_{400mg} values for ET-1 in the presence and absence of the ET_A receptor antagonist FR 139317 in tracheal rings from (A) Control rats and (B) Chronically hypoxic rats. Statistical comparison between values was calculated by Student's t-test. In both control and chronically hypoxic rat trachea, FR 139317 did not alter the sensitivity of the tissue to ET-1.

5.3.3 Antagonism Of ET_B Receptors

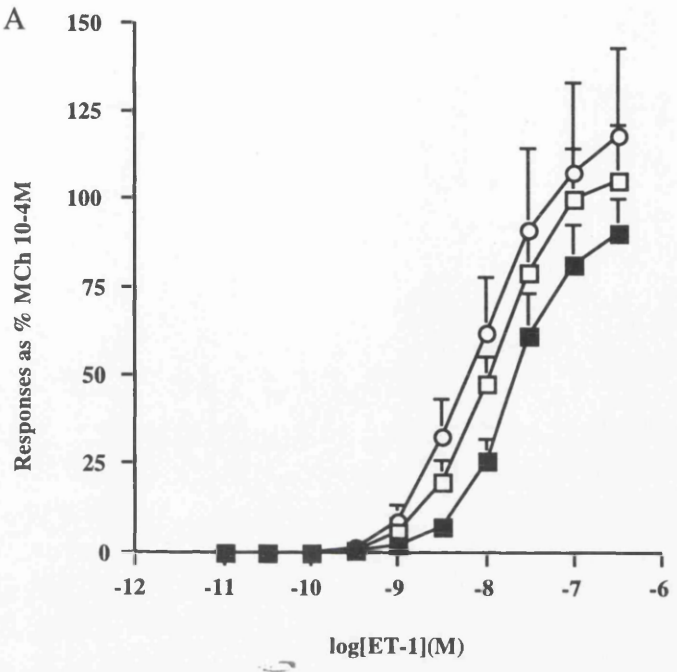
The selective ET_B antagonist BQ 788 failed to inhibit ET-1 mediated contractions in trachea from control rats (Figure 5.3A). Instead it evoked a concentration-dependent enhancement (For data sets, BQ 788 10^{-6}M; $P<0.05$ compared with controls and BQ 788 $3x10^{-6}$M; $P<0.01$ compared with controls) of the ET-1 response. As was the case for 10^{-8}M FR 139317, BQ 788, at the 10^{-6}M level, enhanced ET-1 responses without altering the sensitivity of the tissue (see Table 5.3A for a summary of EC_{200mg} and EC_{400mg} values) When pre-incubated at the $3x10^{-6}$M level however, BQ 788 significantly ($P<0.05$ for data points) enhanced the sensitivity of the tissue to concentrations of ET-1 at the lower end of the ET-1 concentration-response curve whereas the sensitivity of the tissue to higher concentrations of ET-1 was unaltered (see Table 5.3A for a summary of EC_{200mg} and EC_{400mg} values).

The results from chronically hypoxic rats followed a similar pattern (Figure 5.3B). BQ 788, at concentrations of 10^{-6} and $3x10^{-6}$M, significantly ($P<0.05$ and $P<0.01$ for data sets, respectively) enhanced contractions to ET-1. In each case, BQ 788 also significantly ($P<0.05$ for data points, in each case) increased the sensitivity of the tissue to concentrations of ET-1 at the lower end of the ET-1 concentration-response curve. BQ 788 did not alter the sensitivity of the tissue to higher concentrations of ET-1, however (see Table 5.3B for a summary of EC_{200mg} and EC_{400mg} values).

Figure 5.3

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) alone (■), or in the presence of the ET_B receptor antagonist BQ 788 at concentrations of (❑, 10^{-6}M), (○, $3x10^{-6}$M) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. In both control and chronically hypoxic rat trachea, BQ 788 potentiated ET-1 responses in a concentration-dependent manner.

Figure 5.3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Responses as % MCh 10-4M
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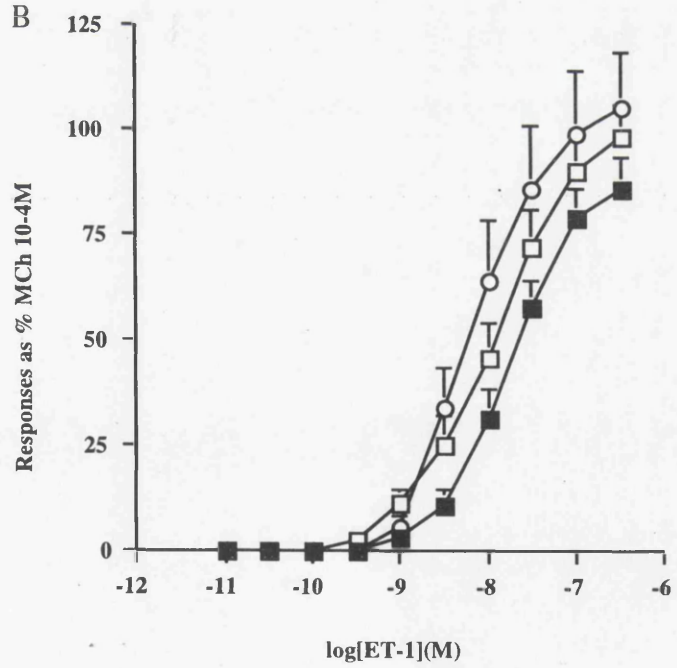
-7
-6
log[ET-1](M)


B
125
100
75
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25
0
Responses as % MCh 10-4M
-12
-11
-10
-9
-8
-7
-6
log[ET-1](M)

Table 5.3

A

Group	Control rat trachea EC_{200mg} (-log M)	EC_{400mg} (-log M)	(n)
ET-1 control	9.03 ± 0.10	8.40 ± 0.10	8
ET-1 plus BQ 788 10^{-6}M	9.36 ± 0.18	8.67 ± 0.16	8
ET-1 plus BQ 788 $3x10^{-6}$M	*9.55 ± 0.21	8.40 ± 0.10	8

B

Group	Chronically hypoxic rat trachea EC_{200mg} (-log M)	EC_{400mg} (-log M)	(n)
ET-1 control	8.85 ± 0.17	7.98 ± 0.16	8
ET-1 plus BQ 788 10^{-6}M	*9.30 ± 0.11	8.05 ± 0.12	8
ET-1 plus BQ 788 $3x10^{-6}$M	*9.39 ± 0.18	8.14 ± 0.31	7

Table 5.3: EC_{200mg} and EC_{400mg} values for ET-1 in the presence and absence of the ET_B receptor antagonist BQ 788 in tracheal rings from (A) Control rats and (B) Chronically hypoxic rats. Statistical comparison between values was calculated by Student's t-test. In control rat trachea, BQ 788 at the $3x10^{-6}$M level, significantly enhanced the sensitivity of the tissue to ET-1 (*$P<0.05$, ET-1 plus BQ 788 $3x10^{-6}$M compared to ET-1 alone). In hypoxic rat trachea, the sensitivity of the tissue to ET-1 was enhanced by BQ 788 at both the 10^{-6}M and the $3x10^{-6}$M level (*$P<0.05$, ET-1 alone compared to either BQ 788 10^{-6}M or BQ 788 $3x10^{-6}$M).

5.3.4 Dual Antagonism Of ET_A/ET_B Receptors

In this study we used two methods of dual antagonism: in the first case, simultaneous addition of both BQ 788 (10^{-6}M) and FR 139317 (10^{-6}M) and in the second, addition of the potent, non-selective ET antagonist SB 209670. Combined treatment with both BQ 788 and FR 139317 significantly ($P<0.01$ for data sets) attenuated ET-1-induced contractions in trachea from control rats (Figure 5.4A), albeit without altering the sensitivity of the tissue (control -log EC_{200mg}, 8.93±0.10, n=8; plus BQ 788 10^{-6}M and FR 139317 10^{-6}M, 8.75±0.10, n=6. Control -log EC_{400mg}, 8.40±0.10, n=8; plus BQ 788 10^{-6}M and FR 139317 10^{-6}M, 8.14±0.19, n=6).

In trachea from chronically hypoxic rats, however, simultaneous addition of both BQ 788 and FR 139317 had no significant effect on ET-1 responses (Figure 5.4B, control -log EC_{200mg}, 8.83±0.17, n=6; plus BQ 788 10^{-6}M and FR 139317 10^{-6}M, 8.65±0.20, n=6. Control -log EC_{400mg}, 7.78±0.16, n=6; plus BQ 788 10^{-6}M and FR 139317 10^{-6}M, 7.83±0.17, n=6).

Incubating the non-selective ET antagonist SB 209670 at a concentration of 10^{-7}M did not alter ET-1 contractions in control rat trachea (Figure 5.4A, control -log EC_{200mg}, 8.92±0.16, n=6; plus SB 209670 10^{-7}M, 8.61±0.19, n=6. Control -log EC_{400mg}, 8.31±0.16, n=6; plus SB 209670 10^{-7}M, 8.05±0.18, n=6.). When the concentration of SB 209670 was increased to 10^{-6}M however, it evoked a significant ($P<0.001$ for data sets) inhibition of the ET-1 concentration-response curve, including a significant ($P<0.01$ for data points) attenuation of the sensitivity of the tissue (control -log EC_{200mg}, 8.89±0.14, n=6; plus SB 209670 10^{-6}M, 7.93±0.18, n=6).

In trachea from chronically hypoxic rats, SB 209670 induced a concentration-dependent inhibition of the ET-1-evoked response (Figure 5.4B). At a concentration of 10^{-7}M, SB 209670 significantly ($P<0.01$ for data sets) inhibited the ET-1 concentration-response curve, including a significant ($P<0.01$ for data points) attenuation of the sensitivity of the tissue (control -log EC_{200mg}, 8.67±0.18, n=6; plus SB 209670 10^{-7}M, 7.84±0.22, n=6). This inhibition of ET-1-induced contractions was increased when the concentration of SB 209670 was increased to 10^{-6}M. We were unable to calculate -log EC_{400mg} values for the effect of 10^{-6}M SB 209670 on ET-1 responses in control trachea or for the effect of 10^{-7}M and 10^{-6}M SB 209670 in trachea from chronically hypoxic rats, since the maximum response to ET-1 in each case was less than 400mg.

Figure 5.4

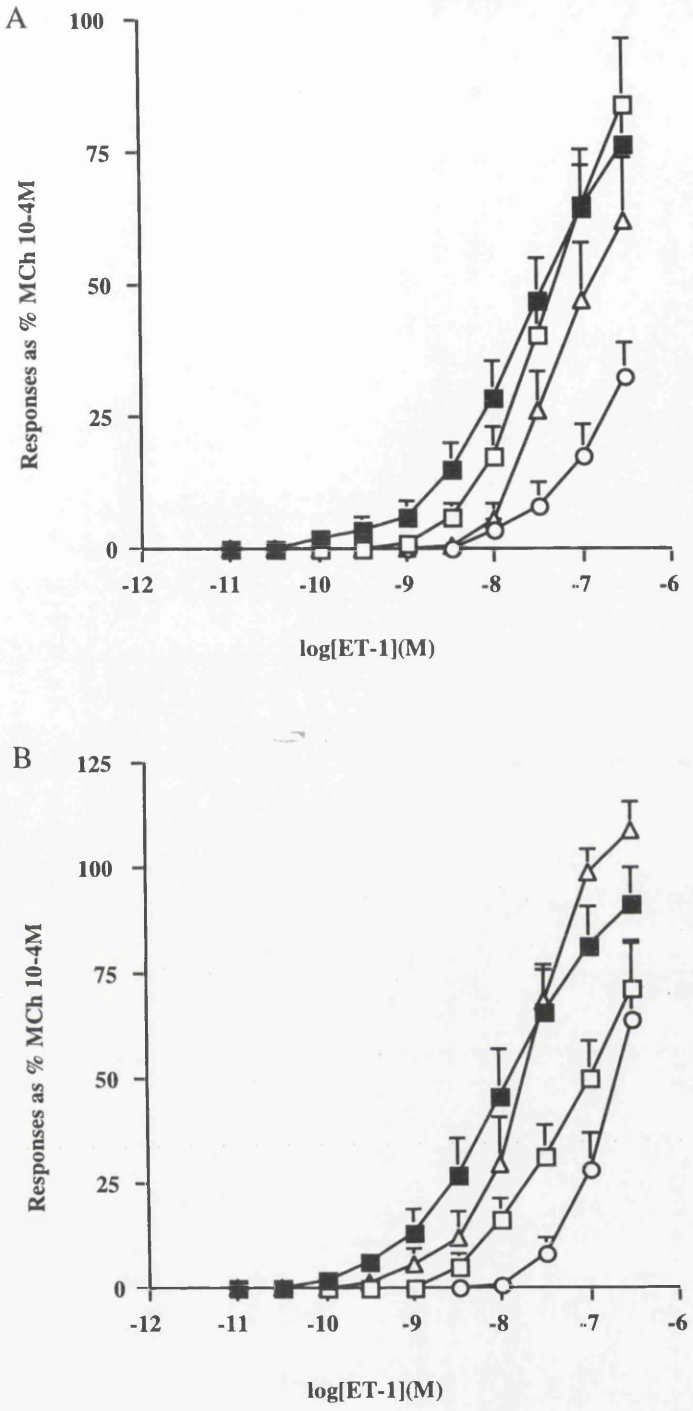


Figure 5.4

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) alone (■), or in the presence of the non-selective ET receptor antagonist SB 209670 at concentrations of (❑, 10^{-7}M) or (○, 10^{-6}M), or in the presence of a combination of the ET_A receptor antagonist FR 139317 (10^{-6}M) and the ET_B receptor antagonist BQ 788 (10^{-6}M, Δ) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. In control rat trachea, SB 209670 at a concentration of 10^{-6}M significantly attenuated ET-1 responses as did a combination of FR 139317 and BQ 788. In chronically hypoxic rat trachea, a combination of FR 139317 and BQ 788 did not alter ET-1 responses, whereas SB 209670 significantly attenuated ET-1 responses in a concentration-dependent manner.

5.3.5 The Effect Of L-Name On ET-1 Responses

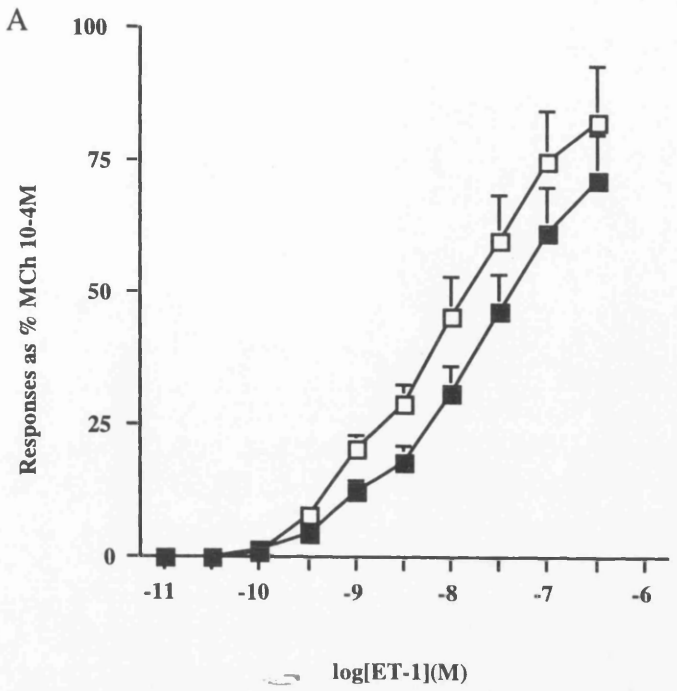
Pre-incubating the nitric oxide synthase inhibitor, L-NAME, at a concentration of 10^{-4}M significantly ($P<0.05$ for data sets) potentiated responses to ET-1 in control rat trachea (Figure 5.5A). L-NAME significantly ($P<0.05$ for data points) enhanced the sensitivity of the tissue to concentrations of ET-1 at the lower end of the ET-1 concentration-response curve (control -log EC_{200mg}, 9.01±0.14, n=6; plus L-NAME 10^{-4}M, 9.49±0.20, n=6), whereas the sensitivity of the tissue to higher concentrations of ET-1 was unaltered (control -log EC_{400mg}, 8.32±0.15, n=6; plus L-NAME 10^{-4}M, 8.64±0.20, n=6).

L-NAME also significantly ($P<0.01$ for data sets) enhanced responses to ET-1 in trachea from the chronically hypoxic rats, in this case with a concomitant increase in the sensitivity at the lower and the higher end of the ET-1 concentration-response curve (control -log EC_{200mg}, 8.81±0.14, n=6; plus L-NAME 10^{-4}M, 9.39±0.16, n=6. Control -log EC_{400mg}, 8.01±0.16, n=6; plus L-NAME 10^{-4}M, 8.49±0.17, n=6, $P<0.05$ in each case, see Figure 5.5B).

Figure 5.5

Cumulative concentration-response curves to ET-1 (10^{-11}-$3x10^{-7}$M) alone (■), or in the presence of the nitric oxide inhibitor L-NAME (10^{-4}M, ❑) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. L-NAME significantly ($P<0.05$ and $P<0.01$ for data sets, respectively) potentiated responses to ET-1 in both control and hypoxic rat trachea. In each case, (n)=6. Each point represents the mean ± SEM.

Figure 5.5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Responses as % MCh 10-4M
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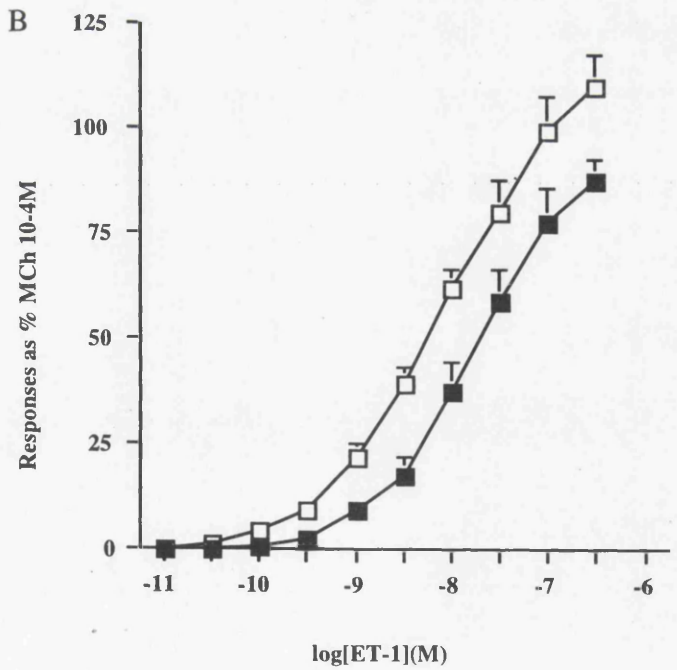
log[ET-1](M)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Responses as % MCh 10-4M
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log[ET-1](M)

5.3.6 ET Receptor-Mediated Relaxation

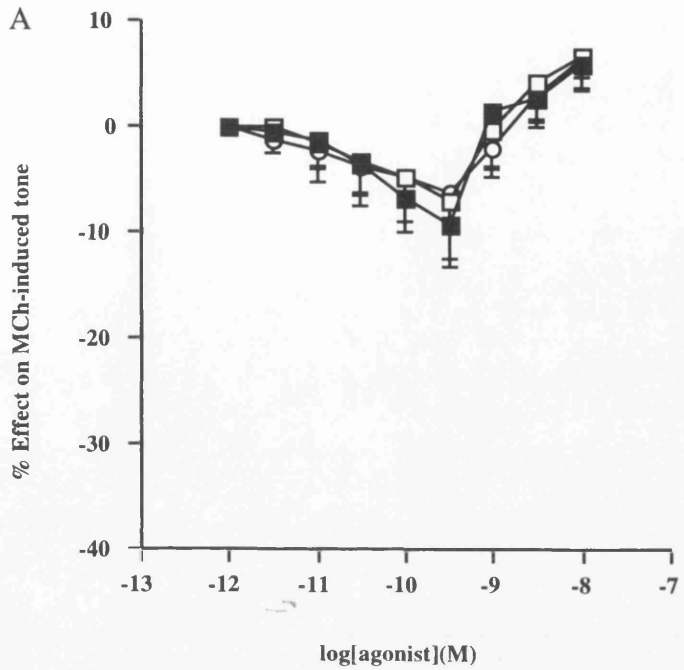
In trachea from control and chronic hypoxic rats, ET-1 and sarafotoxin S6c evoked only very small, non-reproducible relaxatory responses. Furthermore, in the presence of BQ 788, relaxations to ET-1 were again small and non-reproducible (see Figure 5.6).

