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THE UNIVERSITY OF ALBERTA

Pharmacologic and Morphologic Studies of Guinea-pig Trachea and Carotid Sinus

by

Hwa-sup Shin

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

IN

Pharmaceutical Sciences (Pharmacology)

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EDMONTON, ALBERTA

Spring 1990

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"How many are your works, O LORD! In wisdom you made them all;
the earth is full of your creatures." (Psalm 104:24)

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Pharmacologic and Morphologic Studies of Guinea-pig Trachea and Carotid Sinus" submitted by Hwa-sup Shin in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Pharmacology).

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Dr. R. Schellenberg .....
External examiner

Date: November 14, 1989

To my father, Sang-kap Shin, my mother, Seung-wan Bak,

and to my wife, Sung-hee

ABSTRACT

This thesis is in 2 Parts. In Part I, I determined whether postjunctional α_1- and presynaptic α_2-receptors contribute to airway hyperresponsiveness. Using an isolated, innervated guinea-pig tracheal tube preparation, the pressor effects of norepinephrine (NE) and phenylephrine and the inhibitory effects of NE on tracheal responses to pre- (NS) and post-ganglionic (FS) cholinergic stimulation were measured. NS and FS were applied at 2, 8, and 32 Hz, using trains of 64 pulses or for 10 s, and NE pD_2 values were calculated. In control tissues, NE induced dose-dependent inhibition of responses to NS and FS at all frequencies, and its pD_2 values correlated negatively with frequency and were similar for NS and FS at the same frequencies. Yohimbine blocked the inhibitory effects of NE and its pA_2 values were correlated negatively with frequency for FS. Cocaine or desipramine, and chemical sympathectomy with 6-hydroxydopamine significantly increased NE pD_2 values. In tracheas from guinea-pig models of asthma, the α_1- and α_2-receptor-mediated effects were similar to controls. I conclude that α-adrenoceptors do not contribute to airway hyperresponsiveness in asthma.

In Part II, I examined the fine structure of baroreceptors and elastic laminae in the bifurcation region of guinea-pig carotid arteries using transmission and scanning electron microscopy, respectively. Baroreceptors were found close to elastic and collagen

fibers in the adventitia and media of the carotid sinus (CS). Their morphologic features included densely packed mitochondria, osmiophilic lamellated and homogeneous bodies, clear and agranular vesicles, lamellar systems, glycogen particles, neurotubuli, neurofilaments, vacuoles, and translucent cytoplasm. Unlike other areas of the bifurcation, the CS exhibited several unique structures on the luminal surface of the internal elastic laminae - blister-like outgrowths, dense clusters of fenestrations, and a honeycomb-like maze. In the CS, the elastic networks of the adventitial elastic laminae were finer, and the cross-sectional thickness of the EL was less than in its adjacent arteries. These findings demonstrate interspecies homogeneity among the structural features of baroreceptors in the CS, and differences in elastic laminae of the CS and its adjacent arteries. The latter are presumably related to the transducer function of the CS.

ACKNOWLEDGEMENTS

The author wishes to express thanks to Dr. D.F. Biggs for his invaluable guidance and patience during the course of this work. Dr. Biggs' cordial supervision enabled the author to carry on with research in an environment conducive to learning and exploration. The author also would like to thank members of the Supervisory Committee for their encouragement and suggestions at various stages during the research.

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GLOSSARY OF ABBREVIATIONS

ANOVA	Analysis of Variance
NE	Norepinephrine
NS	(Vagal) Nerve Stimulation
FS	Field Stimulation
FEV_1	Forced Expiratory Volume in one second
BAL	Bronchoalveolar Lavage
EPO	Eosinophil peroxidase
MBP	Major basic protein
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
PAF	Platelet Activating Factor
ASM	Airway Smooth Muscle
PMC	Proportion of ASM in the circumference
EpDRF	Epithelium derived relaxing factor(s)
ECF-A	Eosinophil Chemotactic Factor of anaphylaxis
HMW-NCF	High Molecular Weight Neutrophil Chemotactic Factor
SP	Substance P
(i)-NANC	(Inhibitory)-Nonadrenergic Noncholinergic
(e)-NANC	(Excitatory)-Nonadrenergic Noncholinergic
SARs	Slowly Adapting Receptors
RARs	Rapidly Adapting Receptors
FRC	Functional Residual Capacity

ACh	Acetylcholine
DMPP	Dimethylphenylpiperazinium
VIP	Vasoactive intestinal peptide
TTX	Tetrodotoxin
-LI	-Like immunoreactivity (-tive)
CGRP	Calcitonin Gene Related Polypeptide
NKA	Neurokinin A
NPK	Neuropeptide K
NPY	Neuropeptide Y
ELE	Eledoisin
NEP	Neutral endopeptidase
ip	intraperitoneal
o	degrees Celcius
h	hour(s)
min	minute(s)
s	second
ml	Milliliter
kg	kilogram
g	gram
ug	microgram
um	micrometer
n	Number of observations
A	Angstrom
6HD	6-Hydroxydopamine
IEL	Internal Elastic Lamina(e)

SD	Standard Deviation
CS	Carotid Sinus
CCA	Common Carotid Artery
OA	Ovalbumin or Occipital Artery
ECA	External Carotid Artery
AEL	Adventitial Elastic Lamina(e)

1. LITERATURE REVIEW and PROPOSED RESEARCH

1.1 Airway Morphology

The lungs are paired organs that fill most of the pleural cavity. In humans, the right and left lungs are divided into three and two lobes, respectively, and in guinea pigs into four and three lobes, by deep fissures lined by visceral pleura (Cooper & Schiller, 1975). The air spaces of the lungs begin at the trachea at the cricoid cartilage. The trachea descends through the superior mediastinum to the level of the junction of the manubrium and body of the sternum where it bifurcates giving rise to the main bronchi. In guinea pigs, the trachea bifurcates into the right and left main bronchi at the level of the third rib. The two main bronchi enter the lungs at the pulmonary hila. Within the lung, the main bronchi divide into one lobar bronchus for each pulmonary lobe. Lobar bronchi bifurcate, and become narrower, by irregular dichotomy down to the alveolar sacs. Functionally, the airways can be divided into conducting airways (trachea to terminal bronchioles [generations 0-16]), and the acini, the respiratory units of the lung (respiratory bronchioles to alveolar sacs [generations 17-23]). Conducting airways serve as conduits and do not participate in respiratory gas exchange, only the acini are involved in gas exchange.