In contrast, the nitric oxide donor, sodium nitroprusside, reversed methacholine-induced contractions in a concentration dependent manner, producing a maximum relaxant response of 54.5±3.9%, n=6 in control rat trachea and 65.6±7.2%, n=6 in chronically hypoxic rat trachea (see Figure 4.7B). This compares with a maximum relaxant response to ET-1, at a concentration of $3x10^{-10}$M, of 8.9±6.1%, n=6 in control rat trachea and 8.2±5.7%, n=6 in chronically hypoxic rat trachea.

Figure 5.6

The abilty of ET-1 (10^{-12}-10^{-8}M) alone (■), or in the presence of the ET_B receptor antagonist BQ 788 (10^{-6}M, ❑) and also the ability of the ET_B receptor agonist sarafotoxin S6c (10^{-12}-10^{-8}M, ○) to reverse methacholine-induced tone in isolated tracheal rings from (A) control rats and (B) chronically hypoxic rats. Relaxatory responses to ET-1 and sarafotoxin were small and non-reproducible in both control and chronically hypoxic rat trachea.

Figure 5.6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% Effect on MCh-induced tone
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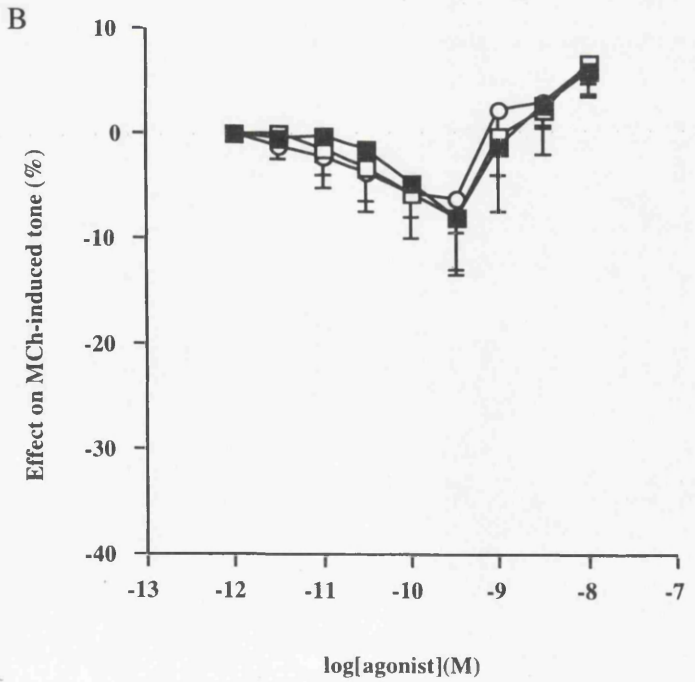
log[agonist](M)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-40
Effect on MCh-induced tone (%)
-13
-12
-11
-10
-9
-8
-7
log[agonist](M)

5.4 DISCUSSION

We have shown that the contractile response to ET-1 is significantly attenuated in tracheal rings from rats previously exposed to chronic hypoxia (see section 4). We aimed, in this study, to determine if this attenuation is due to alterations in the ET receptor subtype population. The ET_B receptor selective agonist, sarafotoxin S6c produced concentration-dependent contractions of isolated trachea from both control and chronically hypoxic rats. Sarafotoxin S6c was significantly more potent than ET-1 in control rat trachea, a finding which is in keeping with a previous study conducted on rat isolated trachea (Henry, 1993). In contrast, in trachea from chronically hypoxic rats, sarafotoxin S6c and ET-1 were equipotent. The results with sarafotoxin S6c suggests that activation of ET_B receptors contributes greatly to the contractile response to ET-1 in both control and hypoxic rat trachea. It was surprising, therefore, that the ET_B receptor selective antagonist, BQ 788, did not inhibit the contractile response to ET-1. In this study, ET-1-induced contractions of isolated trachea from control rats were not antagonized by either BQ 788 or by the ET_A receptor antagonist, FR 139317. A combination of these two antagonists, however, significantly inhibited the ET-1 response in this tissue, as did the mixed ET_A/ET_B antagonist SB 209670, indicating that simultaneous antagonism of both receptors is necessary to attenuate the response to ET-1. In some species, ET-1-induced contraction of airway smooth muscle appears to be mediated primarily via stimulation of either ET_A (for example sheep, Abraham *et al.*, 1993; Goldie *et al.*, 1994) or ET_B (for example, guinea-pig bronchus, Hay, 1992) receptors. The results from the present investigation, which are in accordance with those from previous studies conducted upon rat isolated trachea (Henry, 1993; O'Donnell & Kay, 1995), suggest that both ET_A and ET_B receptors are involved in mediating contraction in rat trachea. This finding is not unique to rat airways; in human isolated bronchus (Fukuroda *et al.*, 1996) and also in the pulmonary arteries from rats (MacLean *et al.*, 1995) and rabbits (Fukuroda *et al.*, 1994), ET-1-induced contractions are thought to be mediated via stimulation of both ET_A and ET_B receptors.

The inability of an ET_A antagonist (in the present study, FR 139317) to block ET-1 contractions has previously been shown in rat isolated trachea (O' Donnell & Kay, 1995) and human isolated bronchus (Fukuroda *et al.*, 1996). In the present study, low concentrations of FR 139317 actually enhanced ET-1-induced contractions in trachea from control rats. Similarly, the ET_B antagonist BQ788 failed to inhibit the ET-1 response in control rat trachea and instead enhanced contractions in a concentration dependent manner. These results suggest the presence of an inhibitory ET_A-mediated and also an inhibitory ET_B-mediated component which, in each case oppose contraction. An inhibitory ET_A receptor-mediated response has been demonstrated in the rat fundic strip (Gray & Clozel, 1993) and more recently, in the rat pulmonary resistance arteries (McCulloch & MacLean, 1995). It is not known if an inhibitory ET_A-receptor mediated response exists in the airways, but a recent study found results

similar to our own, in that the bronchoconstrictor response to ET-1 was significantly augmented in rat isolated perfused lungs by an ET_A receptor antagonist, in this case BQ 123 (Lal *et al.*, 1995).

It is believed that the initial depressor effect of ET-1 in the cardiovascular system is mediated by ET_B receptors (Saeki *et al.*, 1991; Douglas & Hilley, 1991), possibly by the release of vasodilating substances such as nitric oxide from the endothelium (Warner *et al.*, 1989; Filep *et al.*, 1993). This group has previously shown in bovine isolated bronchi that the selective ET_B receptor agonist sarafotoxin S6c relaxed methacholine-induced tone in a concentration-dependent manner and furthermore, desensitization of the ET_B receptor enhanced ET-1-induced contractions in this tissue (Nally *et al.*, 1994c). This suggests that airways may express an inhibitory ET_B receptor similar to that found in the vasculature. Sokolovsky *et al* (1992) reported a subclassification of the ET_B receptor subtype (termed ET_{B1} and ET_{B2} based on differing binding properties. It is now widely accepted that the different cell-type locations of the ET_B receptors are responsible for their opposing effects, with ET_{B2} receptors localized on vascular smooth muscle evoking the contractile responses to ET-1 (Moreland *et al.*, 1992; Sumner *et al.*, 1992), whereas the ET_{B1} receptors which mediate vasodilation are located on the endothelium (Sakuri *et al.*, 1990; Vane; 1990; Shetty *et al.*, 1993). It is not known if a similar system operates in the airways, with the inhibitory ET receptors (if present) being located on the epithelial layer. Removal of the epithelial layer significantly enhances contractile responses to the endothelins in human bronchus (Candenas *et al.*, 1992), indicating that the epithelia does indeed exert an inhibitory effect on ET-1-induced contractions. This inhibitory effect, however, may be due to the degradation of ET-1 by endopeptidases or indeed, the release of an epithelial-derived relaxant factor (Barnes *et al.*, 1985), rather than the actions of an inhibitory ET_B receptor. Indeed, in this present study, the nitric oxide synthase blocker, L-NAME, significantly potentiated responses to ET-1 in trachea from both control and chronically hypoxic rats. This would suggest that activation of ET receptors in these tissues stimulates the release of nitric oxide, which would tend to oppose the contractile response to ET-1. Contractile responses to the cholinergic agonist, methacholine, however, were also potentiated by L-NAME (see section 4.3), indicating that nitric oxide or a nitric oxide-like substance is released in response to contraction of the airway smooth muscle, rather than stimulation of ET receptors. Furthermore, we demonstrated that ET-1 evoked only small, non-reproducible relaxations of methacholine-induced tone. In addition, stimulation of either the ET_A (ET-1 in the presence of BQ 788) or the ET_B (sarafotoxin S6c) receptor subtypes failed to uncover any significant relaxation in trachea from control or chronically hypoxic rats. This is unlikely to be due to a loss of relaxant activity in these vessels as the nitric oxide donor, sodium nitroprusside, produced marked reversal of the induced tone (see Figure 4.7B). This adds evidence to our suggestion that activation of ET receptors *per se* does not stimulate release of nitric oxide from rat isolated tracheal preparations.

Our results show that antagonism of either ET_A or ET_B receptors alone does not inhibit ET-1-induced contractions in control rat trachea, whereas simultaneous blockade of both receptors significantly inhibited the ET-1 response. We have suggested the presence of inhibitory ET_A and ET_B receptors to explain our findings that both ET_A and ET_B receptor antagonists enhanced ET-1-induced contractions. Alternatively, it has been speculated that a cross-talk mechanism exists in the signal-transduction pathways between ET_A and ET_B receptors. For example Fukuroda *et al.*, (1996) postulate a mechanism whereby activation of ET_A receptors by ET-1 leads to moderate inhibition of the ET_B receptor-mediated signal and vice versa. Thus, activation of only ET_A or only ET_B receptors would be sufficient to produce the full signal, whereas simultaneous activation of both ET_A and ET_B receptors by ET-1 would produce a reduced response. One would therefore expect that blockade of either ET_A or ET_B receptors would free the signal transduction pathways from the inhibitory cross-talk mechanism. This was indeed what we found in this study; in the presence of either an ET_A antagonist or an ET_B antagonist, ET-1-induced contractions were significantly enhanced. Furthermore, the ET_B receptor agonist sarafotoxin S6c was significantly more potent than ET-1 in trachea from control rats.

We found that the ability of FR 139317, at the 10^{-8}M level, to enhance ET-1-mediated contractions in rat trachea was not present in the chronically hypoxic rats and furthermore, combined treatment with FR 139317 and BQ 788 attenuated ET-1 responses in control rat trachea but had no effect on responses in trachea from chronic hypoxic rats. In contrast, responses to ET-1 in the presence of BQ 788 alone or in the presence of the non-selective ET receptor antagonist SB 209670 were not altered by chronic hypoxia. Thus chronic hypoxia alters the pattern of responses to ET-1 in the presence of some, but not all, ET receptor antagonists.

Previous studies indicate that chronic exposure to hypoxia enhances the pulmonary vasoconstrictor response to ET-1 (McCulloch & MacLean, 1995; MacLean *et al.*, 1995). The reasons underlying this are unclear, but may be due to a reduction in the vasodilator response to ET-1. For example, Eddahibi and co-workers have shown that ET-1 induces concentration-dependent relaxation in rat pulmonary arteries via stimulation of ET_B receptors, and that this effect is completely abolished in lungs from chronically hypoxic rats (Eddahibi *et al.*, 1993). Alternatively, chronic hypoxia may attenuate the influence of the inhibitory ET_A receptors which are postulated to exist in various tissues including pulmonary arteries. For example, a recent study found that the ET_A receptor antagonist BMS 182874 potentiated responses to ET -1 in control rat pulmonary resistance arteries, but had no effect on ET-1 responses in vessels from chronically hypoxic rats (McCulloch & MacLean, 1995). Interestingly, our results followed a similar pattern in that the ET_A receptor antagonist FR 139317 potentiated ET-1 responses in trachea from control rats whereas it did not alter responses to ET-1 in chronic hypoxic rat trachea.

It remains unclear whether the altered responsiveness to ET-1 by chronic hypoxia is due to changes at the receptor level. Since lung ET-1 levels are increased by chronic hypoxia (Elton *et al.*, 1992), one would expect a relative decrease in ET receptors due to down regulation. Indeed in the monocrotaline lung injury model of pulmonary hypertension, lung ET-1 levels are raised and expression of ET_B receptor mRNA was subsequently found to be decreased (Yorikane *et al.*, 1993). In contrast, however, chronic hypoxia actually increases the level of ET_A receptor mRNA in whole rat lung and raises the level of ET_B receptor mRNA in the rat main pulmonary artery but does not change ET_A receptor mRNA in these vessels (Li *et al.*, 1994). Only one study has assessed the effect of chronic hypoxia on ET binding, finding no change in total or subtype-specific ET receptor binding in pulmonary vessels and airways from chronically hypoxic rats compared with normoxic animals (Eddahibi *et al.*, 1993).

In summary, the results of this study indicate that both ET_A and ET_B receptors mediate ET-1-induced contraction in rat isolated trachea and that simultaneous blockade of both receptor subtypes is required for inhibition of this response. Furthermore, our results suggest the existence of an inhibitory ET_A and ET_B-mediated component since blockade of either ET_A or ET_B receptors by FR 139317 or BQ 788, respectively, actually potentiated ET-1-mediated contractions. It would appear unlikely that these inhibitory components are due to the existence of ET_A or ET_B receptor subtypes which oppose contraction by releasing relaxant factors, since we demonstrated that sarafotoxin S6c or ET-1 in the presence of FR 139317 or BQ 788 produced little or no reversal of methacholine-induced tone in rat isolated trachea. We also found that the inhibitory ET_A receptor mediated, but not the ET_B receptor mediated component was lost in rats exposed to chronic hypoxia.

CHAPTER 6

THE EFFECT OF HYPOXIA ON THE PROLIFERATION OF CULTURED HUMAN AIRWAY SMOOTH MUSCLE CELLS

6.1 INTRODUCTION

The previous results in this thesis have focussed upon the effects of changes in oxygen tension on the pharmacological responsiveness of airway smooth muscle. As stated in section 1.7, changes in oxygen tension may also have an effect on the growth of airway smooth muscle cells. This may be of clinical relevance given that airway remodelling is a pathological feature of many respiratory disorders such as asthma and chronic bronchitis.

In the present series of experiments I examined the mitogenic effects of ET-1 and platelet-derived growth factor (PDGF) on cultured human airway smooth muscle cells under both normoxic and hypoxic conditions. As stated in section 5.1, ET-1 is a potent bronchoconstrictor which evokes its responses by binding to and activating specific ET receptors (ET_A and ET_B). In addition to its contractile responses on airway smooth muscle, there is evidence that activation of ET receptors may also induce proliferation of airway smooth muscle.

As mentioned in section 5.1, ET-1-induced contraction of human bronchial smooth muscle is thought to be mediated predominantly via activation of ET_B receptors (Hay *et al.*, 1993a and b; Goldie *et al.*, 1995). In contrast, the mitogenic effect of ET-1 in this tissue appears to occur through stimulation of ET_A receptors (Tomlinson *et al.*, 1994; Panettieri *et al.*, 1996b). ET-1 *per se* evokes only a modest proliferation of human airway smooth muscle (Tomlinson *et al.*, 1994), however in the presence of another growth factor (for example, epidermal growth factor, Panettieri *et al.*, 1996), the mitogenic effects of ET-1 are significantly potentiated. In this study we attempted to characterise the ET receptor subtype(s) involved in ET-1-induced mitogenesis under normoxic and hypoxic conditions .

PDGF has been shown to be a potent mitogen in a number of cell types, including airway smooth muscle (Hirst *et al.*, 1992). In contrast to ET-1, PDGF acts by stimulating receptors with intrinsic tyrosine kinase activity (see section 1.5.2.1.1).

Our preliminary results showed that hypoxia *per se* stimulated proliferation and also enhanced the mitogenic effect of both ET-1 and PDGF. In an attempt to elucidate the mechanism(s) underlying these findings, I studied the effect of the protein kinase C (PKC) inhibitor, staurosporine, on ET-1 and PDGF-mediated mitogenesis in normoxia and hypoxia.

6.2 METHODS

6.2.1 Airway Smooth Muscle Cell Culture

Human airway smooth muscle cells were harvested from bronchial segments obtained from thoracic surgery (as described in section 2.2.1). The cells used in this study were derived from three individual human patients.

The identity of the bronchial smooth muscle cells was confirmed by immunocytochemical staining as described in section 2.2.3.

6.2.2 Assessment Of Proliferation

Proliferation of human airway smooth muscle cells was assessed by measuring the uptake of radiolabelled thymidine (see section 2.2.4.3).

Cells were stimulated with ET-1 or the ET_B receptor agonist sarafotoxin S6c (10^{-11}M - 10^{-7}M) or PDGF-BB (10ng/ml) for 24 hours, either in a normal CO_2 incubator (5% CO_2) or in a hypoxic incubator (again 5% CO_2) under an environmental O_2 tension of ~30mm Hg.

To characterise the ET receptors involved in mitogenesis in the hypoxic cells, the ET_A receptor antagonist BQ 123 ($3x10^{-7}$M) or the ET_B receptor antagonist BQ 788 ($3x10^{-7}$M) were added 30 minutes prior to addition of ET-1.

To study the involvement of PKC activation in the mitogenic response to ET-1 and PDGF, the PKC inhibitor, staurosporine (10^{-7}M), was added 30 minutes prior to addition of either ET-1 or PDGF, in both normoxic and hypoxic cells.

In all experiments, for the last 4 hours of agonist stimulation, cells were labelled with [^{3}H] thymidine (0.1μCi/ml) before the reaction was stopped by washing in phosphate buffered saline (see section 2.2.4.3). Radioactivity was determined by liquid scintillation counting and results expressed as disintegrations per minute (DPM).

6.2.3 Materials

The following chemicals were used: BQ 123, BQ 788, ET-1, PDGF, sarafotoxin S6c, staurosporine and [^{3}H] thymidine (see section 2.2.6 for full chemical and suppliers names).

6.2.4 Statistics

Results are expressed as the mean ± s.e.mean. Statistical analysis was undertaken as reported in section 2.2.7.

6.3 RESULTS

6.3.1 Agonist response to hypoxia

In normoxia, ET-1 showed no mitogenic effects, evoking a maximum response (mean±sem) (842.6±20.6 DPM, at a concentration of 10^{-7}M) which was not significantly different from basal levels (827.5±84.5 DPM). Under hypoxic conditions, however, ET-1 produced a concentration-dependent increase in DNA synthesis which was significantly ($P<0.001$ for data sets) greater than the concentration-response curve in normoxia. Furthermore, responses to ET-1 at concentrations of 10^{-9}M (1945.1±102.6 DPM), 10^{-8}M (2125.3±114.2 DPM) and 10^{-7}M (2242.0±110.5 DPM) were significantly ($P<0.05$ for data points) greater than basal levels in hypoxia (1175.3±140.9 DPM, see Figure 6.1).

PDGF (10ng/ml) was found to be a potent mitogen under normoxic conditions, stimulating thymidine incorporation to a level (3247.8±112.7) which was significantly greater ($P<0.01$, for data points) than basal levels (887.9±94.1 DPM, see Figure 6.3). This mitogenic effect of PDGF was enhanced in hypoxia, with PDGF (10ng/ml) producing a response (5467.8±269.1 DPM) which was significantly ($P<0.001$, for data points) greater than basal counts in hypoxia (1231.3±168.4 DPM) and significantly ($P<0.05$, for data points) greater than the response to PDGF in normoxia.

6.3.2 Characterisation of the ET receptor subypes involved in mitogenesis

The enhancement of ET-1-induced mitogenesis in hypoxic conditions was significantly ($P<0.01$ for data sets) attenuated by the ET_A receptor antagonist BQ 123 ($3x10^{-7}$M), but not by the ET_B receptor antagonist BQ 788 ($3x10^{-7}$M). In addition, the ET_B receptor-selective agonist sarafotoxin S6c evoked no mitogenic response (see Figure 6.2).

6.3.3 Effect of the PKC inhibitor, staurosporine, on proliferation.

In normoxia, staurosporine (10^{-7}M) did not alter the uptake of [^{3}H] thymidine. In cells quiesced under hypoxia, however, the slight mitogenic effect of hypoxia was completely abolished by staurosporine (In the absence of staurosporine; 1286.7±134.8 DPM, compared to in the presence of staurosporine; 816.5±80.9 DPM, $P<0.05$ for data points).

The mitogenic response to 10^{-7}M ET-1 in hypoxic cells was also abolished by staurosporine (In the absence of staurosporine; 2340.5±107.6 DPM, compared to in the presence of staurosporine; 837.5±88.1 DPM, $P<0.01$ for data points).

Proliferation induced by 10ng/ml PDGF was again inhibited by prior incubation of staurosprine (PDGF in normoxia in the absence of staurosporine; 3247.8±112.7 DPM, and in the presence of staurosporine; 852.6±82.4 DPM, $P<0.01$ for data points. PDGF in hypoxia in the absence of staurosporine; 5467.8±269.1 DPM, and in the presence of staurosporine; 875.6±92.0 DPM, $P<0.001$ for data points).

Figure 6.1

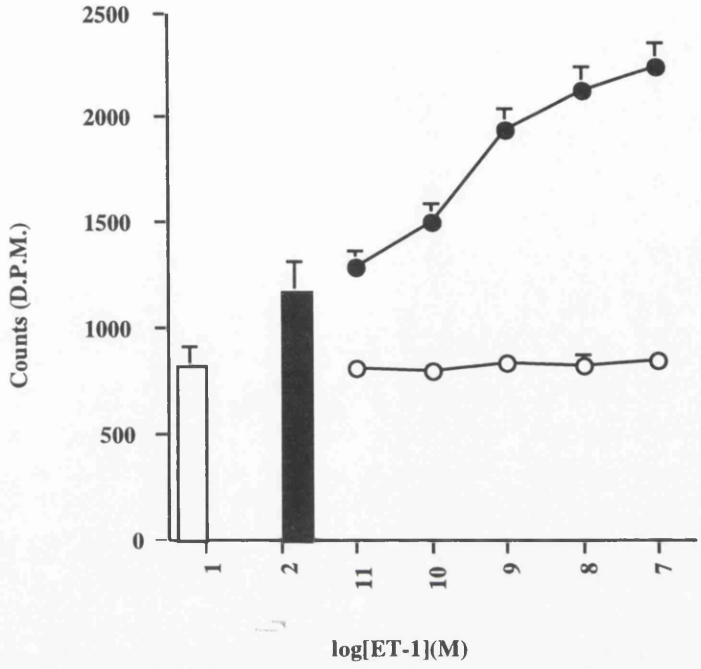


Figure 6.1

Uptake of [^{3}H] thymidine in human airway smooth muscle cells in normoxia (○) and hypoxia (●). Hypoxia stimulates thymidine uptake in basal cells (■) and "uncovers" the mitogenic effect of ET-1. The concentration-response curve to ET-1 was significantly ($P<0.001$ for data sets) greater in hypoxia than in normoxia and at concentrations of 10^{-9}, 10^{-8} and 10^{-7}M, ET-1-induced DNA synthesis was significantly ($P<0.05$ for data points) greater than basal counts. Each data point represents the mean ±s.e.mean of 4 individual wells from three separate experiments.

Figure 6.2

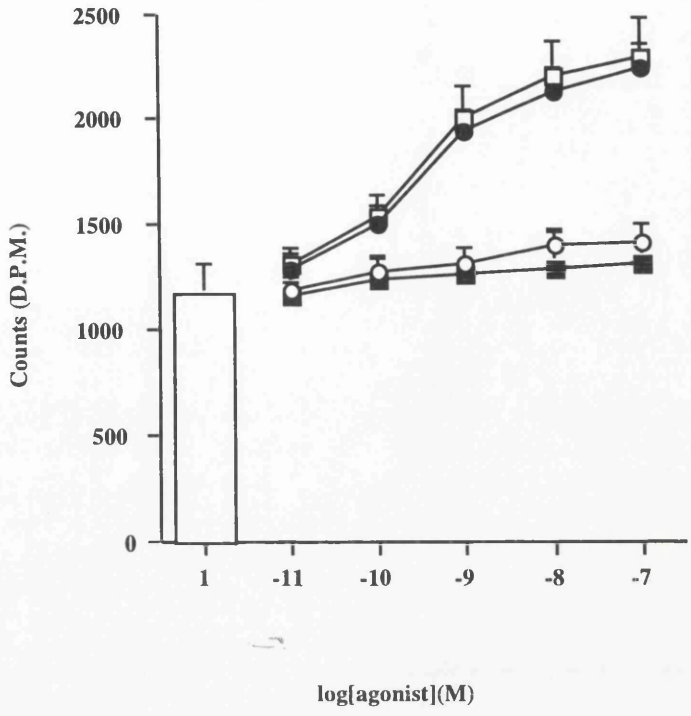


Figure 6.2

Uptake of [^{3}H] thymidine in human airway smooth muscle cells under hypoxic conditions (●). At concentrations of 10^{-9}, 10^{-8} and 10^{-7}M, ET-1 significantly ($P<0.05$) stimulated mitogenesis. This enhancement of ET-1-induced mitogenesis was significantly ($P<0.01$ for data sets) attenuated by the ET_A receptor antagonist BQ 123 ($3x10^{-7}$M, ○), but was not altered by the ET_B antagonist BQ 788 ($3x10^{-7}$M, ❑). Furthermore, the ET_B receptor agonist sarafotoxin S6c (■) evoked no mitogenic response, indicating that this effect of ET-1 is mediated via activation of ET_A receptors. Each data point represents the mean ±s.e.mean of 4 individual wells from three separate experiments.

Figure 6.3

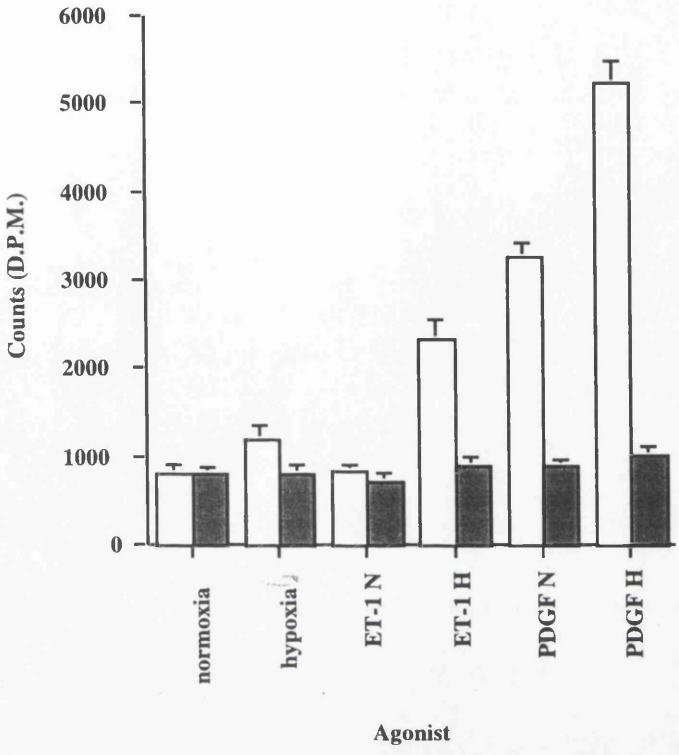


Figure 6.3

Uptake of [^{3}H] thymidine in human airway smooth muscle cells in the presence (stippled columns) and absence (clear columns) of the PKC inhibitor staurosporine (10^{-7}M). Thymidine incorporation, stimulated by either hypoxia alone, ET-1 (10^{-7}M) in hypoxia, PDGF (10ng/ml) in normoxia or PDGF (10ng/ml) in hypoxia was inhibited in the presence of staurosporine. Each data point represents the mean ±s.e.mean of 4 individual wells from three separate experiments. Abbreviations: ET-1 N; ET-1 in normoxia, ET-1 H; ET-1 in hypoxia, PDGF N; PDGF in normoxia, PDGF H; PDGF in hypoxia.

6.4 DISCUSSION

The main findings of the present study were: (1) Hypoxia stimulated thymidine uptake in unstimulated human airway smooth muscle cells; (2) ET-1 alone had little or no mitogenic effects on these cells under normoxic conditions, but in the presence of hypoxia, ET-1 significantly stimulated DNA synthesis (3) PDGF was a potent mitogen in normoxic conditions and this effect was enhanced under hypoxia; (4) the mitogenic effect of ET-1 in hypoxia appears to be mediated via activation of ET_A receptors; (5) the protein kinase C inhibitor, staurosporine, completely abolished thymidine uptake stimulated by either hypoxia alone, ET-1 or PDGF.

The mitogenic effects of ET-1 in vascular smooth muscle have been well documented (Komuro *et al.*, 1988; Nakaki *et al.*, 1989; Eguchi *et al.*, 1992), although in human and animal cultured airway smooth muscle, ET-1 on its own has only weak mitogenic effects (Tomlinson *et al.*, 1994; Panettieri *et al.*, 1996b; Noveral *et al.*, 1992; Stewart *et al.*, 1994; Glassberg *et al.*, 1994). Interestingly, Panettieri and co-workers (1996b) found that, although ET-1 produced little mitogenic effect on its own, it significantly augmented the mitogenic effect of the growth factor EGF in human airway smooth muscle. Furthermore, this enhancement of the mitogenic effect was blocked by the ET_A receptor antagonist, BQ 123 (Panettieri *et al.*, 1996b). This is in keeping with our present study, in that ET-1, in the presence of hypoxia, stimulated DNA synthesis in cultured human airway smooth muscle cells via activation of ET_A receptors.

While it has previously been shown that ET-1 can potentiate the mitogenic effect of growth factors such as EGF (Panettieri *et al.*, 1996b) and PDGF (Fujitani & Bertrand, 1996), we believe that this is the first report of a synergistic relationship between ET-1 and hypoxia in the proliferation of airway smooth muscle. The precise mechanism(s) by which ET-1 synergises with hypoxia or, for that matter, growth factors remain unknown, but may reflect interactions at certain points on the intracellular pathways through which these mediators transduce their mitogenic effects.

In this current study I examined the effect of protein kinase C inhibition on hypoxia- and agonist-induced mitogenesis. Various lines of evidence point to the involvement of protein kinase C in cellular growth. For example, proliferating cells exhibit greater PKC activity than do quiescent cells (Adamo *et al.*, 1986). In addition, direct activation of PKC by phorbol esters induces smooth muscle cell growth, whereas PKC inhibitors reduce cell replication (Owen, 1985; Newby *et al.*, 1994; Panettieri *et al.*, 1993).

Previous studies in airway smooth muscle cells demonstrated that PKC inhibition markedly attenuates the proliferation induced by either ET-1 or PDGF (Malarkey *et al.*, 1995). The results from the present study are in agreement with this and in addition, the small mitogenic effect of hypoxia alone was blocked by staurosporine. This indicates that hypoxia induces its effect on cell growth via stimulation of PKC.

While G protein receptor agonists such as ET-1 are thought to be almost exclusively PKC-dependent, receptor tyrosine kinase-mediated cell proliferation is believed to

include both PKC-dependent and independent pathways (Stumpo & Blackshear, 1983). It was perhaps surprising therefore, to find that PDGF-induced mitogenesis, under both normoxic and hypoxic conditions, was completely abolished by staurosporine. It is possible that the concentration of staurosporine used was too high, leading to inhibition of other intracellular kinases (for example, MAP kinases). It may be, however, that PKC inhibition is indeed the principle event in both ET-1 and PDGF mediated mitogenesis in these cells.

Interestingly, Dempsey *et al* (1991) showed that PKC was a required step in allowing cultured pulmonary artery smooth muscle cells to proliferate in response to hypoxia. In contrast to my findings, however, Dempsey and co-workers found that pulmonary artery smooth muscle cells did not proliferate in response to hypoxia *per se*, but rather PKC stimulation was essential to "prime" the cells to respond to hypoxia. Our findings are more in keeping with the response of fibroblasts, since cultured fibroblasts have been shown in several studies to have a direct proliferative response to hypoxia (Taylor *et al.*, 1978; Storch & Talley, 1988).

It is important to be cautious in considering the potential relevance of these *in vitro* observations to events occuring *in vivo*. Several points must be considered. Firstly, the cells used in this study were quiesced before stimulation, which would not relate to the proliferative state *in vivo*. Secondly, the homogeneous population of airway smooth muscle cells used in this study has been removed from the influence of other cell types such as epithelial and mast cells. Thirdly, our measurements were limited to proliferation, whereas other forms of response such as hypertrophy are also potentially important in a remodelling process. Fourthly, the definitions of hypoxia and normoxia were by convention and may not relate well to the oxygen tension surrounding airway smooth muscle cells *in vivo*. Lastly, the cells used in this study were cultured from patients undergoing thoracic surgery for bronchial carcinoma. As a rule, these patients were of poor health, and the effect of bronchial carcinoma on the lungs would be expected to involve some degree of hypoxia. Thus the cells cultured from these patients may have become adapted to a more hypoxic environment.

On a further cautionary note, as mentioned in section 2.2.4.3, there are a number of potential artefacts in relating thymidine uptake to cellular proliferation. While, in the present study, I have taken the increase in thymidine uptake under hypoxic conditions to be indicative of cell proliferation, one may speculate that hypoxia may increase the incorporation of thymidine into macromolecules other than DNA or increase the synthesis of DNA for reparative purposes. This would lead to an increase in assimilation of tritiated thymidine without any significant enhancement of cell proliferation. Since we had no means of testing this experimentally, this remains purely speculative and I would tentatively conclude that our measurements of thymidine uptake under hypoxic conditions are indeed representative of cellular proliferation.

In summary, the present data indicates that hypoxia stimulates mitogenesis in cultured human airway smooth cells and enhances the mitogenic effect of ET-1 and PDGF on these cells. ET-1-induced mitogenesis of these cells in hypoxia is mediated by activation of ET_A receptors and PKC inhibition appears to abolish hypoxic-, ET-1 and PDGF-induced proliferation.

CHAPTER 7

THE EFFECT OF ACUTE ALTERATIONS IN OXYGEN TENSION ON SALBUTAMOL-INDUCED RESPONSES IN HUMAN ISOLATED BRONCHI AND ASTHMATIC PATIENTS *IN VIVO*

7.1 INTRODUCTION

As reported in section 3, changing the O_2 tension of the environment can alter the ability of various bronchodilators, including salbutamol, to reverse induced tone in bovine isolated bronchial rings. While bovine bronchial tissue is thought to be a reasonable model for human bronchial tissue, it is obviously of importance to conduct key experiments on human airways wherever possible. In this group of experiments, the ability of salbutamol to reverse methacholine-induced tone was studied in human isolated bronchial rings under hyperoxic, normoxic and hypoxic conditions.

Measuring isometric contractions and relaxations in isolated tissue provides useful information on the pharmacology and physiology of the airways at the level of the airway smooth muscle. As mentioned in section 1.6, however, the effects of hypoxia on airway smooth muscle *in vitro* do not always predict what would happen to the airways *in vivo*. Thus, there is a need to measure the effect of changes in inspired oxygen tension on bronchial reactivity in human subjects *in vivo*.

This group has previously shown that the bronchodilator effects of salbutamol are not altered by changing from a normoxic to a hyperoxic gas mixture in asthmatic patients (Dagg *et al.*, 1996). This previous study did not examine the effect of hypoxia on bronchodilator responses, therefore in this present series of experiments, we compared the ability of salbutamol to induce bronchodilation under hyperoxic and hypoxic conditions.

7.2 METHODS

7.2.1 *In-vitro* study

7.2.1.1 Tissue collection and preparation

Macroscopically normal human bronchi (2nd to 4th order, internal diameter 3-5mm) were obtained from patients undergoing thoracic surgery and dissected as described in section 2.1.2.3. Rings of human bronchi were suspended in vertical organ baths (as described in section 2.1.4) under a resting tension of 2g wt. Alterations in gas tension from hyperoxia to either normoxia or hypoxia were achieved by substituting oxygen with nitrogen as stated in section 2.1.5. Hyperoxic, normoxic and hypoxic gas mixtures produced final bath O_2 tensions of 520-525mm Hg, 142-145mmHg and 24-28mmHg, respectively.

7.2.1.2 Experimental Protocol

Tissues were pre-incubated with 10^{-6}M methacholine (approximately the EC_{50} for methacholine in this tissue, personal observations) and the contraction allowed to reach a plateau. Cumulative concentration-response curves were then constructed to salbutamol (10^{-9}-10^{-4}M). To avoid tachyphylaxis, only one concentration-response curve was conducted to salbutamol in each tissue, therefore the three different oxygen tensions were imposed upon three separate bronchial rings. In each experiment one tissue acted as a time control to ensure that the methacholine-induced contraction was maintained.

7.2.1.3 Materials

The following chemicals were used: methacholine and salbutamol (see section 2.1.8 for full chemical and suppliers names). Concentration in the text refers to the salts, with the exception of salbutamol which is expressed as the base. Stock solutions of drugs were prepared in distilled water and subsequent dilutions made in Krebs-Henseleit solution.

7.2.2 *In-vivo* study

7.2.2.1 Patient Information

Twelve adult asthmatic patients (2 female) with a mean (SD) age 39 (8) years agreed to participate in the study (see Table 7.1). All of the patients gave a history of asthma and none had any other significant cardiac or respiratory disease. All of the patients were taking inhaled $ß_2$-agonists as required and eleven regularly inhaled corticosteroids. Three were also receiving the long acting $ß_2$-agonist salmeterol and one had been provided with a home nebuliser to take salbutamol on an "as required" basis. One patient was receiving a short acting oral theophylline. All of the patients gave written

informed consent to the study protocol which had the approval of the West Ethics Committee.

7.2.2.2 Study design

Patients were asked to attend the study laboratory on five separate days at approximately the same time each day. Prior to each study day patients were asked to withhold their inhaled and nebulised $ß_2$-agonists for 8 hours and inhaled salmeterol for 24 hours. Oral theophyllines were discontinued 48 hours prior to each study day. Patients were asked to continue taking their inhaled corticosteroids as usual. Patients who at an initial visit, following 30 minutes of supine rest, demonstrated an improvement in FEV_1 of more than 15% after inhaling salbutamol were deemed eligible for the remaining four study days.