For a better understanding of pathophysiology of asthma, it is essential to understand airway morphology, which has been the subject of many reviews (Nash, 1976; Gil, 1982; Richardson and Ferguson, 1980; Weibel, 1985). This section will be dedicated only

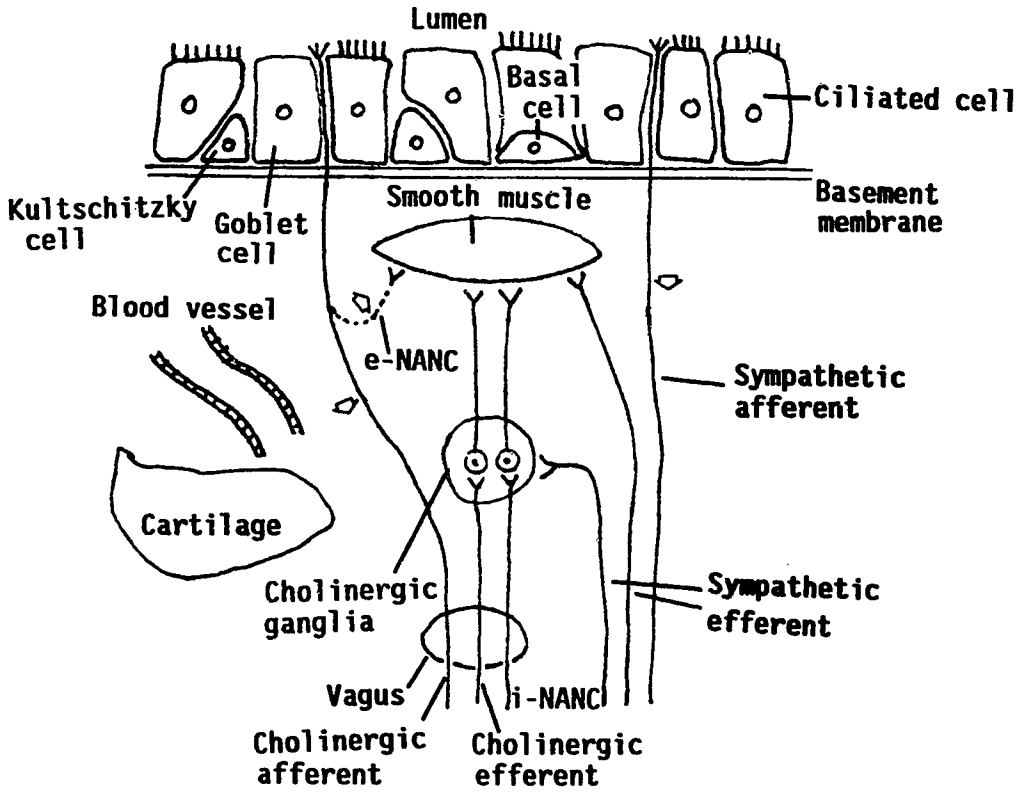

Fig. 1-1. The anatomy of guinea-pig trachea

to the morphology of the trachea. A schematic representation of the structure of the trachea is shown in Fig. 1. The trachea consists of three layers: mucous, submucous, and fibrocartilaginous. The mucosa consists of a continuous epithelial cell sheet which forms an uninterrupted barrier between the underlying interstitial (connective tissue) space and the air space. This lining is "tight" because well-developed terminal bars or tight junctions seal the narrow intercellular clefts (Green et al., 1977; Schneeberger, 1980). The epithelium comprises a mosaic of cells specialized for different functions. The essential functional property of the epithelium is that it forms and maintains a continuous stream of

fluid and mucus which flows towards the pharynx by coordinated ciliary motion. This process is termed mucociliary clearance.

In all species examined, three distinct cell types have been described in the epithelium: ciliated, basal, and goblet. Ciliated cells and goblet cells are slender columnar cells that extend from the basement membrane to the surface; smaller basal cells that appear to be poorly differentiated are restricted to the lower part of the epithelium abutting against the basement membrane. Ciliated cells are most common. The anatomical evidence for the production of mucus by these cells is uncertain; they neither contain secretory granules nor stain for mucin (Nadel et al., 1985). The nature and function of the ciliated cell product(s) are unknown (Richardson & Somerville, 1988). Their main function is to maintain the coordinated movement of cilia (Sleigh, 1981). Goblet cells have slender bases and broad apices, the latter containing dense-cored secretory granules. They are presumably the primary source of mucus in species such as goose, rat, rabbit, and guinea pig, which have few or no submucosal glands (Nadel et al., 1985). They produce mucus that contributes to normal mucociliary clearance, and, if the mucus becomes impacted, it can contribute to disease (Mygind et al., 1987). Basal cells are small cells with oval nuclei that can differentiate into either ciliated or nonciliated cells.

In humans, Kultschitzky (neuroendocrine) cells, members of the APUD (amine precursor uptake and decarboxylation) series of cells found throughout the body, are found in higher density in trachea than in bronchi (see Laitinen, 1988). Their characteristic feature is the presence of small basal granules with a dense core. They may

have a receptor function: cell bodies send a long narrow "fingertip" through thick epithelium to reach the lumen, and these fingertips may act as receptor units for stimuli entering the airways (Laitinen, 1988). Their close association with basal sensory intraepithelial nerves (King et al., 1974; McDonald, 1977) appears to support a receptor function. Also, they may have a neurosecretory function and secrete monoamines and/or bioactive peptides (Pearse, 1976; Reichlin, 1980) into the microcirculation through fenestrated capillaries located nearby.

All the epithelial cells are attached to basement membrane that forms a continuous sheet. This membrane consists of a true basal lamina about 80 nm thick which is visible only with the electron microscope. This basal lamina is associated with collagen, and in patients with asthma it is thickened characteristically due to an increase in its collagen content and the deposition of IgG, IgA, albumin and fibrinogen (Hogg, 1988).

The submucosa is defined as the tissue space between the basement membrane of the mucosa and the cartilage, and contains submucosal glands, smooth muscle, and connective tissue. In healthy adult humans, the volume of the submucosal glands is much greater than goblet cell volume, probably indicating that submucosal glands make the greater contribution to the production of mucus (Nadel et al., 1985).

Beneath the submucosa, providing support for the wall, are a series of U-shaped cartilages. The dorsal aspect of the trachea, termed Paries membranaceus contains a flat layer of smooth muscle fibers that fills the gap between the tips of U-shaped cartilage

rings (Nash, 1976). In humans and guinea pigs, the muscle is inserted on the concave surface of each cartilage at some distance from the ends of the cartilage and it is therefore much longer than the gap between cartilage ends (Amiri & Gabella, 1988). Isotonic contraction of the muscle brings the ends of a cartilage very close to one another; in the cervical trachea the gap between the ends disappears, while in the thoracic trachea the cartilage ends are bent and overlap extensively. Muscle fibers are arranged in bundles and run transversely. The tracheal smooth muscle of all species studied have gap junctions, which are composed of arrays of membrane proteins and provide a basis for cell-to-cell communication of electrical and metabolic signals (Daniel, 1988). Gap junctions vary in density among species; they are found in higher density in tracheal muscle from humans than from guinea pigs. However, tracheal muscle from humans and guinea pigs is of the "multiunit" type described by Burnstock (1972). Each unit consists of a single muscle cell with its own innervation (Stephens, 1987). The ganglia of the tracheal nerve plexus, blood vessels (bronchial circulation), and large lymphatic vessels are located in a connective tissue stroma over the dorsal surface of the muscle between the cartilage ends (Amiri & Gabella, 1988). Autonomic nervous control of tracheal cells such as smooth muscle and ganglia is complex and involves sympathetic, parasympathetic, and nonadrenergic noncholinergic mechanisms (Richardson, 1979; Nadel and Barnes, 1984; Barnes, 1984). Epithelial cells appear to receive only afferent innervation (Laitinen, 1988).

1.2. Asthma

1.2.1. Definition and Types

Bronchial asthma is a disease recognized clinically as generalized airway obstruction which is reversible either spontaneously or with treatment (Holgate, 1983). This definition of asthma deliberately avoids any reference to pathogenesis because the cause is unknown (Reed, 1988). Asthma covers a broad clinical spectrum, ranging from mild, readily-reversible bronchospasm to severe, chronic, intractable obstruction of airflow (Kay, 1987). Asthmatic patients can be divided into three subgroups: allergic or extrinsic asthmatics, cryptogenic or intrinsic asthmatics, and asthmatic bronchitics (Holgate, 1983), but there are many "classifications".

Classic allergic asthma often has its onset in childhood and is associated with an increased prevalence of allergic rhinitis, food allergies, conjunctivitis and eczema, high circulating levels of the reaginic antibody (IgE) directed against common environmental antigens such as house dust mites and pollen, and positive immediate skin reactions to those specific antigens. However, skin reactivity to allergens does not parallel the severity of asthma.

Non-allergic asthma occurs more often later in life (late-onset asthma). It occurs occasionally in children. There is no association with clinical or immunological markers of allergy and it tends to be more resistant to treatment than the allergic form of the disease.

Finally, a group of patients, having chronic obstructive lung disease, become "asthmatic". The most likely explanation for the development of "asthmatic bronchitis" in adults and some form of

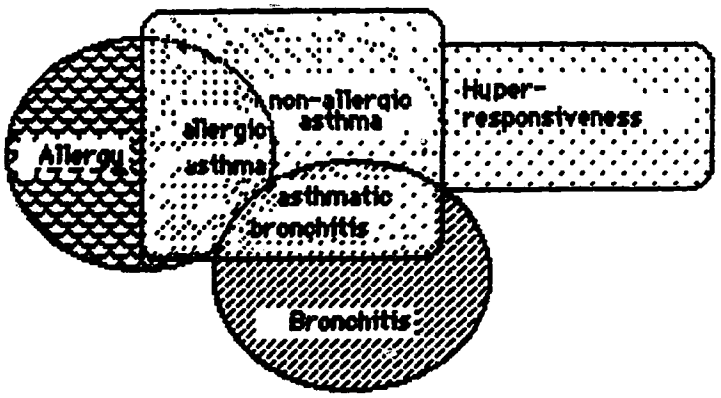

Fig. I-2. Venn diagram representing the clinical, physiological and immunological overlaps in bronchial asthma (modified from Holgate, 1983)

"wheezy bronchitis" in children is that there is an altered airway responsiveness after repeated viral and bacterial infections.

The complicated physiological and clinical overlaps which appear to contribute to bronchial asthma may be better appreciated via a Venn diagram (Fig. 2).

1.2.2. Phases of Asthma

It has been clearly established that the events which follow challenge with an appropriate stimuli, allergic or non-allergic, in susceptible asthmatic subjects can be divided into three main phases: (1) rapid, spasmogenic; (2) late, sustained; and (3) a subacute/chronic inflammatory phase (Kay, 1987; see Hutson et al., 1988).

Rapid phase refers to an increase in airways' resistance to airflow which is rapid in onset with a peak fall in forced

expiratory volume in one second (FEV_1) occurring 10 to 20 min after bronchial challenge with common environmental allergens in sensitized subjects.

Late (sustained) phase refers to the second often more severe rise in airways' resistance to airflow that occurs after bronchial challenge and is maximal 6 to 8 h after exposure. Compared with the rapid phase, the onset and resolving time of the late phase is longer (more sustained, sometimes several days). About 50% of adults who develop the rapid phase appear to develop the late phase, and it may be more common in children (O'Byrne et al., 1987). In a subset of sensitized subjects, inhaled antigen does not cause any early response but is followed 3 to 8 h later by a late asthmatic phase ("isolated late phase") (O'Byrne et al., 1987). Multiple late phase reactions are common after allergen challenge in asthmatic humans (see Hutson et al., 1988). The late phase is of clinical interest because it has been associated with prolonged airway hyperresponsiveness (see section 1.2.3. for the details). Some of the features of early and late asthmatic phase after allergen inhalation are summarized in Table 1 (O'Byrne et al., 1987).

The subacute/chronic inflammatory phase is believed often to be associated with asthma in untreated or poorly controlled patients. Infiltration of the bronchi by large numbers of eosinophils and mononuclear cells is a characteristic finding at autopsy (Kay, 1987).