On subsequent study days on arrival at the laboratory, patients were rested in a supine position for 30 minutes following which they were connected to a closed breathing circuit for the delivery of all study gases. After breathing air (FiO_2 0.21) for 10 minutes through the closed breathing circuit, baseline measurements of oxygen saturation (SaO_2%), heart rate (HR), respiratory rate (RR), inspired oxygen and carbon dioxide levels (insp O_2%, insp CO_2%) and end-tidal oxygen and carbon dioxide levels ($pETO_2$%, $pETCO_2$%) were made (see section 2.3.3).

After the baseline measurements had been recorded patients breathed either a hypoxic gas mixture (FiO_2 0.15) on two days or oxygen (FiO_2 1.0) on two further study days for the remainder of that day. All gases were administered in a randomised double blind manner by a second operator obscured from the vision of both the patient and the doctor performing the salbutamol test. Ten minutes after commencing the study gas for that day the measurements made at baseline were repeated. Subsequently at fifteen minute intervals in a randomised double blind fashion patients received either three incremental doses of nebulised salbutamol: (0.05 mg/ml, 0.17 mg/ml, 5 mg/ml) on two days or placebo (nebulised saline) on two further days. Measurements were repeated thirteen minutes after each nebulisation until completion of the study day. The solutions of salbutamol and saline were made up by the pharmacy department of our hospital and were given through a micro cirrus nebuliser driven by a hypoxic gas mixture (British Oxygen Corporation, Special Gases Division, Manchester) or oxygen (FiO_2 1.0) at a predetermined flow rate to produce a nebuliser output of 0.13 mls/min.

7.2.2.3 Measurements

Heart rate, oxygen saturation, inspired and expired oxygen and carbon dioxide levels were measured as reported in section 2.3.3.1 and FEV_1 values were measured as stated in section 2.3.3.2. Study gases were generated as described in section 2.3.4.

7.2.3 Statistical analysis

For the *in-vitro* studies results for the reversal experiments are expressed in terms of the mean maximum inhibition. Results are expressed as mean (SEM). Statistical significance between the data sets was tested by two-way analysis of variance (ANOVA). Significance between mean maximum inhibitions was calculated by Students t-test (see section 2.1.9.3). Number of observations (n) refers to the number of patients from whom tissues were obtained.

For the *in-vivo* studies, analysis of Variance (ANOVA) corrected for multiple comparisons was used to compare measurements made at baseline and following each dose of nebulised saline or salbutamol between study days.

Table 7.1: Patient characteristics

Patient No	Age (years)	Sex	FEV_1 absolute value (L) pre-salb	FEV_1 absolute value (L) post-salb	FEV_1 % predicted	Current treatment
1	44	M	2.14	2.82	83	Aprn, B
2	52	M	2.70	3.15	91	Aprn, B
3	39	M	3.12	3.60	90	Aprn
4	52	M	2.31	2.72	66	Aprn, B
5	34	F	3.02	3.52	106	Aprn, B
6	35	M	3.15	3.80	88	Aprn,B
7	49	M	2.08	2.75	77	Aprn, B, S, Neb
8	36	M	2.85	3.29	98	Aprn
9	29	M	2.94	3.59	79	Aprn, B
10	32	F	2.33	2.73	107	Aprn, B, S
11	29	M	3.34	3.93	87	Aprn, B
12	36	M	2.52	2.97	75	Aprn, B, S, Th
Mean		**39**	**2.71**	**3.24**	**87**	
(SD)		**(8)**	**(0.42)**	**(0.44)**	**(11)**	

Aprn = inhaled salbutamol as required, B = inhaled steroid (beclomethasone or budesonide), S = inhaled salmeterol twice daily, Th = oral theophylline, Neb = nebulised salbutamol and ipratropium bromide as required, pre-salb = pre-salbutamol FEV_1, post-salb = post-salbutamol FEV_1.

7.3 RESULTS

7.3.1 *In-vitro* study

The time control studies indicated that contractions evoked by methacholine (10^{-6}M) were sustained throughout the experimental day (data not shown). Contractions evoked by the single concentration of methacholine did not differ significantly between the three oxygen tensions: in hyperoxia; 761.3±178.9mg wt, in normoxia; 613.8±156.2 mg wt and in hypoxia; 483.5±149.9mg wt.

Salbutamol evoked a marked, concentration dependent reversal of the methacholine-induced contraction (see Figure 7.1A). Under hyperoxic and hypoxic conditions, responses to salbutamol were initiated at between $3x10^{-9}$M and 10^{-8}M and between 10^{-9}M and $3x10^{-9}$M in normoxia (Figure 7.1B). Lowering the oxygen tension from 95% to 20% oxygen did not significantly alter the ability of salbutamol to reverse methacholine-induced tone (mean maximal inhibition at 10^{-4}M: 103.02 ± 9.04 % in hyperoxia and 74.07 ± 11.65 % in normoxia, n=8). In contrast, responses evoked by salbutamol were significantly ($P<0.001$ for data sets) attenuated in hypoxia when compared to hyperoxia (mean maximal inhibition at 10^{-4}M: 103.02 ± 9.04 % in hyperoxia and 59.36 ± 7.85 % in hypoxia, $P<0.01$ for data points, n=8). Responses to salbutamol were also significantly ($P<0.01$ for data sets) less in hypoxia compared with normoxia, although there was no significant difference between the maximum responses (mean maximal inhibition at 10^{-4}M: 74.07 ± 11.65 % in normoxia and 59.36 ± 7.85 % in hypoxia, n=8).

Figure 7.1A

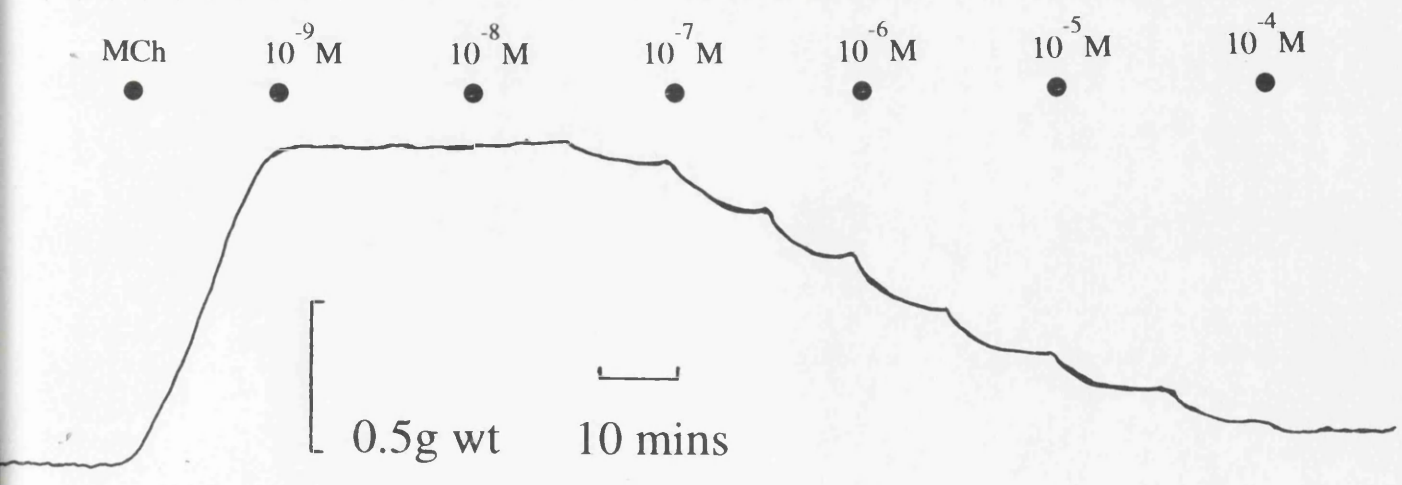


Figure 7.1A

Representative trace depicting the ability of salbutamol to reverse methacholine-induced contraction in human bronchial rings. After a stable contraction to methacholine (10^{-6}M) was produced, salbutamol was added cumulatively with each concentration being added after the previous response had reached a plateau.

Figure 7.1B

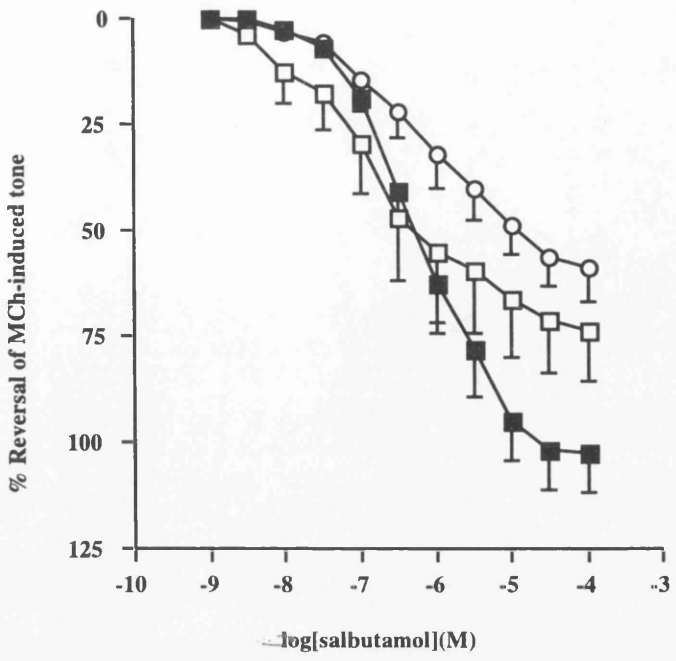


Figure 7.1B

The ability of salbutamol to reverse methacholine-induced tone in human isolated bronchi in hyperoxia (■), normoxia (❑) and hypoxia (○). Salbutamol was added cumulatively to give final bath concentrations of 10^{-9}-10^{-4}M. Responses are expressed as a percentage reversal of the methacholine contraction. Salbutamol was significantly ($P<0.001$) more effective in hyperoxia than in hypoxia and significantly ($P<0.01$) more effective in normoxia than in hypoxia. Number of observations (n)=8 in each case.

7.3.2 *In-vivo* study

Baseline measurements: There were no significant differences in the baseline measurements made between any of the study days (Table 7.2).

Table 7.2: Baseline measurements

	Oxygen (FiO_2 1.0)		Hypoxia (FiO_2 0.15)	
Baseline measurements	Placebo	Salbutamol	Placebo	Salbutamol
FEV_1 (L)	2.49 (0.12)	2.54 (0.10)	2.52 (0.12)	2.36 (0.12)
Heart rate (beats/min)	73 (2.5)	72 (2.8)	71 (2.6)	70 (2.9)
Resp. rate (breaths/min)	14 (1.7)	16 (1.3)	15 (1.3)	15 (1.4)
SaO_2 (%)	96 (0.3)	96 (0.3)	96 (0.2)	96 (0.2)
Inspired O_2 (%)	21 (0.2)	21 (0.0)	21 (0.1)	21 (0.1)
Expired CO_2 (%)	4.8 (0.1)	4.7 (0.1)	4.7 (0.2)	4.7 (0.2)

Mean (SEM) baseline measurements of FEV_1, respiratory rate, heart rate, oxygen saturation, inspired oxygen % and expired carbon dioxide % for each study day.
There was no significant differences between any of the study days

% change in FEV_1 from baseline: The mean (SEM) maximum % change in FEV_1 from baseline was significantly greater (P<0.05) on the days on which nebulised salbutamol was administered: hyperoxia/salbutamol 21 (4.7) %, hypoxia/salbutamol 18 (3.6) % when compared to the study days on which nebulised saline was administered: hyperoxia/saline 1 (2.1) %, hypoxia/saline -2 (2.9) %. There was however no significant difference in the mean maximum % change in FEV_1 from baseline at any time point between the study days on which nebulised salbutamol was given (see Table 7.3, Figure 7.2). There was no significant difference in the mean % change in FEV_1 from baseline on any study day 10 minutes after being commenced on the study gas for that day but prior to inhalation of nebulised salbutamol or saline. Therefore, changing the inspired oxygen tension did not appear to alter the airway calibre.

The mean (SEM) % change in FEV_1 from baseline 10 minutes after commencing each study gas was: hyperoxia/salbutamol: -3 (3.5)%, hypoxia/salbutamol: -2 (3.6)%, hyperoxia/saline 4: (2.1)% and hypoxia/saline: 3 (2.5)%.

Oxygen saturation: Oxygen saturation was significantly higher (P<0.05) on the days on which oxygen was administered when compared to the days on which the hypoxic gas mixture was inhaled at all time points (see Table 7.3, Figure 7.3).

Change in heart rate from baseline: The fall in heart rate from baseline was significantly greater (P<0.05) on the days that patients inhaled oxygen when compared to the days on which they inhaled the hypoxic gas mixture (Table 7.3).

End-tidal carbon dioxide: There were no significant differences in end-tidal carbon dioxide levels between any of the study days at any time point (Table 7.3).

Respiratory rate There were no significant differences in respiratory rate between study days at any time point (Table 7.3).

Table 7.3:

Measurement (Post agonist challenge)	Oxygen (FiO_2 1.0)		Hypoxia (FiO_2 0.15)	
	Placebo	Salbutamol	Placebo	Salbutamol
FEV_1 (L)	2.54 (0.10)	*3.01 (0.14)	2.47 (0.12)	*2.78 (0.11)
Heart rate (beats/min)	+65 (2.1)	+69 (2.8)	77 (3.6)	79 (3.1)
Resp. rate (breaths/min)	14 (1.7)	15 (1.3)	16 (1.3)	16 (1.4)
O_2 saturation (%)	+98 (0.3)	+98 (0.3)	91 (0.1)	90 (0.2)
Expired CO_2 (%)	4.9 (0.1)	4.8 (0.1)	4.8 (0.2)	4.7 (0.3)

Table 7.3.

The effect of nebulised salbutamol or saline on FEV_1, oxygen saturation, heart rate and end-tidal CO_2 levels whilst breathing either oxygen (FiO_2 1.0) or a hypoxic gas mixture (FiO_2 0.15) in twelve stable asthmatic patients. FEV_1 values were significantly higher on salbutamol days compared to placebo days (* denotes $P < 0.05$ for salbutamol versus placebo). Heart rate was significantly lower on hyperoxia days than on hypoxia days (+ denotes $P < 0.05$ for hyperoxia versus hypoxia). Oxygen saturation values were significantly higher on hyperoxia days compared to hypoxia days (+ denotes $P < 0.05$ for hyperoxia versus hypoxia). There was no significant difference in expired CO_2 levels or respiratory rate between the diferent study days.

Figure 7.2

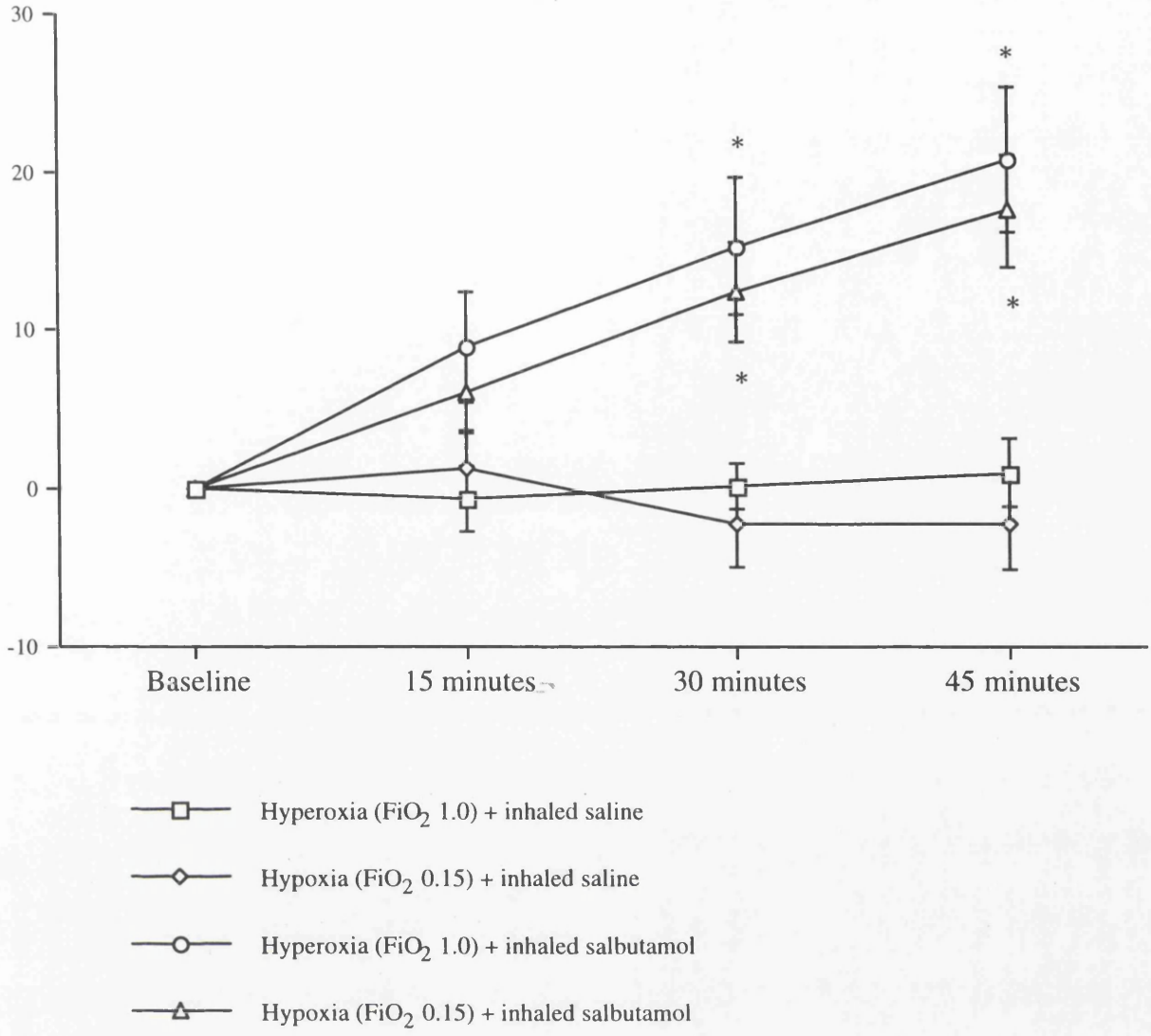


Figure 7.2

Effect of nebulised salbutamol or saline (inhalation 1: salbutamol 0.05 mg/ml or saline, inhalation 2: salbutamol 0.17 mg/ml or saline, inhalation 3: salbutamol 5 mg/ml or saline) on maximum % change in FEV_1 from baseline (%) whilst breathing oxygen (FiO_2 1.0) or hypoxia (FiO_2 0.15) in twelve asthmatic patients.

* denotes $P < 0.01$ for salbutamol versus saline at each time point.