Mechanisms responsible for the rapid and late asthmatic phases have been reviewed (Abraham, 1987; O'Byrne et al., 1987; Larsen, 1988; Holgate, 1983, 1987). Recent evidence appears to indicate a

Table 1-1. Features of rapid and late asthmatic reactions following allergen inhalation (modified from O'Byrne et al., 1987)

	Rapid Reaction	Late Reaction
Onset	< 10 min	3-4 h
Peak	10-30 min	8-12 h
Duration	1.5-3 h	> 12 h
Prolonged increase in nonspecific airway hyperresponsiveness	-	+
Premedication		
Indomethacin	No effect	May inhibit
Glucocorticoids	Can inhibit if given long enough	Can inhibit
Sodium cromoglycate	Can inhibit	Can inhibit
β_2-agonists	Inhibit	No effect

major role for mast-cell mediators in the early phase. Mast cells' number is increased in the airways of allergic and nonallergic asthmatics, and those isolated from allergic asthmatics show an enhanced ability to release preformed mediators (Holgate, 1987). Thus, during the rapid phase, blood levels of histamine and high-molecular-weight-neutrophil-chemotactic factor (HMW-NCF) are correlated negatively with FEV_1 (see Kay, 1987; Holgate, 1987). Other possible mediators include lipid-derived products such as PGD_2, $PGF_{2\alpha}$, TXA_2, LTC_4, LTD_4, and platelet-activating factor (PAF) (Holgate, 1987; Hutson, 1988; Skoner, 1988).

The late phase may be associated with a reactivation of mast cells as a second elevation in blood levels of NCA and histamine has been observed during it (Kay, 1987). However, the absence of PGD_2, a component of mast cells but not basophils, during the late phase

may indicate that NCA and histmaine come from recruited basophils as has been suggested for late nasal reactions (Pearce, 1988). Important determinants of whether a person will develop the late phase are the severity of the early phase and the responsiveness of the airways of the subject at the time of allergen inhalation (O'Byrne et al., 1987). The level ofcirculating IgE antibody is another determinant of the late phase although IgE cannot be demonstrated during the late phase after exercise or exposure to occupational chemical sensitizers in intrinsic asthma (see O'Byrne et al., 1987; Cockcroft 1988). The persistence of the late phase and its resistance to inhaled bronchodilators suggests that it is likely caused by the effects of mediator release on airway smooth muscle and airway inflammatory responses, e.g., mucosal edema and increased secretions.

Evidence from humans and animal models of asthma appears to suggest the involvement of airway inflammatory responses in the late asthmatic phase. In humans, bronchoalveolar lavage (BAL) during the late phase suggests that acute airway inflammation may be responsible for the late phase, with eosinophils and neutrophils being the predominant cell type (O'Byrne et al., 1987). In atopic patients with mild asthma, the relative fall in peripheral blood eosinophil counts during the late phase correlates strongly with its severity and with coincidental changes in airway responsiveness to histamine (Cookson et al., 1989). These findings suggest that eosinophils may have been recruited to the lung to contribute to the development of the late phase after inhalation of allergen.

In a guinea-pig model of asthma, BAL demonstrated a significant

increase in neutrophils followed by an increase in eosinophils during the late phase, without any change in mononuclear cells or lymphocytes (Hutson et al., 1988). BAL has revealed eosinophilia in allergic sheep that develop antigen-induced late phase reactions but not in allergic sheep that have only rapid phase reactions after antigen challenge (Abraham et al., 1988). Methylprednisolone succinate and antiallergic agents that were effective in blocking the late phase prevented the recruitment of eosinophils but not neutrophils into the airways. These findings in guinea pigs and allergic sheep suggest that the recruitment of granulocytes, especially eosinophils, is associated with the late phase.

Inflammatory mediators recruited into the lungs may contribute to late response by releasing different types of mediators. The possible mediators derived from eosinophils include major basic protein (MBP), eosinophil cationic protein (ECP), and peroxidase, LTC_4, and active species of oxygen (i.e., hydrogen peroxide, superoxide anion, hydroxyl radical) (Hutson et al., 1988). Free radicals released from neutrophils may also be involved in the late phase by exaggerating the inflammatory response (see Holgate, 1983). Studies in sheep have suggested strongly that lipoxygenase products of arachidonic acid metabolism released during the rapid phase play an important role in the development of the late phase (Abraham, 1987). However, an oral LTD_4 antagonist had no significant effect on the development of the late phase after antigen challenge in atopic asthmatics (Britton et al., 1987). It has been shown that PAF leads only to rapid phase reactions in humans (Cuss et al., 1986), although in many species including humans, PAF has induced airway

hyperresponsiveness, known as late asthmatic sequelae together with late phase. At present, the relative importance of various mediators in the late phase is unknown.

1.2.3. Pathophysiology of Asthma

Despite differences in clinical patterns and associated factors, all three categories of asthma (see section 1.2.1.) is symptomatically similar, varying greatly in severity and in time-course from infrequent isolated episodes (with symptom-free intervals) to frequent episodic exacerbations (with persistent symptoms) (Scadding, 1976). Symptoms of asthma include cough (commonest), wheezing, chest pain (often pleuritic), nocturnal misery, and tightness of the chest. More than two of the above would suggest and confirm the presence of asthma. Asthma is divided into three types based on severity and the medical regimens required: 1) mild - controlled by bronchodilators and avoidance of known precipitating factors; does not interfere with normal activities; 2) moderate - occasionally interferes with normal activities; may require use of corticosteroids; and 3) severe asthma - seriously interferes with normal activities (Scadding, 1976).

The descriptions of the pathophysiologic features of bronchial asthma have been based largely on autopsy studies of the lung (conducting airways) of patients who died from status asthmaticus, a severe, life-threatening exacerbation of bronchial asthma that does not respond to therapy and persists for days and even weeks (Wanner, 1986; Don, 1986). Microscopically, there are destruction of the bronchial epithelium, mucosal edema, hypersecretion of mucus, mucous

plugging, mucus gland hyperplasia, infiltration of the airway wall with eosinophils and neutrophils, thickening of the subepithelial basement membrane, and hypertrophy of airway smooth muscle (Holgate 1983; Murlas, 1988). Although it is not known how closely these features correlate with severity of asthma, the pathological changes in patients with stable asthma or nonfatal exacerbations of asthma seem to differ only quantitatively from those seen in status asthmaticus (Wanner, 1986). In bronchial biopsies of mild allergic asthmatic subjects, Beasley et al. (1989) described shedding of the ciliated epithelium, partial mast cell degranulation, collagen deposition beneath the epithelial basement membrane and eosinophil infiltration of the lamina propria. Thus, extensive inflammatory changes occur in the airways even in mild clinical or subclinical disease. Also, these authors observed a significant increase in ciliated epithelial cells in BAL fluid from these asthmatics, confirming the findings of Laitinen et al. (1985).