Figure 7.3

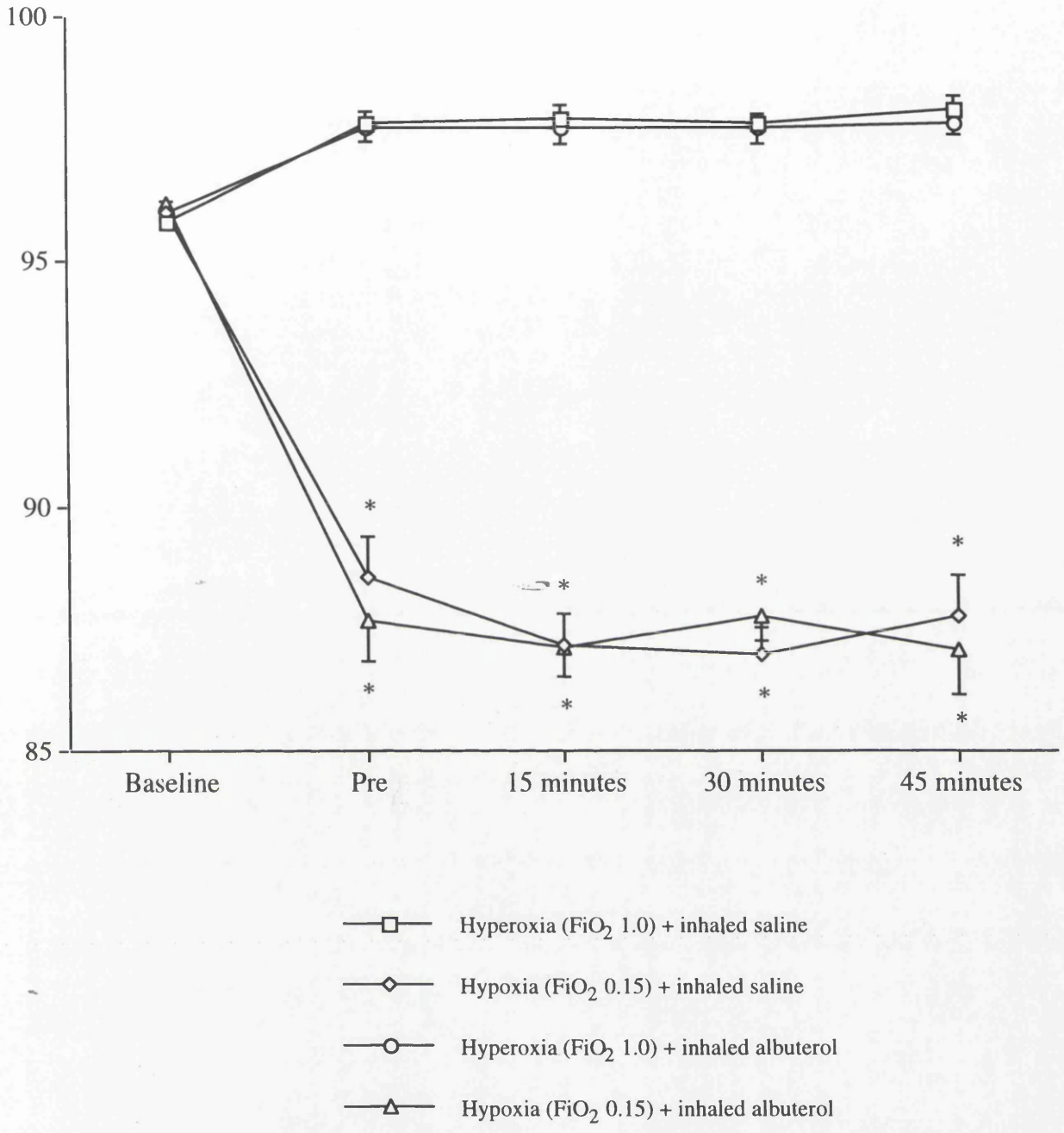


Figure 7.3

Effect of nebulised salbutamol or saline on oxygen saturation (SaO_2%) whilst breathing either oxygen (FiO_2 1.0) or hypoxia (FiO_2 0.15) in twelve asthmatic patients.

* denotes $P< 0.01$ for hyperoxia versus hypoxia at each time point.

7.4 DISCUSSION

The results from our *in-vitro* experiments suggest that the ability of salbutamol to reverse methacholine-induced tone in human isolated bronchial rings is dependent upon the ambient oxygen tension of the environment. This is in keeping with our previous study in bovine isolated bronchial rings, although the pattern of responses differed slightly between the two studies (see section 3). In bovine bronchi, there was no difference between responses in hyperoxia and hypoxia, whereas in human bronchi responses to salbutamol were significantly greater in hyperoxia than hypoxia. A few notable features were in common between the two studies, namely that there was no difference between responses to salbutamol in hyperoxia and normoxia and also that lowering the oxygen tension from normoxia to hypoxia significantly attenuated the relaxatory effect of salbutamol.

Several possible factors for this hypoxic attenuation, such as ß-adrenoceptor-down regulation or uncoupling of the ß-adrenoceptors from their regulatory G-proteins, have been discussed in section 3. It remains possible, however, that hypoxia may have other effects on airway smooth muscle. For example, hypoxia has been shown to induce opening of membrane-bound potassium channels in isolated bronchial rings (Lindemann *et al.*, 1994). Under an oxygen tension of 26 mm Hg it is likely that the majority of these channels will be open. Salbutamol is thought to mediate smooth muscle relaxation, at least partly, by opening membrane-bound potassium channels (Miura *et al.*, 1992). If the majority of potassium channels are already open then the ability of salbutamol to cause further smooth muscle relaxation will be significantly attenuated under hypoxic conditions.

In contrast to our *in-vitro* experiments, it was found that acute hypoxia has no effect on the bronchodilator response to salbutamol in patients with asthma. The changes we observed in oxygen saturation would suggest that our closed breathing circuit had achieved significant changes in vascular oxygen tensions. Hypoxia and hyperoxia in man may cause increases in minute ventilation (Dripps & Comroe, 1947) and a subsequent fall in end-tidal carbon dioxide levels leading to bronchoconstriction (O'Cain *et al.*, 1979; Newhouse *et al.*, 1964; Sterling, 1968; Elshout *et al.*, 1991). In our study we have observed no significant differences in end-tidal carbon dioxide levels suggesting this was not a factor in influencing our results. Prior to the study the nebuliser output for both the hypoxic and hyperoxic gas mixtures was found to be 0.13 mls/min at a flow rate of 7 l/min.

There are several possible explanations for the differences we have observed between our *in vitro* and *in vivo* studies. The lowest concentration of O_2 in the gas mixture used in the *in-vitro* study was 0%, whereas in the *in vivo* study, for reasons of safety, the lowest concentration was 15%. As stated in section 1.6 changes in circulating humoral

factors or neural innervation caused by hypoxia and hyperoxia alone may have influences on airway tone *in vivo* which may offset the effects of the bronchodilator salbutamol. For example, circulating catecholamine levels may affect airway tone *in vivo* (see section 1.4.2). This would appear to be unlikely, however, since we have shown in previous *in vivo* studies using the same closed breathing circuit and inspired hyperoxic gas mixture that no significant changes in circulating catecholamine levels occurs (Dagg *et al.*, 1996, see also section 8). It must also be remembered that the actual concentration of nebulised salbutamol administered to the asthmatic patients is not known, but is likely to be several orders of magnitude less than the concentrations of salbutamol used in the organ baths and this may go some way to explaining the differences between the *in vivo* and *in vitro* results.

As pointed out in section 1.6.2, isolated airway preparations are useful for studying the direct effect of hypoxia on airway smooth muscle function. In the *in vivo* situation, however, the effect of hypoxia on the airway smooth muscle may be opposed by various factors, with perhaps the most important control mechanism being neural reflexes. The inability to isolate these various other control mechanisms makes the task of elucidating the response all the more difficult.

We conclude that acute hypoxia attenuates the bronchodilator response to salbutamol in human isolated bronchial rings but has no effect on salbutamol induced bronchodilation in patients with stable asthma.

CHAPTER 8

THE EFFECT OF ACUTE ALTERATIONS IN OXYGEN TENSION ON METHACHOLINE- AND HISTAMINE-INDUCED RESPONSES IN HUMAN ISOLATED BRONCHI AND ASTHMATIC PATIENTS *IN VIVO*

8.1 INTRODUCTION

In the previous section we investigated the effect of changes in oxygen tension on salbutamol-induced bronchodilation in human airways. As reported in section 3, changing the O_2 tension of the environment can also alter contractile responses, at least in bovine bronchial rings. In this present study, responses to the bronchoconstrictors methacholine and histamine were measured in human isolated bronchial rings under hyperoxic, normoxic and hypoxic conditions. In addition, the ability of methacholine and histamine to induce bronchoconstriction in patients with asthma was compared after inhalation of hyperoxic, normoxic and hypoxic gas mixtures.

8.2 METHODS

8.2.1 *In-vitro* study

8.2.1.1 Tissue collection and preparation

Rings of human bronchi were dissected and mounted in organ baths as described in section 7.2.1.1.

8.2.1.2 Experimental Protocol

Initial experiments were conducted to ascertain the optimum level of applied resting tension for contractile responses in human bronchial rings. Under hyperoxic conditions, an initial resting tension of 0.5g wt was applied to each bronchial ring. Tissues were allowed to equilibrate for 15 minutes before addition of a single concentration of methacholine (10^{-4}M). This concentration was chosen as it evoked an approximately maximal contractile response. When the contraction had reached a plateau, the tissues were washed three times with fresh Krebs-Henseleit solution over a 15 min period, or until the tension returned to baseline. Tension was then removed before increasing the degree of initial tension to the next increment (1.0g wt).

Human bronchial tissue tends to be of variable condition, therefore the viability of each ring was ascertained by the addition of a single concentration (10^{-4}M) of methacholine to evoke a reference contractile response. Once the response reached a plateau, the vessels were than washed 3 times with fresh Krebs-Henseleit solution and allowed to return to baseline tensions. Alterations in gas tension from hyperoxia to either normoxia or hypoxia were achieved by substituting oxygen with nitrogen as stated in section 2.1.5. Tension was adjusted if necessary and the vessels were left for a further 45 minutes before the addition of any drugs.

Cumulative concentration-response curves (CCRCs) were then conducted to either methacholine (10^{-9}-$3x10^{-4}$M) or histamine (10^{-9}-$3x10^{-4}$M) in each of the oxygen tensions. In the case of methacholine, four consecutive concentration response curves were conducted upon the same bronchial ring; first in hyperoxia, then normoxia followed by hypoxia or vice versa. Lastly, a second concentration response curve was conducted in hyperoxia. In addition, on each study day, one ring acted as a time control whereby three consecutive concentration response curves to methacholine were conducted in hyperoxia alone.

In the case of histamine, to avoid tachyphylaxis, only one concentration response curve could be conducted in each bronchial tissue, therefore the three different oxygen tensions were imposed upon three separate bronchial rings. Results for histamine are expressed both in absolute terms and as a % of the initial reference contraction to methacholine.

8.2.1.3 Materials

The following chemicals were used: methacholine and histamine (see section 2.1.8 for full chemical and suppliers names). Stock solutions of drugs were prepared in distilled water and subsequent dilutions made in Krebs-Henseleit solution.

8.2.2 *In-vivo* study

8.2.2.1 Patient Information

Due to the large number of visits required to complete this study, patients were randomly divided into three study groups. All of the patients gave a history of asthma and none had any other significant cardiac or respiratory disease. All of the patients gave written informed consent to the study protocol which had the approval of the West Ethics Committee.

Study Group 1

Eleven mild asthmatic patients (five male) mean (SD) age 42 (12) years were recruited into the study (see Table 8.1). All eleven patients were receiving inhaled $ß_2$-agonists as required and ten regular inhaled corticosteroids. Two were taking regular inhaled salmeterol and one a long acting oral theophylline. This group was used to study responses to methacholine in normoxia and hypoxia.

Study Group 2

Fourteen adult mild asthmatic patients (five male) mean (SD) age 36 (9.2) years were recruited into the study (Table 8.1). All patients were taking inhaled $ß_2$-agonists on an as required basis. Ten were receiving regular inhaled corticosteroids and two were taking regular oral theophyllines and one patient inhaled salmeterol. This group was used to study responses to methacholine in normoxia and hyperoxia.

Study Group 3

Fourteen adult mild asthmatic patients (eight female) mean (SD) age 39 (13) years were recruited into the study (Table 8.1). All patients were taking inhaled $ß_2$-agonists on an as required basis. Twelve were receiving regular inhaled corticosteroids and of these, two were also receiving salmeterol. This group was used to study responses to histamine in hyperoxia, normoxia and hypoxia.

For each group, inhaled $ß_2$-agonists were discontinued for 8 hours prior to attendance, salmeterol for 24 hours and oral theophyllines for 48 hours prior to attendance. Patients were asked to continue their inhaled corticosteroids as usual. Patients were asked to refrain from caffeine containing products for 8 hours prior to each study day. All patients had been stable for a period of two months prior to entry into the study with no significant change in their asthma symptoms or medication. Patients were excluded from the study if there was any significant change in their asthma symptoms or medication between visits. The maximum period between each visit was seven days.

Table 8.1 Patient characteristics

Study No.	No. of patients	Mean age (years)	Mean FEV1 litres	Mean FEV1 % predicted	Geometric mean PC20 (agonist) mg/ml
1	11	42 (12.1)	2.76 (0.49)	86 (7.7)	1.13 (0.04 - 7.90)
2	14	36 (9.2)	2.80 (0.60)	90 (8.6)	0.84 (0.04 - 7.90)
3	14	39 (12.8)	3.04 (0.77)	91 (9.2)	1.79 (0.29 - 6.80)

Table 8.1 Patient characteristics

Abreviations: FEV1; forced expiratory volume in 1 second, PC_{20} (agonist); concentration of agonist (methacholine in studies 1 and 2, and histamine in study 3) provoking a fall in FEV1 of 20%. Data is expressed as mean (S.D).

8.2.2.2 Study design

In Study Groups 1 and 2, patients were asked to attend the study laboratory on three separate days (one screening visit and two study days) at approximately the same time each day. In Study Group 3, patients attended on four separate days (one screening visit and three study days). During the initial screening visit, the patients in Study Groups 1 and 2 underwent a methacholine inhalation challenge test to determine a PC_{20} value ie: that concentration of methacholine causing a 20% fall in FEV_1. Patients in Study Group 3 inhaled histamine to determine a PC_{20} value for this agonist. On the subsequent study days, after thirty minutes of supine rest, the patients were commenced on a closed breathing circuit. Following a ten minute run in period breathing air (FiO_2 0.21) baseline measurements of FEV_1, respiratory rate (RR), heart rate (HR), oxygen saturation (SaO_2%) inspired oxygen and carbon dioxide levels (insp O_2%, insp CO_2%) and expired oxygen and carbon dioxide levels ($PETO_2$%, $PETCO_2$%) were made (see section 2.3.3). In Study Groups 1 and 2, venous blood was also taken for assay of plasma catecholamines.

In Study Group 1, patients then received either air (FiO_2 0.21) or a hypoxic gas mixture (FiO_2 0.15) and in Study Group 2, either air (FiO_2 0.21) or oxygen (FiO_2 1.0) for the remainder of the study day. Patients in Study Group 3 received either hyperoxia, air or hypoxia for the remainder of the study day. All gases were administered in a randomised double blind manner by a second operator obscured from the vision of both the patient and the doctor performing the bronchoconstrictor challenge. Ten minutes after commencing the study gas, all measurements made at baseline, except venous blood sampling, were repeated prior to commencing the bronchoconstrictor inhalation challenge. The study day was terminated when a PC_{20} value had been obtained and the measurements made at baseline were then repeated.

8.2.2.3 Measurements

Heart rate, oxygen saturation, inspired and expired oxygen and carbon dioxide levels were measured as reported in section 2.3.3.1 and FEV_1 values were measured as stated in section 2.3.3.2. Study gases were generated as described in section 2.3.4.

Bronchoconstrictor inhalation challenge: Baseline FEV_1 was measured by taking the best of three recordings, thereafter a saline inhalation was administered and then doubling doses of either nebulised methacholine (made up in normal saline), or nebulised histamine (made up in normal saline), were administered at 5 minute intervals. Each concentration was given for 2 minutes via a (micro-cirrus) nebuliser. All challenge tests were performed by the same person throughout both studies. The nebuliser output for all gases in Study Group 1 and Study Group 3 at a flow rate of 7 Litres per minute was 0.13 mls/min and in Study Group 2 at a flow rate of 6 Litres per minute the nebuliser output was 0.12 mls/min for both gases. The FEV_1 was measured at 0.5, 1.5 and 3 minutes after each inhalation until a fall in FEV_1 of at least 20% was achieved as determined by linear interpolation from the logarithmic dose-response curve. This result was then expressed as the methacholine PC_{20} (provocation concentration 20%) or the histamine PC_{20} (provocation concentration 20%). The methacholine and histamine solutions were made up by the sterile unit of our pharmacy department.

Plasma catecholamines: Adrenaline and noradrenaline levels in the plasma were measured as described in section 2.3.3.3.

8.2.3 Statistical analysis

For the *in-vitro* studies the results for methacholine are expressed as a % of the maximum response in the initial concentration-response curve, in hyperoxia, while results for histamine are expressed in mg wt. Results are expressed as mean (SEM). Statistical significance between the data sets generated by concentration response curves was tested by two-way analysis of variance (ANOVA). Significance between data points (mean maximum responses) was calculated by Students t-test (see section 2.1.9.3). Number of observations (n) refers to the number of patients from whom tissues were obtained.

For the *in-vivo* studies, analysis of variance (ANOVA) corrected for multiple comparisons was used to compare measurements between study days. The PC_{20} values were logarithmically transformed before analysis. A P-value below 0.05 was accepted as significant.

8.3 RESULTS

8.3.1 *In-vitro* study

Optimisation Of Initial Resting Tension

Changing the degree of applied (passive) tension significantly altered the contractile (active) response evoked by a maximal concentration of methacholine (10^{-4}M). The measured contractile response increased as the applied tension was increased from 0.5g wt to 2.0g wt, reaching a maximum contraction at 2.0g wt. When the applied tension was increased beyond 2.0g wt, however, the contractile responses appeared to decline. The maximum response to methacholine, at a resting tension of 2.0g wt, 2109.5±535.9 mg wt, was significantly greater ($P<0.05$ for data points) than the response to methacholine at a resting tension of 1.5g wt, 1178.2±312.8 mg wt. Number of observations (n) =6 in each case.

Figure 8.1

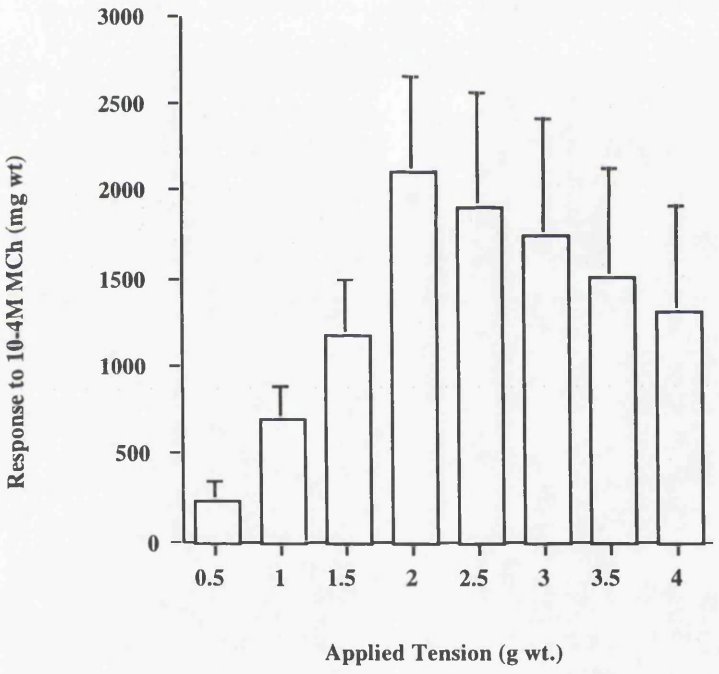


Figure 8.1

The effect of changing the degree of initial resting tension on contractile responses. Contractions evoked by a single concentration (10^{-4}M) of methacholine were measured in human bronchial rings in hyperoxia. Responses are expressed in milligrams weight (mg wt). Contractile responses were significantly ($P<0.05$ for data points) greater at a resting tension of 2.0g wt than at a resting tension of 1.5 g wt. Number of observations (n) = 6 in each case.