Compared with non-asthmatic subjects, the airways of asthmatic patients are hyperresponsive to the constrictor effects of a large number of different stimuli, such as exercise, cold air, hyperventilation and chemical agents (Simmonson, 1983; Kay, 1987). This enhanced airway responsiveness is often termed nonspecific, as most, if not all, asthmatics bronchoconstrict to these stimuli. This nonspecific airway hyperresponsiveness will be discussed in more detail below because of its relevance to this thesis.

Airway hyperresponsiveness can be demonstrated by a bronchoprovocation challenge test with methacholine or histamine. Two techniques are most frequently used for the generation and

delivery of aerosols. One is an intermittent generation and inhalation of the aerosol. The time for each aerosol generation is adjusted to 0.6 sec and patients take 5 consecutive slow, deep inspirations starting at the functional residual capacity level. The dosage administered is expressed in terms of breath units (which equals the concentration of the drug multiplied by the number of inhalations). The second method is one of continuous aerosol generation and continuous inhalation of the aerosol for a fixed duration of time using tidal volume breathing. The dosage is expressed in terms of the concentration of the solution.

The test is performed by measuring baseline and control (after inhalation of an aerosol consisting only of the diluent containing 0.5% NaCl, 0.275% $NaHCO_3$, and 0.4% phenol, pH 7.0) spirometry followed by inhalation of serially increasing concentrations of methacholine or histamine from the recommended lowest concentration to the highest concentration. Spirometry is performed 1-5 min after inhalation of each concentration. A positive test is a 20% or greater reduction of FEV_1 (forced expiratory volume at 1 sec) from the control which sustains for 3 min. The diagnosis of bronchial hyperresponsiveness is made after PD_{20}- or PC_{20}-FEV_1 (the provocation dose [the cumulative number of breath units] or concentration causing a 20% fall in FEV_1) is less than or equal to the highest cumulative value of breath units used in the study or the highest scheduled concentration (Kanner & Watanabe, 1986). The methods and techniques for investigating spirometric lung functions have been recommended by the American Thoracic Society (ATS) (1979, 1988); the second method is preferred by the ATS.

Murlas (1988) differentiated acute and chronic airway hyperresponsiveness in terms of mechanism and time course of their development. The early onset increase in responsiveness after immunologic and nonimmunologic airway injury occurs soon after the early phase and appears to be related to normal lung cell constituents including mucosal cells (neuroepithelial bodies, mast and epithelial cells) and mediators generated by them. LTD_4, LTC_4, LTE_4, and LTB_4, 5-lipoxygenase products of arachidonic acid, could induce acute hyperresponsiveness by increasing airway neuromuscular responses via prejunctional or postjunctional actions (see Murlas, 1988).

Chronic airway hyperresponsiveness, termed late inflammatory sequelae together with the late asthmatic phase, is more important than acute hyperresponsiveness in the pathogenesis of atopic allergic and occupational asthma (Cockcroft, 1988). Airway hyperresponsiveness can last days, weeks, or months (Abraham, 1987; O'Byrne et al., 1987) and its magnitude correlates with the clinical severity of the disease (Cockcroft et al., 1977). This nonspecific airway hyperresponsiveness also can worsen asthmatic symptoms via a vicious cycle (Cockcroft, 1988; Larsen, 1988): in perennial asthmatics, a cycle may be established when natural exposure to an allergen leads to airway hyperresponsiveness, which in turn causes an increased response to subsequent exposure to the allergic and nonallergic triggers, leading to further airway hyperresponsiveness. Thus, airway hyperresponsiveness appears to be fundamental to the pathogenesis of asthma or a result of the disease (Barnes, 1987). This abnormal response in asthma has been attributed to an increased

sensitivity and an increased contractility to a given stimuli (i.e., a higher maximal response) or a combination of these two, as the airway dose-response of asthmatic subjects in vivo is characterized both by a leftward shift and by an increased maximal response (Woolcock et al., 1984; Moreno et al., 1986; Sterk & Bell, 1989).

The potential mechanisms for airway hyperresponsiveness have been the subject of many previous reviews (Barnes, 1985; Boushey, 1985; Boushey et al., 1980; Holtzman, 1982; Moreno et al., 1986; Nadel, 1983; Nadel & Holtzman, 1984; O'Byrne et al., 1987; Sterk & Bel, 1989). The mechanisms proposed include: (1) mechanical factors; (2) alteration in airway smooth muscle (ASM); (3) epithelial damage; (4) airway inflammation; (5) disorders of neural mechanisms. Each of these mechanisms will be discussed in detail below.

Possible mechanical factors underlying airway hyperresponsiveness may include airway geometry (wall thickness, baseline airway caliber) and proportion of ASM in the circumference (PMC). The effect of wall thickness in exaggerated airway narrowing has been well demonstrated by James et al. (1989) who measured dimensions of the "contracted" airways, specimens of lung obtained from asthmatics postmortem. They calculated the "relaxed" airway dimensions and the amount of muscle shortening required to occlude the airway lumen. Their calculation was based on the earlier finding that airway internal perimeter and wall area remained constant at different lung volumes and with different degrees of airway smooth muscle shortening despite substantial changes in luminal area (James et al., 1988; James et al., 1987). They showed that airway

resistance increased more rapidly with muscle shortening in the asthmatic airways although the baseline resistance was only slightly increased. Thus, occlusion of the lumen requires less muscle shortening in asthmatic than nonasthmatic airways. Asthmatic airways showed an increase in wall area with increased areas of epithelium, muscle, and submucosa. Their findings indicate that airway wall thickening caused by chronic inflammation could be as important as smooth muscle shortening in determining the airway responsiveness in asthmatics. Thus, chronic inflammatory processes in the airway wall of asthmatics, that are associated with cellular infiltration, deposition of connective tissue, hypertrophy of smooth muscle, goblet cell metaplasia of the epithelium, and airway mucous plugging, can lead to excessive airway narrowing without excessive smooth muscle contraction, resulting in sustained hyperresponsiveness (Moreno et al., 1986; James et al., 1989). Also, these findings suggest that airway wall thickening caused by edema, cellular infiltration, and the hyperemia of acute inflammation could explain fluctuations in airway responsiveness in individual subjects.

Decreased baseline airway caliber may contribute to airway hyperresponsiveness because, in vivo, airway resistance is inversely proportional to the fourth power of the radius when flow in the airway is laminar (Barnes et al., 1988). Baseline caliber can be decreased by at least 4 mechanisms (Moreno et al., 1986). (1) Normal airway smooth muscle tone could excessively narrow airways in which the resting "preload" is decreased due to cartilage softening and a decrease in lung elastic recoil because of low lung volume or loss

of elastin and collagen. (2) Increased baseline tone could shorten the smooth muscle to lengths below its resting length. (3) Despite normal "tone" and preload, a hyperplastic, hypertrophied, or more efficient smooth muscle could excessively narrow the airways and increase baseline airway resistance. (4) Airway wall thickening or secretions within the airway lumen associated airway inflammation could increase baseline resistance.

ASM occupies a variable proportion of the airway circumference, being minimal in the trachea and maximal in small bronchi and bronchioles where the muscle completely surrounds the airway circumference (von Hayek 1960). It has been shown that for a given degree of ASM shortening, external diameter decreases linearly as PMC increases (Moreno et al., 1986). This finding indicates that the increased PMC could result in more airway narrowing for any degree of ASM shortening.

Increased ASM contractility could be related to airway hyperresponsiveness in asthma as asthmatics develop airway narrowing more rapidly during challenge tests than normal subjects. Also, asthmatics respond more rapidly to bronchoconstrictors (Moreno et al., 1986). Contractility essentially denotes the maximal ability to contract and its measurement requires measurement of maximal isometric force, maximal shortening capacity, and maximal velocity of shortening (Stephens, 1987). Increased ASM contractility could result in increased ASM shortening for a given degree of stimulation and increased maximal response (Moreno et al., 1986). This nondeviation supersensitivity has been suggested as a mechanism for airway hyperresponsiveness as asthmatics demonstrate an increased

maximal response to histamine (Woolcock et al., 1984). To try and explain airway hyperresponsiveness, some authors suggest that there is defect in the cellular processes that govern contraction of asthmatic airway smooth muscle (Barnes et al., 1988). Two different, yet similar, theories have been proposed. Firstly, it has been proposed that there is an increase in electrical excitability (conduction abnormality) in airway smooth muscle cells. This could be due to increased electrical coupling among smooth muscle cells resulting from greater numbers of gap junctions being present than in normal ASM. Thus, the normally multi-unit ASM has been converted to a single unit (see section 1.1. for gap junctions). How multi-unit smooth muscle becomes a single unit is unknown. However, there is evidence that potassium channel blockers (e.g. 4-aminopyridine, tetraethylammonium chloride) and inflammatory mediators released during antigen-antibody reactions can alter the density of gap junctions (Kannan et al., 1983; Mansour & Daniel, 1988). Secondly, it has been proposed that the excitation-contraction coupling-uncoupling process in asthmatic ASM is disturbed in some way. Thus, the regulation of myoplasmic Ca^{2+} concentrations by the ASM cells could be altered as in hypertension. However, there is no convincing evidence for a defect of Ca^{2+} regulation in asthma (Barnes, 1988). In asthma, ASM undergoes hypertrophy and/or hyperplasia (James et al., 1989) and this could influence airway responsiveness. In vitro, studies of human ASM demonstrated a positive correlation between the maximal tension induced by histamine (Armour et al., 1984) or LTD4 (Lewis et al., 1986) and the amount of ASM in bronchial strips. Re[illegible]he

properties of hyperresponsive ASM have been studied in asthmatics undergoing thoracic surgery (Roberts et al., 1985; Cerrina et al., 1986; Schellenberg et al., 1985; De Jongste et al.,1987). Interestingly, despite enhanced responsiveness *in vivo*, tissue responses to mediators were similar to normals *in vitro*, except in a few instances. The absence of a correlation between the responses to agonists of asthmatic ASM *in vivo* and *in vitro* suggests that intrinsic ASM function is not the major determinant of the airway responsiveness, *in vivo*. However, we cannot exclude the possibility that the descrepant results regarding asthmatic airway smooth muscle responses *in vitro* could be attributed to problems of methodology. Therefore, more studies are required to confirm these conclusions.

Epithelial damage is a prominent feature, postmortem, in patients who died from asthma (Dunnill, 1960). Recently, bronchial biopsies from mild clinical and subclinical asthmatics have revealed epithelial damage at all levels of the airways (Laitinen et al., 1985a; Beasley et al., 1989). It has been suggested that epithelial damage is important in the pathogenesis of airway hyperresponsiveness in asthma (Hogg & Eggleston, 1984; Vanhoutte, 1987). This hypothesis is supported by the correlation between epithelial cell counts in lavage fluid and the degree of airway responsiveness to inhaled histamine in mild asthmatics (Beasley et al., 1989). Studies with ozone, and after viral infections of the respiratory tracts, indicate that conditions inducing reversible airway epithelial damage result in transient airway hyperresponsiveness (Empey et al., 1976; Golden et al., 1978).

The mechanisms that cause airway hyperresponsiveness following

epithelial damage could be: Firstly, airway permeability is increased, thereby allowing higher concentrations of inhaled antigens or inflammatory mediators to reach "target" cells (e.g., submucosal mast cells, ASM). Recent studies, in which the clearance of an inhaled radiolabelled tracer was used as an index of permeability, showed that mucosal permeability increased in asthmatic humans, and that this was associated with bronchial hyperresponsiveness (Ilowite et al., 1989). Secondly, sensory nerve endings found in epithelium and subepithelial tissue (Laitinen et al., 1985) could be exposed to specific or nonspecific stimuli and mediate reflex bronchoconstriction via cholinergic reflexes and/or local axon reflexes (Barnes, 1986). Thirdly, removal of epithelium could decrease production of epithelium derived relaxing factor(s) (EpDRF) (Vanhoutte, 1988). This could enhance the constrictor effects of several spasmogens such as acetylcholine, histamine, $PGF_{2\alpha}$, and LTD_4 (Aizawa et al., 1988; Fedan et al., 1988), or reduce the effects of bronchodilators (Cuss & Barnes, 1987). Epithelial damage could reduce the activity of airway neutral endopeptidase and result in decreased metabolism of tachykinins (Sheppard et al., 1988). Fourthly, epithelial cell damage could release chemotactic factors such as LTB_4 (Holtzman et al., 1983) and 15-HETE (Hunter et al., 1985), that induce secondary cell recruitment and an augmented inflammatory response. Lastly, epithelial damage could result in the inability to control the osmolarity and ion concentration of the fluid lining the airway surface. This could lead to disruption of epithelial tight junctions and stimulation of sensory nerve endings close to these junctions

(Hogg & Eggleston, 1984).