Methacholine evoked contractile responses in human isolated bronchial rings in a concentration-dependent manner (see Figure 8.2). In both hyperoxia and normoxia, responses to methacholine were initiated at between 10^{-9}M and $3x10^{-9}$M and between $3x10^{-8}$M and 10^{-7}M in hypoxia (see Figure 8.3A). Reducing the oxygen fraction in the gas mixture from 95% to 20% significantly ($P<0.05$, for data sets) attenuated the contractions evoked by methacholine. Lowering the oxygen tension further, from hyperoxic to hypoxic levels, produced a further reduction in the contractile response to methacholine (responses in hyperoxia were significantly, $P<0.001$ for data sets, greater than responses in hypoxia). Responses to methacholine in normoxia were significantly ($P<0.01$, for data sets) greater than in hypoxia. The maximum response to methacholine in hyperoxia was not significantly different from the maximum response in normoxia (maximum response to methacholine in hyperoxia, at the 10^{-4}M level; 2015.1±503.7mg wt, maximum response to methacholine in normoxia, at the 10^{-4}M level; 1468.8.±609.3mg wt). The maximum response in hypoxia, however (maximum response to methacholine in hypoxia, at the 10^{-4}M level; 816.4±321.0mg wt), was significantly less ($P<0.01$ and $P<0.05$, for data sets, respectively) than in either hyperoxia or normoxia.

In the time control studies, there was no significant difference between the three consecutive concentration response curves to methacholine in hyperoxia (Figure 8.3B), indicating that the contractile responses to methacholine in this tissue were not altered by time.

Figure 8.2

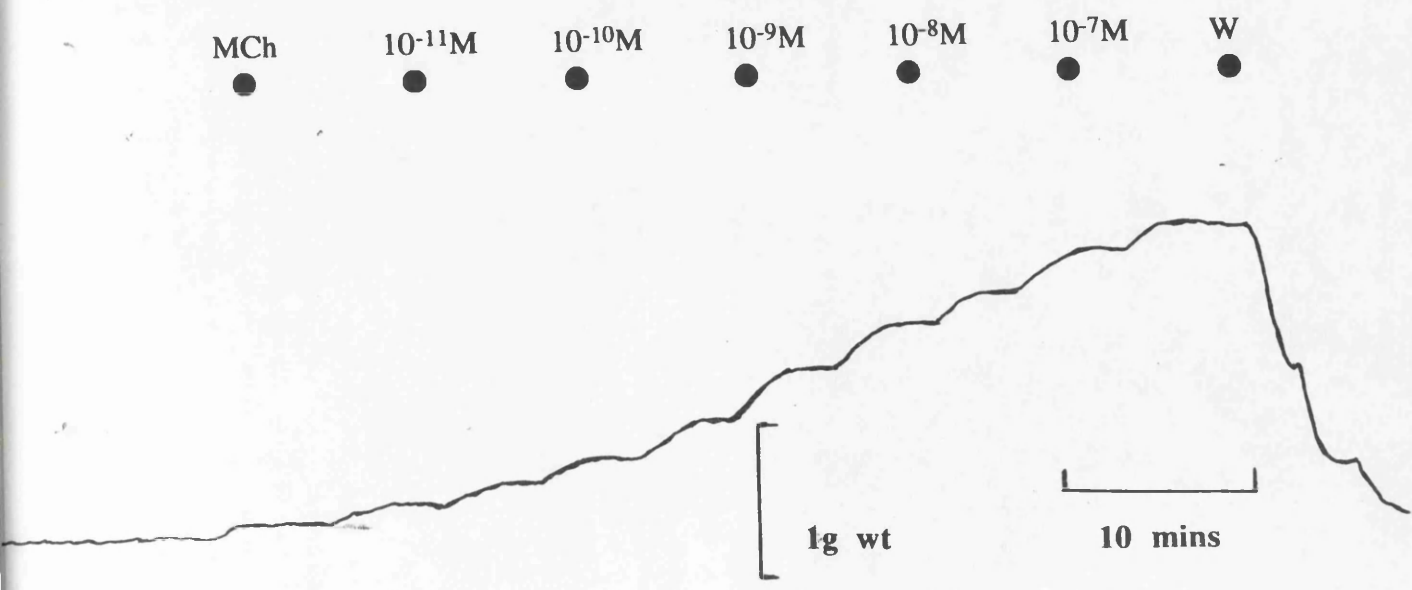


Figure 8.2

Representative trace depicting methacholine-induced contractions in human bronchial rings. Methacholine was added cumulatively to give final bath concentrations of 10^{-9}M-$3x10^{-4}$M. Each concentration was added after the previous response had reached a plateau.

Figure 8.3A

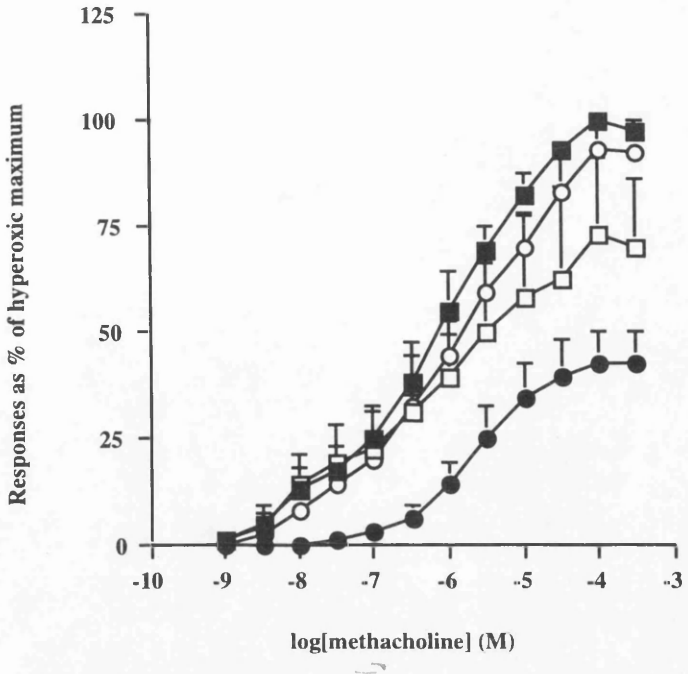


Figure 8.1A

Contractions evoked by methacholine in human isolated bronchi in hyperoxia (■), normoxia (❑) and hypoxia (●), followed by a final concentration-response curve in hyperoxia again (○). Methacholine was added cumulatively to give final bath concentrations of 10^{-9}M-3x10^{-4}M. Responses are expressed as a % of the maximum response in hyperoxia. Methacholine was significantly ($P<0.05$ and $P<0.001$, respectively) more effective in hyperoxia than in normoxia and hypoxia and significantly ($P<0.01$) more effective in normoxia than in hypoxia. There was no difference between the first and the last concentration-response curves in hyperoxia. Number of observations (n)=8 in each case.

Figure 8.3B

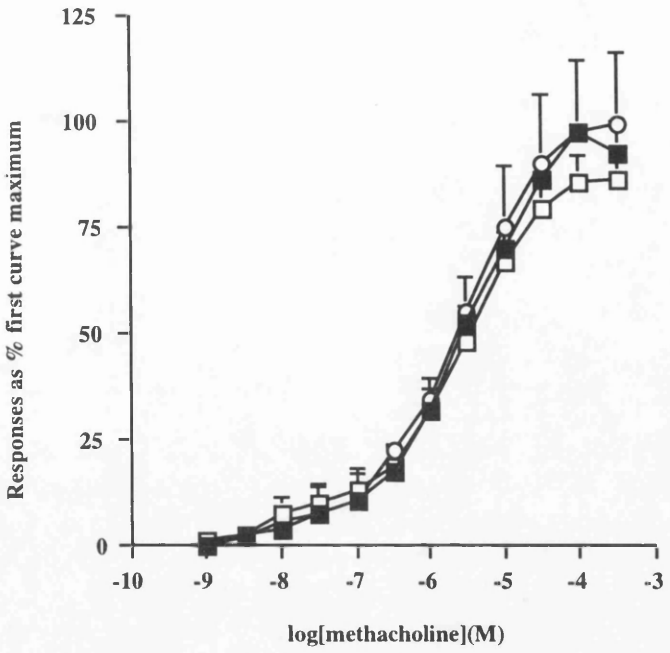


Figure 8.3B

Time control studies for methacholine in human isolated bronchi. Contractions evoked by methacholine in human isolated bronchi in the first curve (■), second curve (❑) and third curve (○). Methacholine was added cumulatively to give final bath concentrations of 10^{-9}M-3x10^{-4}M. Responses are expressed as a % of the maximum response in the first curve. There was no significant difference between the three consecutive concentration response curves to methacholine in hyperoxia. Number of observations (n)=8 in each case.

Histamine also induced concentration-dependent contractions of the human isolated bronchial rings. In each of the oxygen tensions, responses to histamine were initiated at between 10^{-7} M and $3x10^{-7}$ M, indicating that changes in oxygen tension did not alter the sensitivity of the tissue to histamine (see Figure 8.4). Throughout the whole concentration-response curve, contractions evoked by histamine were significantly ($P<0.01$ and $P<0.001$ for data sets, respectively) greater in hyperoxia than in normoxia or hypoxia, and responses in normoxia were significantly ($P<0.05$ for data sets) greater than in hypoxia (n=8 in each case). There was no significant differences between the maximum response (at a concentration of $3x10^{-4}$M) in hyperoxia (1995±509.7mg) and normoxia (1076.7±347.4mg), or between the maximum response in normoxia and hypoxia (683.3±199.2mg). The maximum response in hyperoxia, however, was significantly ($P<0.05$ for data points) greater than the maximum response in hypoxia.

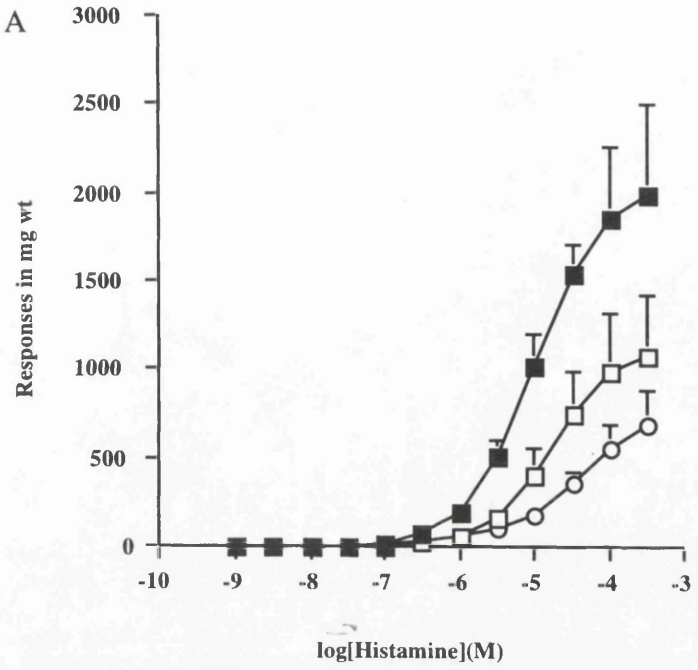
In addition to expressing the results in absolute terms (mg wt.), I also expressed the results as a % of the initial reference contraction evoked by 10^{-4}M methacholine for comparison (see Figure 8.4B). As shown in Figure 8.4B, expressing the results as a % of the methacholine contraction did not alter the pattern of responses. Responses to histamine were still significantly ($P<0.01$ and $P<0.001$ for data sets, respectively) greater in hyperoxia than in normoxia or hypoxia, and responses in normoxia were significantly ($P<0.05$ for data sets) greater than in hypoxia (n=8 in each case).

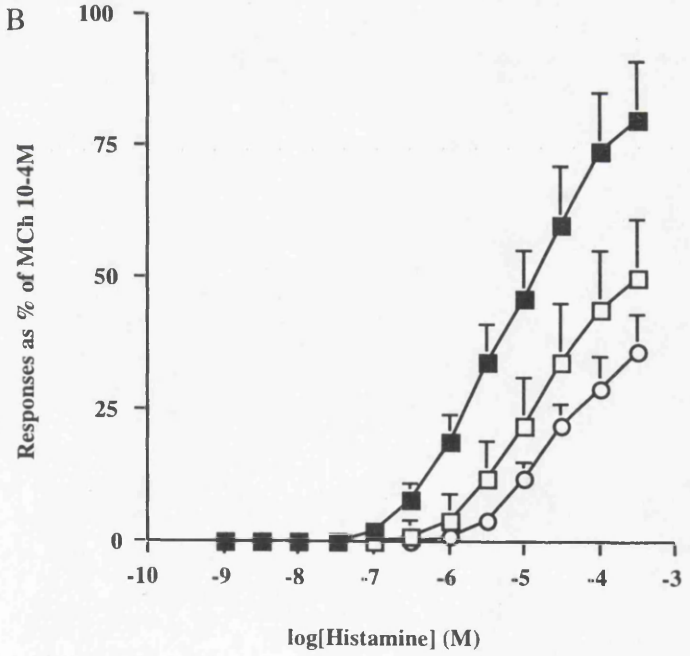
Furthermore, expressing the results in either way did not alter the calculated EC_{50} values (EC_{50} values for histamine in hyperoxia; 5.1±0.7 for results expressed as mg wt, 5.2±0.8 for results expressed as % methacholine. EC_{50} values for histamine in normoxia; 4.9±0.8 for results expressed as mg wt, 4.7±0.7 for results expressed as % methacholine. EC_{50} values for histamine in hypoxia; 4.6±0.6 for results expressed as mg wt, 4.4±0.8 for results expressed as % methacholine.).

Figure 8.4

Contractions evoked by histamine in human isolated bronchi in hyperoxia (■), normoxia (❑) and hypoxia (○). Histamine was added cumulatively to give final bath concentrations of 10^{-9}M-$3x10^{-4}$M. Responses are expressed as (A) mg wt. and (B) as a % of the initial contractile response to methacholine 10^{-4}M. In both cases, histamine was significantly ($P<0.01$ and $P<0.001$, respectively) more effective in hyperoxia than in normoxia and hypoxia and significantly ($P<0.05$) more effective in normoxia than in hypoxia. Number of observations (n)=8 in each case.

Figure 8.4


A
3000
2500
2000
1500
1000
500
0
Responses in mg wt
-10
-9
-8
-7
-6
-5
-4
-3
log[Histamine](M)


B
100
75
50
25
0
Responses as % of MCh 10-4M
-10
-9
-8
-7
-6
-5
-4
-3
log[Histamine] (M)

8.3.2 *In-vivo* study

Baseline measurements: For Study Groups 1 and 2, there were no significant differences in baseline measurements between study days in either study (see Table 8.2). For Study Group 3, there were no significant differences in the baseline measurements between the three study days (Table 8.3).

Table 8.2

	Study 1		*Study 2*	
Baseline measurement	Normoxia study day	Hypoxia study day	Normoxia study day	Hyperoxia study day
FEV1 (L)	2.67 (0.14)	2.63 (0.16)	2.71 (0.14)	2.71 (0.17)
Heart rate (bpm)	71.0 (4.0)	71.0 (4.7)	77.0 (4.3)	75.0 (3.7)
Respiratory rate (breaths/min)	15.0 (1.5)	16.0 (1.6)	14.0 (1.4)	16.0 (1.3)
SaO2 (%)	96.5 (0.2)	96.3 (0.2)	96.5 (0.3)	96.7 (0.4)
Inspired O2%	20.8 (0.2)	21.0 (0.0)	21.0 (0.0)	20.9 (0.1)
End-tidal CO2%	4.66 (0.11)	4.64 (0.14)	4.49 (0.17)	4.47 (0.21)
Plasma noradrenaline (nmol/l)	1.72 (0.14)	1.61 (0.29)	2.14 (0.58)	1.80 (0.26)
Plasma adrenaline (nmol/l)	0.10 (0.02)	0.10 (0.02)	0.11 (0.02)	0.07 (0.02)

Table 8.2:
Baseline measurements for patients in Study Groups 1 and 2. Mean (SEM) baseline respiratory, heart rate and plasma catecholamine measurements for Study 1 (hypoxia v normoxia n=11) and Study 2 (hyperoxia v normoxia n=14). No significant differences between study days in either study.

Table 8.3

Baseline measurement	Normoxia study day	Hyperoxia study day	Hypoxia study day
FEV_1 (Litres)	2.73 (0.2)	2.65 (0.21)	2.65 (0.20)
Heart rate (beats/min)	71 (3.7)	71 (3.0)	70 (3.0)
Respiratory rate (breaths/min)	16 (1.2)	16 (1.2)	16 (0.8)
Oxygen saturation (%)	96 (0.20)	96 (0.17)	96 (0.31)
Inspired oxygen (%)	20.8 (0.1)	20.8 (0.1)	20.9 (0.1)
Expired oxygen (%)	15.4 (0.3)	15.3 (0.2)	15.6 (0.1)
End-tidal CO2 (%)	4.9 (0.1)	4.8 (0.1)	4.8 (0.1)

Table 8.3

Baseline measurements for patients in Study Group 3. Mean (SEM) baseline measurements of FEV_1, oxygen saturation, heart rate, respiratory rate, inspired and expired oxygen tension and end-tidal carbon dioxide levels on the normoxic, hyperoxic and hypoxic study days. There were no significant differences between any baseline measurements on any study day.

Change from baseline

Study 1

Methacholine PC_{20} values: The geometric mean PC_{20} methacholine value was significantly lower (P<0.05) on the hypoxic study day when compared to the normoxic day (Figure 8.5A). The mean difference in PC_{20} methacholine values (mg/ml) between the hypoxic and normoxic study days was 2.88 mg/ml (95% CI 1.40 to 5.3 mg/ml).

Oxygen saturation: Oxygen saturation was significantly lower (P<0.01) following hypoxia [mean (SEM) SaO_2%: baseline 96.3 (0.24)%, pre-methacholine 91.0 (0.56)%, post-methacholine 90.5 (1.0)%] when compared to normoxia [mean (SEM) SaO_2%: baseline 96.5 (0.16)%, pre-methacholine 96.3 (0.27)%, post-methacholine 96.0 (0.43)%] (Figure 8.6A).

Heart rate: There was no significant difference in heart rate when the hypoxia and normoxia study days were compared at any time point (data not shown).

Forced expiratory volume in one second: The mean FEV_1 after 10 minutes breathing the hypoxic gas mixture alone, prior to inhalation of methacholine was not significantly different when compared to the normoxic study day. The mean FEV_1 (SEM) following 10 minutes of hypoxia alone was 2.40 (0.15) Litres compared to 2.38 (0.14) Litres on the normoxic study day.

Study 2

Methacholine PC_{20} values: There was no significant difference in the geometric mean PC_{20} methacholine values between the normoxic and hyperoxic study days (Figure 8.5B). The mean difference in PC_{20} methacholine values (mg/ml) between the hyperoxic and normoxic study days was 1.45 mg/ml (95% CI 0.83 to 2.51 mg/ml).

Oxygen saturation: Oxygen saturation was significantly higher (P<0.01) following hyperoxia [mean (SEM) SaO2%: baseline 96.7 (0.35)%, pre-methacholine 98.1 (0.23)%, post-methacholine 98.1 (0.20)%] than during the normoxic study day [mean (SEM) SaO2%: baseline 96.5 (0.33)%, pre-methacholine 96.7 (0.37)%, post-methacholine 96.0 (0.52)%] (Figure 8.6B).