Recently, much attention has been focused on airway inflammation as critical to the pathogenesis of asthma. Studies of the pathology of asthma, as discussed in section 1.2.3., showed typical inflammatory reactions in the airways, even in mild clinical and subclinical disease (Laitinen et al., 1985, Beasley et al., 1989). Other studies have shown a close association between the amount of inflammation in the airway walls and the degree of bronchial hyperresponsiveness (Chung, 1986). This section will discuss evidence for the involvement of inflammatory cells and mediators in the development and maintenance of hyperresponsiveness (see previous section for their role in the phases of asthma).

Inflammatory cells. Inflammatory cells involved in lung pathophysiology are divided into two types: cells normally found in the lungs, such as mast cells, alveolar macrophages, epithelial and endothelial cells (primary effector cells), and cells recruited to the lungs, such as platelets, basophils, neutrophils, monocytes, lymphocytes, and eosinophils (secondary effector cells) (Raphael & Metcalfe, 1986; Kay, 1986). Each of these cell types are discussed in detail elsewhere (Pearce, 1988; MacDermot & Fuller, 1988; Dahl et al., 1988; Ford-Hutchinson, 1988; Fedan et al., 1988; Kay, 1986).

The role of the cellular phase of inflammation in the pathogenesis of the airway hyperresponsiveness is suggested by the finding that stimuli which are known to induce bronchial hyperresponsiveness (i.e., sulphur dioxide, cigarette smoke, viral infections, and allergen inhalation) all induce airway inflammation.

This is characterized by an increase of the numbers of neutrophils, eosinophils, and monocytes in bronchoalveolar lavage (BAL) (O'Byrne et al., 1987; Hargreave et al., 1986; Reed, 1988; Pretolani et al., 1988). Inflammatory cells that have been implicated in bronchial hyperresponsiveness in asthma include mast cells, neutrophils, eosinophils, platelets, and macrophages.

In human airways, the greatest number of mast cells is found adjacent to the basement membrane and between the epithelial cells, with occasional cells abutting onto the lumen of the airway (Lamb & Lumsden, 1982; Kay, 1983). Although their location in the airways dose not indicate heterogeneity of mast cells, lumenal mast cells might play an important role in initiating the asthmatic response as mediator release from these cells opens the tight junctions between the bronchial epithelial cells and allows penetration of antigen to mast cells located deeper in the tissues (Simani et al., 1974; Hogg, 1981). Mast cells are involved in the early asthmatic phase after allergen inhalation (Holgate, 1987) and probably in the response to other non-IgE-involving stimuli (Holgate et al., 1986). Also, it is possible that mast cell degranulation is neurally mediated (see later in this section). Their reactivation could result in the late phase (Durham et al., 1974). Whether these cells are important for the development of the bronchial hyperresponsiveness is uncertain. β-Agonists and mast cell-stabilizing drugs (such as oxatomide and loperamide) that prevent mediator release from mast cells, are effective in preventing the acute bronchoconstrictor response to allergen, but they are ineffective in preventing any subsequent bronchial hyperresponsiveness (Kraan et al., 1985; Kerrebijn et al.,

1987; Cockcroft & Murdock, 1987). This has led to attention being focused on other cells.

Neutrophils are involved in the initial host defence mechanism against injurious stimuli. They migrate to the point of injury, usually in response to the production of various chemotactic factors. Their main function is to ingest and digest the injurious stimuli (Ford-Hutchinson, 1988). There have been conflicting reports as to whether neutrophil infiltration is related to airway hyperresponsiveness after challenge with allergens or noxious stimuli (e.g. ozone). Exposure to antigen or ozone results in airway hyperresponsiveness which has been associated temporally with airway inflammation and the influx of neutrophils into the airways of dogs (Holtzman et al., 1983b; Fabbri et al., 1984; Chung et al., 1985) and humans (Seltzer et al., 1984). Confirmation of the importance of neutrophils in hyperresponsiveness was obtained in studies with two chemotactic factors, LTB_4 and PAF. LTB_4 and PAF were given by aerosol to dogs. Hyperresponsiveness occurred and this was prevented by neutrophil depletion. Hyperresponsiveness, but not neutrophil migration, was attenuated by pretreatment with indomethacin or a thromboxane (Tx) synthetase inhibitor (O'Byrne et al., 1985; Chung et al., 1986). These findings suggest that the recruitment of neutrophils to the airways and the possible release of mediators (TxA_2) are necessary for the development of airway hyperresponsiveness in dogs. However, in guinea pigs (Murlas & Roum, 1985a; Hulbert et al., 1985) and rats (Evans et al., 1988), induction of hyperresponsiveness after exposure to inhaled cigarette smoke or ozone is not related temporally to neutrophil migration.

Also, in guinea pigs, ozone-induced airway hyperresponsiveness is not prevented by neutrophil depletion (Murlas & Roum, 1985b). These findings indicate that the effector cells for the development of airway hyperresponsiveness differ among species.

Eosinophils could play a key role in both allergic and intrinsic asthma. Eosinophils are the major inflammatory cells that infiltrate the mucosa in the airways of patients with severe or mild asthma (Beasley et al., 1989). Interestingly, eosinophil basic proteins are localized to areas of epithelial damage. Increased levels of eosinophils and eosinophil cationic proteins have been found in BAL fluid during allergen-induced late phases (De Monchy et al., 1985). Also, increases in eosinophils in peripheral blood correlate closely with any increases in nonspecific bronchial hyperresponsiveness (Durham & Kay, 1985; Taylor & Luksza, 1987). Unlike neutrophils, eosinophils are poorly phagocytic, but, can be activated by mast cell or macrophage secretions to release eosinophil granule components such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO). These can damage airway epithelium *in vitro* (Gleich et al., 1979; Frigas et al., 1980; Motojima et al., 1989), and this epithelial damage could lead to hyperresponsiveness via increases in airway permeability, exposure of sensory nerve endings, decreases in EpDRF concentrations, release of chemotactic factors, and an inability to control osmolarity in the fluid lining airway surface, as described above.

Alveolar macrophages belong to the mononuclear phagocyte system and comprise more than 90% BAL cells (Lee, 1987). Their numbers and

their ability to generate mediators (e.g. LTs, PGs, and Txs) suggest that they merit consideration in the pathogenesis of bronchial asthma. Alveolar macrophage-derived chemotactic mediators can recruit activated inflammatory cells to the lung. These, in turn, can elaborate more pro-inflammatory mediators (Kay, 1987). Pulmonary macrophages could be involved in bronchial hyperresponsiveness as they have a role in antigen recognition in type I hypersensitivity. Also, mediator release (LTB_4, TxB_2) can be prevented from these cells by corticosteroids, drugs which prevent the development of bronchial hyperresponsiveness (MacDermot & Fuller, 1988). Lastly, "activated" cells can be obtained from asthmatic patients by BAL (Godard et al., 1982).