Heart rate : Heart rate was significantly lower (P<0.05) on the hyperoxic study day both before and after the methacholine inhalation test when compared to the normoxic study day. The mean (SEM) HR (bpm) on the hyperoxic study day was: baseline 75 (4), pre-methacholine 71 (4), post-methacholine 71 (4) and on the normoxic study day: baseline 77 (4), pre-methacholine 75 (5), post-methacholine 77 (4).

There were no significant differences in respiratory rate, end-tidal carbon dioxide % or plasma catecholamine levels between study days in either study (Table 8.4 post methacholine values on each study day).

Table 8.4

	Study 1		*Study 2*	
Post methacholine inhalation measurement	Normoxia study day	Hypoxia study day	Normoxia study day	Hyperoxia study day
Respiratory rate (breaths/min)	15.0 (1.6)	14.0 (2.0)	14.0 (1.0)	15.0 (1.1)
End-tidal CO2%	3.70 (0.18)	3.86 (0.17)	3.94 (0.18)	3.87 (0.22)
Plasma noradrenaline nmol/l	1.60 (0.16)	1.40 (0.25)	1.66 (0.29)	1.63 (0.28)
Plasma adrenaline nmol/l	0.08 (0.02)	0.06 (0.02)	0.07 (0.02)	0.07 (0.02)

Table 8.4

Mean (SEM) measurements of respiratory rate, end-tidal carbon dioxide % and plasma catecholamine levels following methacholine inhalation in study 1(hypoxia v normoxia) n=11and study 2 (hyperoxia v normoxia) n=14.

No significant differences were found between study days in either study.

Figure 8.5A

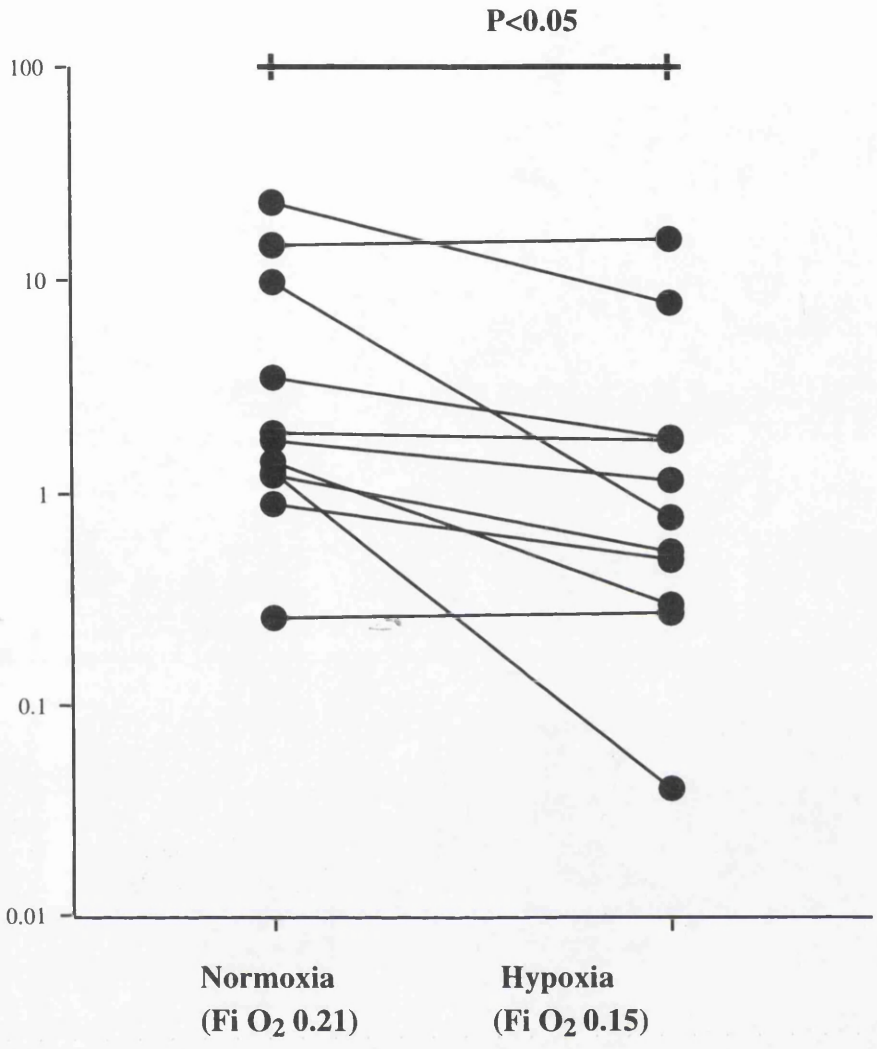


Figure 8.5A

Effect of hypoxia and normoxia on PC_{20} methacholine values in patients with asthma (Study Group 1 n=11). The y-axis represents PC_{20} methacholine values in mg/ml. The geometric mean PC_{20} methacholine value was significantly lower ($P<0.05$) on the hypoxic study day when compared to the normoxic day.

Figure 8.5B

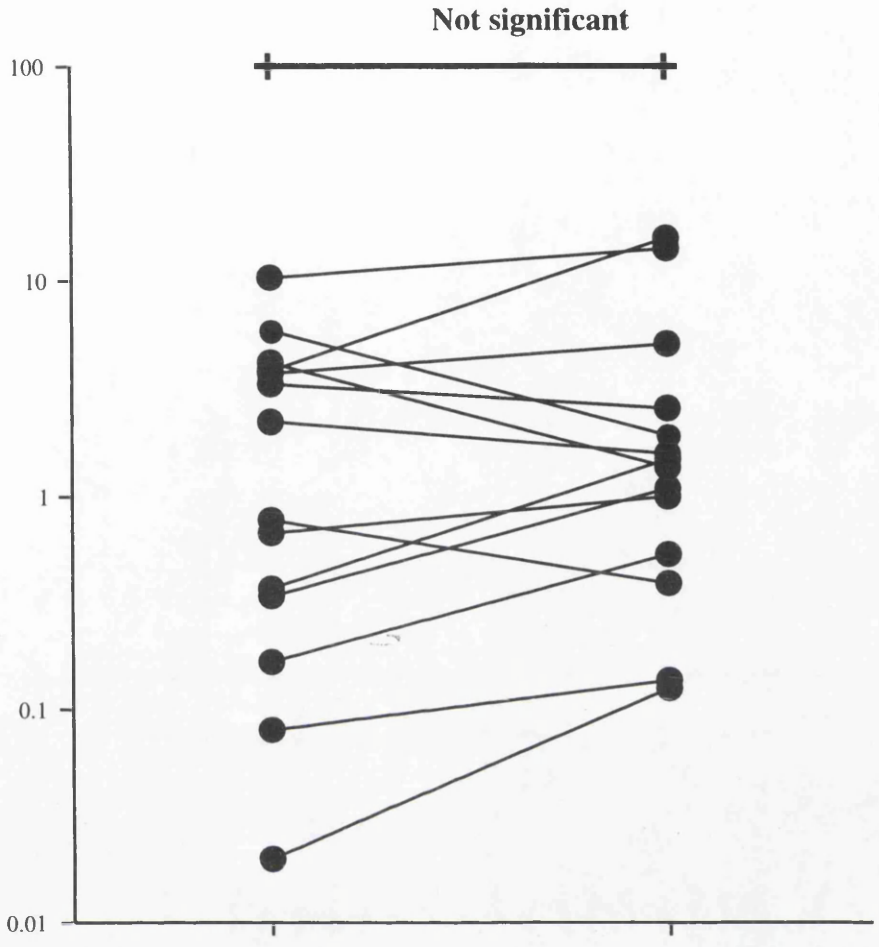


Figure 8.5B

Effect of hyperoxia and normoxia on PC_{20} methacholine values in patients with asthma (Study Group 2, n=14). The y-axis represents PC_{20} methacholine values in mg/ml. There was no significant difference between the geometric mean PC_{20} methacholine values on the hyperoxic and hypoxic study days.

Figure 8.6A

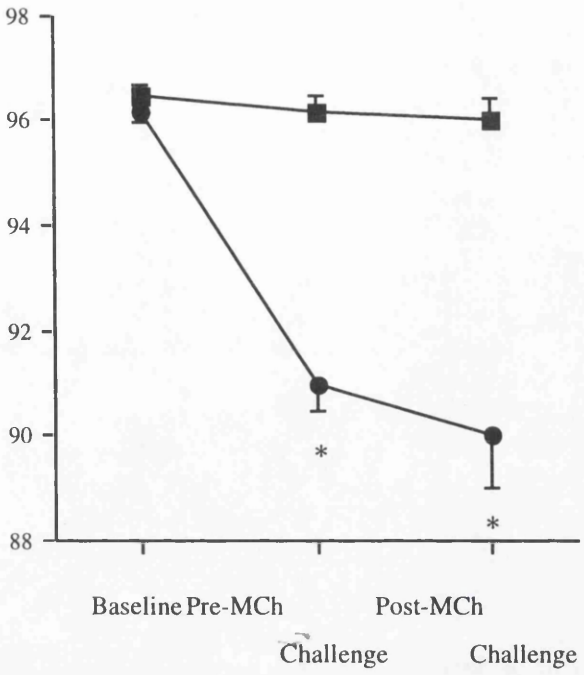


Figure 8.6A

Effect of hypoxia and normoxia on oxygen saturation (SaO_2) in patients with asthma (Study Group 1 n=11). The y-axis represents SaO_2 values in %. The SaO_2 values, both before and after methacholine challenge, were significantly lower ($P<0.01$) on the hypoxic study days (●) when compared to the normoxic days (■). * denotes $P<0.01$ for data points.

Figure 8.6B

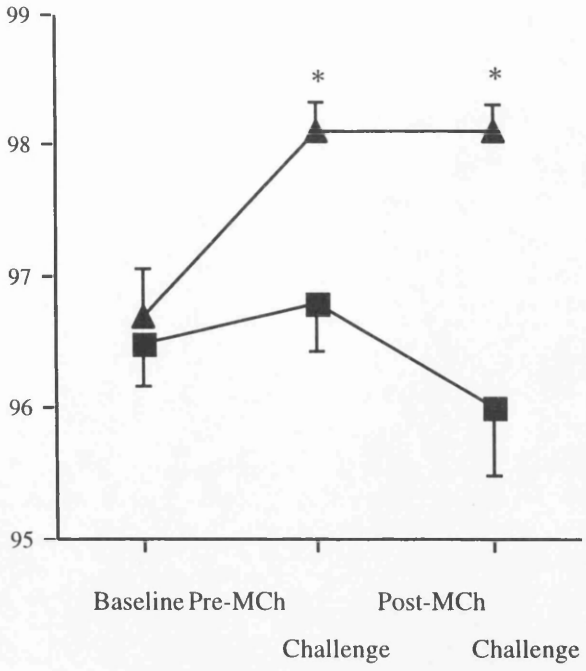


Figure 8.6B

Effect of hyperoxia and normoxia on oxygen saturation (SaO_2) in patients with asthma (Study Group 2 n=14). The y-axis represents SaO_2 values in %. The SaO_2 values, both before and after methacholine challenge, were significantly higher ($P<0.01$) on the hyperoxic study days (▲) when compared to the normoxic days (■). * denotes $P<0.01$ for data points.

Figure 8.7

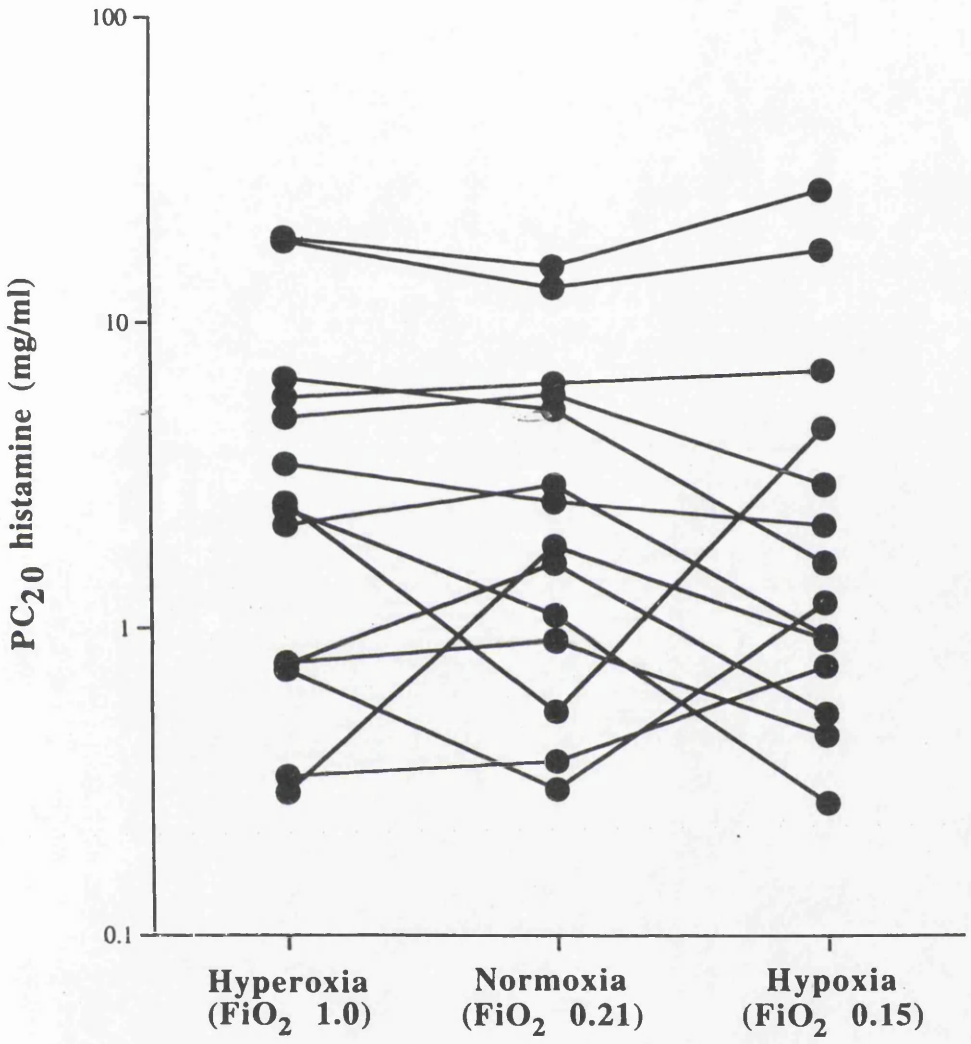


Figure 8.7

Effect of hyperoxia, normoxia and hypoxia on PC_{20} histamine values in patients with asthma (Study Group 3, n=14). The y-axis represents PC_{20} histamine values in mg/ml. There was no significant difference between the geometric mean PC_{20} histamine values between the hyperoxic, normoxic and hypoxic study days.

Figure 8.8

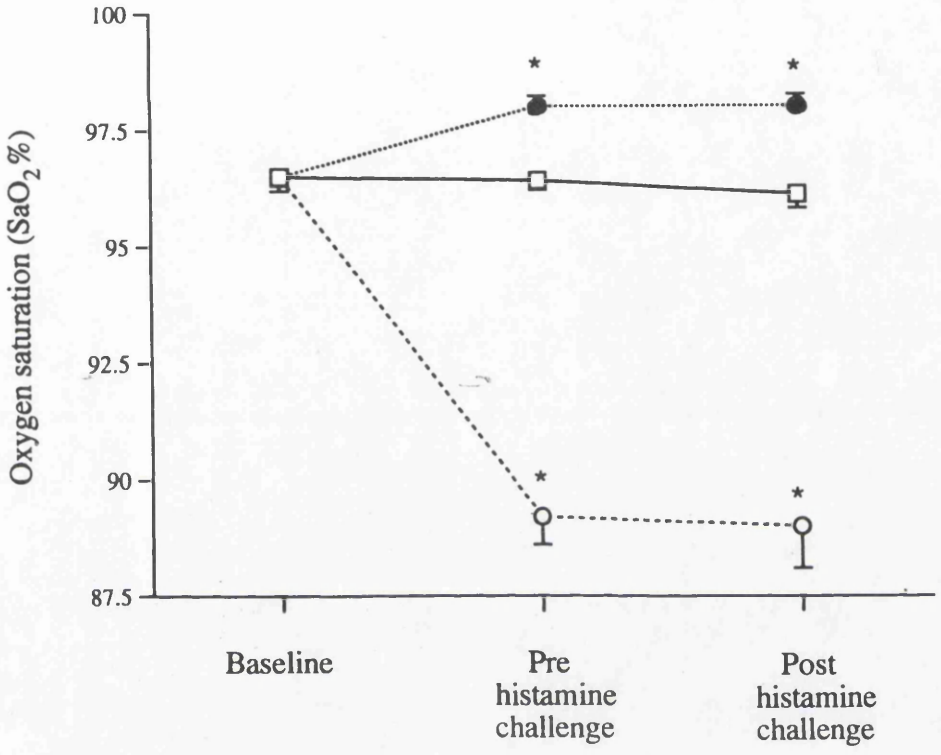


Figure 8.8

Effect of hyperoxia, normoxia and hypoxia on oxygen saturation (SaO_2) in patients with asthma (Study Group 3 n=14). The y-axis represents SaO_2 values in %. The SaO_2 values, both before and after methacholine challenge, were significantly higher ($P<0.01$) on the hyperoxic study days (●) when compared to the normoxic (❑) and hypoxic days (○). * denotes $P<0.01$ for data points.

Study 3

Histamine PC_{20} values: Mean histamine PC_{20} values mg/ml were not significantly different between the three study days (Figure 8.7). The mean (range) PC_{20} value mg/ml on each study day was: hypoxia day; 1.91 (0.27-27.4) mg/ml, hyperoxia day; 2.32 (0.29-19.0) mg/ml, normoxia day; 2.19 (0.3-15.4) mg/ml.

Oxygen saturation: Oxygen saturation was significantly higher (P<0.01) on the hyperoxic study day when compared to the normoxic and hypoxic study days both before and after histamine inhalation. Oxygen saturation was significantly lower (P<0.01) on the hypoxic study day [mean (SEM) SaO2%: baseline 96.1 (0.31)%, pre-histamine 96.3 (0.23)%, post-histamine 96.0 (0.20)%] when compared to the normoxic study day [mean (SEM) SaO2%: baseline 96.7 (0.20)%, pre-histamine 97.1 (0.23)%, post-histamine 97.1 (0.20)%]. Oxygen saturation was significantly higher on the hyperoxic study days [mean (SEM) SaO2%: baseline 96.1 (0.17)%, pre-histamine 99.3 (0.13)%, post-histamine 99.2 (0.11)%] than the hypoxic days. (Figure 8.8).

Heart rate: Heart rate was significantly lower (P<0.01) on the hyperoxic study day when compared to both the normoxic and hypoxic study days both before and after inhalation of histamine. The mean (SEM) heart rate (bpm) on each study day at baseline, prior to histamine inhalation and after histamine inhalation was: hypoxia day 70 (3), 74 (3) and 72 (3): hyperoxia day 71 (3), 65 (3) and 67 (3): normoxia day 71 (4), 71 (4) and 74 (4). Heart rate was not significantly different when the hypoxic and normoxic study days were compared.

FEV_1: There were no significant differences in FEV_1 values between the study days 10 minutes after commencing the study gases prior to inhalation of histamine. The mean (SEM) FEV_1 value litres 10 minutes after commencing the study gas on each day was: hypoxia day 2.65 (0.18) litres, hyperoxia day 2.65 (0.18) litres and normoxia day 2.73 (0.16) litres.

End-tidal carbon dioxide %, respiratory rate: End-tidal carbon dioxide (%) and respiratory rate measurements were not significantly different between study days both before and after histamine inhalation (data not shown).

8.4 DISCUSSION

In this present study, we found that lowering the oxygen tension from hyperoxic to normoxic levels significantly attenuated contractile responses to methacholine and histamine in human isolated bronchi. Reducing the oxygen tension further, from normoxic to hypoxic levels, evoked a further reduction in contractile responses to these two spasmogens.

A previous study by Vidruk & Sorkness (1985) in anaesthetized dogs showed that histamine-induced tracheal constriction was potentiated by hypoxia and attenuated by hyperoxia. These responses were eliminated by denervation of the trachea, indicating that the bronchomotor responses were reflex rather than direct in nature. In contrast, the responses evoked by histamine in our *in vitro* studies reflect the direct effect of histamine on airway smooth muscle.

The prevailing pattern of histamine receptors determines the effect of histamine on airway smooth muscle. Since it varies considerably between species and even between different sites in the same airway (Chakrin & Krell, 1980), extrapolation of results from animal models to man has created considerable confusion. In most species, the predominance of bronchoconstricting H_1-receptors over bronchodilating H_2-receptors ensures that histamine produces bronchoconstriction. In man, H_1-receptor mediated contraction of airway smooth muscle has been extensively demonstrated (Casterline & Evans, 1976; Woenne *et al.*, 1978; Thomson & Kerr, 1980), although the existence of H_2 receptors remains controversial. An early hypothesis (Chand, 1980) suggested that an absence of H_2-receptors might be the cause of bronchial hyperrresponsiveness in asthma. The balance of evidence, however, indicates that bronchodilating H_2-receptors are present in insignificant numbers if at all in human airways (see Eiser, 1992).

Given that responses to methacholine (which of acts via muscarinic post-junctional M_3 and possibly M_2, see section 1.4.1) were altered in a similar manner by changes in oxygen tension, the attenuation in histamine responses as the oxygen tension was decreased is therefore unlikely to be due to a change in the balance between excitatory H_1 and inhibitory H_2-receptors.

Another potential explanation is that lowering the oxygen tension from hyperoxic to normoxic and hypoxic levels increases the rate of cell death for the airway smooth muscle cells. Given that the attenuation of methacholine responses seen in normoxia and hypoxia were reversed when the oxygen tension was returned to hyperoxic levels, this remains unlikely. It is perhaps more plausible that the attenuation of contractile responses as the oxygen tension was reduced reflects an impairment of the airway smooth muscle contractile processes. Previous studies have shown that hypoxia reduces the force of contraction in airway smooth muscle (Stephens & Kroeger, 1970), possibly by impairing energy metabolism (Stephen & Chiu, 1970) or by inhibiting the entry of extracellular calcium (Fernandes *et al.*, 1993, see section 1.6).

In our *in vivo* studies, we found that, compared to responses in normoxia, hypoxia potentiated methacholine-induced bronchoconstriction in asthmatic patients. Hyperoxia, however, did not alter methacholine-induced responses compared to normoxia. Histamine produced a different pattern of results in that bronchoconstrictor responses to histamine were not altered by changing the inspired oxygen tension from normoxia to either hyperoxia or hypoxia.

The rise in oxygen saturation and fall in heart rate seen on the hyperoxic study day and the fall in saturation on the hypoxic study day indicate that our closed breathing circuit has achieved the required changes in airway and vascular oxygen tension (Jennet, 1964; Watt *et al.*, 1942; Dripps & Comroe, 1947). It could be postulated that hypoxia may cause an elevation of plasma adrenaline levels, which may then influence bronchial tone indirectly (see section 1.4.2). The absence of a significant rise in heart rate on the hypoxic study day, however, suggests that the level of hypoxia we have produced has not resulted in an increase in resting sympathetic tone. This is supported by our finding that plasma catecholamine levels were not altered on hypoxic study days.

Both hypoxia and hyperoxia cause an increase in minute ventilation and subsequent fall in end-tidal carbon dioxide levels (Jennet, 1964; Watt *et al.*, 1942; Dripps & Comroe, 1947). Hypocapnia *in-vivo* is associated with bronchoconstriction (Newhouse *et al.*, 1964; O'Cain *et al.*, 1979; Sterling, 1968; Elshout *et al.*, 1991), therefore it could be proposed that our results were influenced by a hypocapnia-mediated increase in airway tone. We, however, found no significant difference in end-tidal carbon dioxide levels between study days either before or after agonist-inhalation, suggesting that this factor did not influence our results.

Nebuliser output may be affected by the molecular weight of the gas used to drive the nebuliser, therefore in all the studies the nebuliser output was calculated on two occasions for each study gas. In all of the studies, the nebuliser output was within acceptable limits (Studies 1 and 3; 0.13 mls/min at a flow rate of 7 l/min and in study 2; 0.12 mls/min at 6 l/min).

Our double blind study has demonstrated an increase in airway reactivity to methacholine following acute hypoxia in keeping with a previous single blind study reported by Denjean *et al.* (1988). Previous animal studies have suggested that the potentiation of methacholine-induced bronchoconstriction by hypoxia is attenuated by prior surgical chemodenervation (Denjean *et al.*, 1991), suggesting that the effect is mediated via peripheral chemoreceptors. In our study we have been unable to detect an increase in airway tone following 10 minutes of hypoxia as one would expect if this effect occured as a consequence of bronchoconstriction due to hypoxia alone. Thus, this

appears to be an enhancement of bronchial reactivity to methacholine, without any change in baseline tone.

Other animal studies have shown that potentiation of histamine and carbachol-induced bronchoconstriction in sheep is significantly reduced by both intravenous cromolyn sodium (Ahmed & Marchette, 1985) and FPL57231, (D'Brot & Ahmed, 1988) a leukotriene receptor antagonist, suggesting that alveolar hypoxia may stimulate release of inflammatory mediators. Hypoxia may also act on vagal nerve endings to stimulate neurotransmitter release and hence cause bronchoconstriction.

These findings may be of clinical relevance as the level of hypoxia we have induced is compatible with those seen in patients admitted to hospital with acute severe exacerbations of asthma. It would appear therefore that hypoxia occurs not only as a consequence of acute severe asthma but may also increase airway responsiveness to bronchoconstrictor stimuli.

We have not, in man demonstrated attenuation of methacholine-induced bronchoconstriction by hyperoxia in contrast to the findings of the *in-vivo* animal studies of Vidruk & Sorkness (1985). This difference may best be explained by species variation or by differences in methodological approach and study design. Our results support and extend those of Wollner *et al* (1991). We have however also examined the potential influences of hypocapnia and circulating catecholamines on airway reactivity during hyperoxia and also demonstrated that our patients show the typical cardiovascular and respiratory responses to hyperoxia. Our results differ from those of Inoue *et al* (1989) who found that hyperoxia attenuated methacholine-induced bronchoconstriction in asthmatic subjects. They however used an inspired oxygen concentration of 30% whereas we used 100%. Six of their patients had arterial oxygen tensions below 10 Kpa, in keeping with resting hypoxaemia, and since inspiring 30% oxygen relieves hypoxic bronchoconstriction (Libby *et al.*, 1981; Astin, 1970) this effect may have falsely influenced their results.

In addition, this study has also shown no change in airway hyperresponsiveness to histamine at various inspired oxygen tensions in patients with asthma. This contrasts with the findings of Vidruk & Sorkness (1985) who found hypoxia potentiated and hyperoxia attenuated histamine induced tracheal constriction in mongrel dogs. It is worth noting that the aforementioned study used an inspired oxygen tension of 12% which is less than our own and that the animals in his studies had been anaesthetised. As stated in section 1.6, the use and level of anaesthesia in these studies can significantly alter the outcome of the study. The anaesthetic drugs in combination with the altered oxygen tensions used may have effects on circulating humoral factors or chemoreceptor activity which have influenced their results. Our study results also differ from those of Ahmed & Marchette (1985) and D'Brot & Ahmed (1988) who, using

conscious sheep, also showed potentiation of histamine induced bronchial reactivity by hypoxia. As stated earlier, the distribution of histamine receptor subtypes in the airways varies from species to species, thus it is possible that species variation could account for these differences.

Our findings for histamine also differ from the methacholine results, in which we demonstrated that hypoxia potentiates methacholine induced bronchoconstriction in patients with asthma. The study protocols were similar and performed using the same breathing circuit. In addition, the *in vitro* studies for methacholine and histamine produced a similar pattern of responses. It is difficult therefore to account for this difference. Clearly the precise mechanisms of these effects is complex and will require further work. We have shown no effect of hyperoxia on histamine induced bronchoconstriction which is in keeping with our own study examining the effect of hyperoxia on methacholine hyperresponsiveness.

In conclusion, we have shown that contractile responses to both methacholine and histamine in human isolated bronchi are attenuated by lowering the oxygen tension. Furthermore these results demonstrate that acute hypoxia potentiates methacholine-induced bronchoconstriction in patients with stable asthma, whereas raising the inspired oxygen tension to hyperoxic levels did not alter bronchial responsiveness to methacholine. In contrast, histamine-induced bronchoconstriction in patients with stable asthma was not altered by acute alterations in inspired oxygen concentration.

CHAPTER 9

GENERAL DISCUSSION

9 GENERAL DISCUSSION

It should be apparent from the results chapters in this thesis that changes in oxygen tension can have a marked effect on airway smooth muscle function. I have shown that hypoxia can:

(i) alter the responsiveness of isolated bronchial rings to both contractile and relaxatory agonists.

(ii) induce proliferation of cultured human airway smooth muscle cells and enhance the mitogenic effects of certain agonists on these cells.

(iii) potentiate the bronchoconstrictor response to methacholine, but not histamine, in asthmatic patients.

Closer examination of the results indicates that the effect of hypoxia on airway smooth muscle responsiveness is not always consistent between different species. The most notable example is the contractile response to methacholine in isolated bronchial rings. In bovine bronchi, lowering the oxygen tension from hyperoxic to either normoxic or hypoxic levels significantly enhanced responses to methacholine. In human and rat isolated airway preparations, the converse was true in that contractile responses were attenuated by hypoxia. This apparent conflict may be due to an underlying difference in the pharmacology or biochemistry of the airway smooth muscle of the various species. Alternatively, the difference may be due to the different order of airways used in the *in vitro* studies. For rats, the trachea was used, in humans the "upper bronchi" (2nd - 4th order) and in bovine, relatively "lower" bronchi (4th - 5th). As stated in section 1.6, the response of the airways to hypoxia may differ between the upper and lower regions of the respiratory tract. Thus, this may account for the experimental differences between the species. It would be interesting to conduct a study comparing various regions of the respiratory tract from each species. Unfortunately this was impractical for a number of reasons: firstly, the supply of human tissue was limited both in quantity and frequency. Secondly, the rat airways rapidly decrease in size after the tracheal stage and become beyond the reasonable limits of resolution for organ bath experiments. The opposite is true for bovine bronchi since bronchi of 2nd-4th generation, which would compare to generation of the human tissue, would be too large for practical use.

While the effect of hypoxia differs between species, there are also differences between various agonists in the same species. For example, in bovine bronchi salbutamol was more effective at reversing induced tone in normoxia than hypoxia, whereas the other dilators were either equipotent (isosorbide dinitrate) or more effective (atrial natriuretic peptide) in 0% than 20% O_2. In contrast, sodium nitroprusside was more effective in hyperoxia than normoxia.

I have postulated that this may be due to the different second-messenger pathways utilised by each of these agents. As stated in section 1.3.4, there are several intracellular

mechanisms thought to be involved in producing relaxation of airway smooth muscle. The idea of "multiplex" signalling suggests that a single agonist may activate more than one pathway simultaneously. It is possible that changes in oxygen tension affects these different pathways to varying degrees, which would perhaps explain the disparity between the various dilators.

This may also go some way to explaining why hypoxia potentiated methacholine-induced bronchoconstriction in asthmatic patients but did not alter histamine-induced bronchoconstriction. While histamine and methacholine are both thought to act via similar second-messenger mechanisms (G protein-linked hydrolysis of PIP_2), there may be slight differences in the intracellular circuitry stimulated by each of these agonists. Evidence against this, however, comes from the *in vitro* results, whereby the pattern of responses to methacholine in the different oxygen tensions was similar to that of histamine. Thus the results from the *in vitro* experiments did not predict the outcome of the *in vivo* studies.

As stated in section 1.4, airway responsiveness is dependent upon the balance between neural and humoral pathways as well as the intrinsic control of the airway smooth muscle. In this body of work, I used isolated airway preparations to examine the importance of the intrinsic muscular control elements in determining airway responsiveness under hyperoxic, normoxic and hypoxic conditions.

Previous studies have compared the results from human airways *in vitro* and *in vivo*. For example, Roberts and coworkers (1985) measured airway responses to histamine in 20 patients prior to undergoing lung resection. The results were compared with the subsequent responses to histamine in isolated bronchi taken from the same patients during thoracotamy. There was a wide variation in the *in vivo* responses, with relatively small variation between the *in vitro* results. No correlation was found, however, between the *in vitro* and *in vivo* studies, a finding which is in agreement with other studies comparing *in vitro* and *in vivo* responses (Vincenc *et al.*, 1983; Armour *et al.*, 1984; Cerrina *et al.*, 1986).

This is not unique to histamine, since several other studies have failed to demonstrate any correlation between *in vivo* and *in vitro* airway responsiveness to methacholine (Roberts *et al.*, 1984). Furthermore, *in vivo* airway responsiveness to LTD_4 varied more than 1000 fold whereas *in vitro* responses were much less variable. As was found with histamine and methacholine, there was no correlation between the *in vivo* and *in vitro* results (Roberts *et al.*, 1987).

There are several possible reasons which may explain the lack of correlation between the *in vivo* and *in vitro* airway responses. For example:

(i) As mentioned earlier, it is unknown whether responsiveness to agonists is uniform throughout the airways. For example, Raffestin and coworkers (1985) reported that the *in vitro* smooth muscle responses to constrictor agents were less in large airways (internal diameter, 6mm) than in smaller airways (internal diameter, 2mm). It has been postulated that *in vivo* airway responsiveness is representative of the average of the

range of responsiveness within the lung. Thus, it is possible that tissues studied *in vitro* are not responsible for the *in vivo* responses.

(ii) Studies comparing *in vivo* with *in vitro* responsiveness have measured *in vitro* sensitivity under conditions of isometric tension, as indeed was the case for my studies. *In vivo*, however, airway narrowing involves shortening of smooth muscle and it is possible that an assessment of isotonic shortening of airway smooth muscle would be more relevant to *in vivo* conditions.

In addition to the effects of acute changes in oxygen tension on airway responsiveness, I have shown that chronic exposure to hypoxia can also significantly alter responses to various agents. Using the hypobaric model of chronic hypoxia, I demonstrated that isolated airway preparations from rats exposed to 14 days of moderate hypoxia responded significantly less than controls to the contractile agents methacholine, endothelin-1 and potassium chloride. In chapter 5, I reported that chronic hypoxia altered the involvement of ET_A and ET_B receptors in the the contractile response to ET-1 in rat trachea. This suggests that chronic hypoxia may induce alterations at the level of the membrane-bound receptor. Whether this represents a change in the expression of the receptor subtypes, or uncoupling of the receptors from their regulatory G proteins is unclear. It is possible that the behaviour of other G protein-linked receptors in airway smooth muscle, such as muscarinic recptors, may be altered by chronic hypoxia. As stated in chapter 4, however, this would not explain why responses to potassium chloride were also altered, since this agent does not act via receptor-stimulation. We have postulated in chapter 4 that the attenuation in responsiveness to contractile agents is due to some alteration in the intrinsic pharmacology/biochemistry of the airway smooth muscle. For example, we found responses to the dilator, sodium nitroprusside but not salbutamol, to be enhanced in chronic hypoxia, suggesting that chronic hypoxia may cause an increase in the responsiveness of airway smooth muscle to cGMP-mediated relaxation.

These findings are in opposition to the effect of chronic hypoxia on the responsiveness of pulmonary arteries to contractile agents. Several authors have reported that contractile responses to a wide variety of agents are enhanced in pulmonary arteries from chronically hypoxic rats (Eddahibi *et al.*, 1991; MacLean *et al.*, 1995). This enhancement is purported to be due to the increase in vascular smooth muscle mass in the blood vessels from the hypoxic rats. Interestingly, our cell culture studies showed that proliferation of airway smooth muscle cells was also stimulated by hypoxia. From this finding, one may expect the airways of the chronic hypoxic rats to exhibit an increased muscularisation. This in turn would be expected to enhance contractile responses, as in the pulmonary arteries, due to an increase in the airway smooth muscle mass. While this has been shown to occur in rats exposed to chronic hyperoxia (Szarek

et al., 1995; Hershenson *et al.*, 1992a and b, see section 1.7), I found that chronic hypoxia actually attenuated the contractile responses.

Given that we did not compare the ultrastructure of the airways from control and chronically hypoxic rats, I cannot make any statements about the degree of muscularization in the airways of the chronically hypoxic rats. However, since chronic hyperoxia has been shown to induce thickening of the airway smooth muscle in rats with a concomitant increase in contractile responses, and since we found an attenuation in contractile responses in the hypoxic rats, one may tentatively conclude that thickening of the airway wall is unlikely to occur in the airways of our chronically hypoxic rats.

This would appear to be at odds with our cell culture studies, since I found hypoxia to potentiate mitogenesis in the cultured airway smooth muscle cells.

It may be difficult, however, to make a direct comparison between the cell culture studies and the chronically hypoxic rat studies for a number of reasons. Firstly, the cells used in the cell culture studies were human airway smooth muscle cells, thus there may be an underlying species difference in terms of the proliferative response to hypoxia. Secondly, the duration and intensity of the hypoxic stimulus was clearly different between the two studies. The cultured cells were exposed to extremely low O_2 tensions (~30mm Hg) for a relatively short time (24 hours), whereas the rats received a less severe hypoxic exposure for a longer time period (~330 mm Hg for 14 days). It is possible that the effect of hypoxia on cell proliferation is dependent on both the intensity and the duration of the hypoxic stimulus, but this remains to be addressed by scientific investigation.

In conclusion, the responsiveness of airway smooth muscle may be significantly altered by either acute or chronic changes in oxygen tensions. This may be of clinical significance given that hypoxia can often be a feature of various respiratory disorders. The full mechanisms underlying the effect of hypoxia on airway smooth muscle function remain to be elucidated.

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Robert Clayton: PhD examination March 17th 1998

Changes required by the examiners are listed here (as discussed at the viva exam). These changes are to be completed to the satisfaction of Dr T C Muir (Internal Examiner), within 6 weeks.

a) Include illustrative original traces of isometric tension records from preparations exemplifying each of the animal species used and a representative example of some, at least, of the agents studied.

✓ **b)** Explain those aspects of your protocol that defined initial resting tension

✓ **c)** Illustrate the comparability of (*e.g.*) ED_{50} estimates whether based on absolute or normalised tension values

✓ **d)** Discuss the assumption that radiolabelled thymidine uptake is a reliable indicator of cell proliferation under conditions of hypoxia

✓ **e)** Provide an explicit statement of the aims of the experimental work

✓ **f)** Discuss the usefulness of sodium nitroprusside as an analogue of nitric oxide

✓ **g)** Make clearer the extend of your involvement in the clinical trials

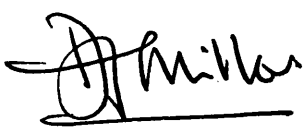
✓ **h)** Correct the table in the Introduction (agents and cell types)

✓ **i)** Correct the wording in the bracket and associated text on p168.

j) Acknowledge the sources for Figs 1.1 and 1.3

✓ **k)** Discuss cell viability and recovery (by explaining the reproducibility/reversibility of your protocols, for example)

l) Correct the typographical and other minor errors given in the accompanying list (provided by Dr Muir)

Dr D J Miller (Convenor)  18. March '98.

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