Platelets possess low affinity receptors for IgE on their cell membrane, suggesting that they could behave similarly to mast cells, basophils and macrophages in allergic disease (Morley et al., 1984). Administration of platelet activating factor (PAF) induces airway hyperresponsiveness in many species including humans.

Studies in human subjects suggest that the cellular response is determined by the stimulus that causes the airway hyperresponsiveness (see O'Byrne et al., 1987). As many inflammatory cells have been implicated in the induction of hyperresponsiveness in asthma, it is likely that there are complex interactions among them, and amplification and modulation of the final effect (Barnes et al., 1988b).

The inflammatory mediators released from cell types are divided into two categories: preformed and newly-generated. Their exocytosis or generation is mediated via biochemical mechanisms triggered via

IgE-dependent and IgE-independent stimuli (Holgate, 1983; Kay, 1983; Cockcroft, 1988; Lazarus, 1987). Mediator-release mechanisms have been much studied in mast cells. Mast cells are activated by the binding of specific antigen to IgE fixed to their surface. This interaction causes bridging of the IgE-Fc receptors with activation of a serine esterase, stimulation of phospholipid metabolism in the cell membrane, and opening of calcium channels. The influx of extracellular calcium into the cell couples the activation signal to the energy-dependent secretory mechanism. The membrane-bound granules swell with partial solubilization of their contents - the preformed mediators. Then, the granules move towards each other and the cell surface. Fusion of perigranular and plasma membranes exposes the granule matrix to the extracellular space into which the various mediators are released by ion exchange with sodium (Holgate, 1983). The preformed, granule-associated mediators include amines (histamine), chemotactic factors (ECF-A, HMW-NCF), neutral proteases (tryptase, carboxypeptidase B), exoglycosidases (aryl sulphatase B, β-glucuronidase, β-galactosidase), and proteoglycan (heparin) (Kay, 1983). As well as initiating degranulation, calcium entry into the mast cell after the binding of allergens with cell-bound IgE activates intramembraneous phospholipase A_2 to release arachidonic acid. This arachidonic acid is metabolized by the cyclooxygenase pathway to form the prostaglandins (PGs), prostacyclin, or thromboxanes (Txs), or by the 5-lipoxygenase pathway to form 5-hydro-peroxy-eicosatetraenoic (5-HPETE) acids, 5-hydroxy-eicosatetraenoic acid (5-HETE), and leukotrienes (LTs) The metabolic pathways that produce newly-generated mediators are shown in Fig. 3.

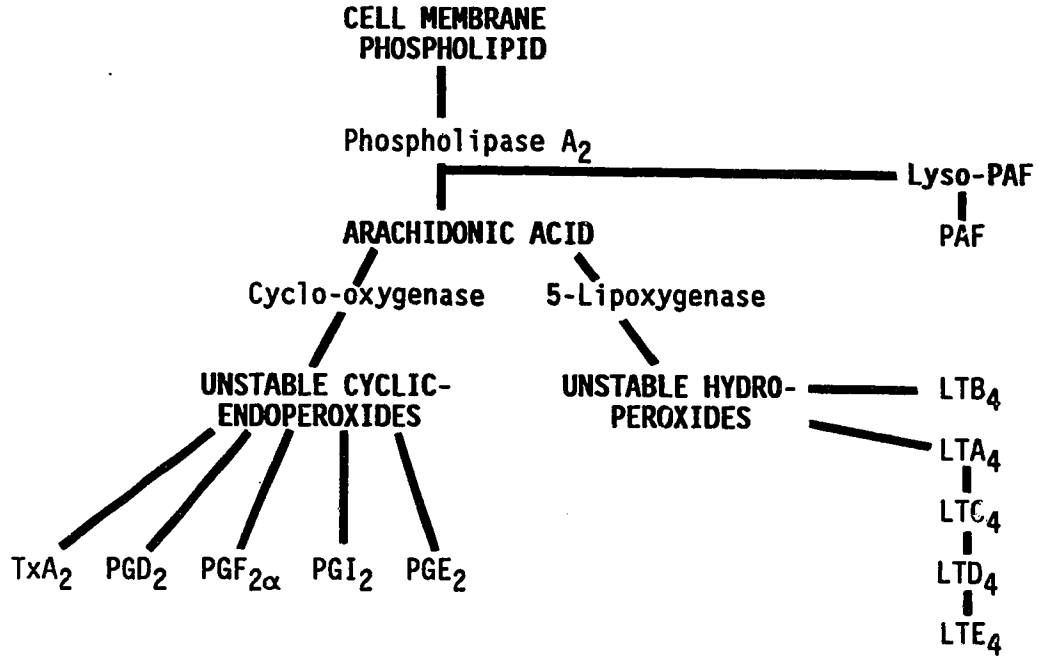

Fig. 1-3. The generation of secondary membrane-derived mediators

Each mediator can have effects that could contribute to the pathology of asthma: smooth muscle contraction, increased airway microvascular leakage, mucus hypersecretion, epithelial damage, cellular infiltrates, or thickening of the basement membrane. Individual mediators that have been implicated in asthma are discussed in detail elsewhere (Barnes, 1987; Brink, 1988; Dahlen, 1988; White & Kaliner, 1988; Page, 1988; Lee, 1988; Nijkamp & Henricks, 1988), and this section will only discuss evidence for their involvement in airway hyperresponsiveness.

Hyperresponsive asthmatics (assessed by methacholine testing)

have high baseline histamine levels that increase only slightly after antigen challenge, while less responsive asthmatics have low baseline plasma histamine levels that increase 2- to 3-fold after antigen challenge (Howarth et al., 1983). These findings support the idea that after antigen challenge less intrapulmonary mediator release is required to produce equivalent bronchoconstriction in patients with hyperresponsive airways than in those with less responsive airways, probably due to a lowered threshold for ASM contraction induced by the products of inflammatory cells (Kay, 1987). Much evidence indicates that inflammatory mediators are involved in the development of airway hyperresponsiveness, as discussed below.

Histamine induces or increases airway hyperresponsiveness in guinea-pig model of asthma (Dorsch et al., 1984).

$PGF_{2\alpha}$ induces or increases airway hyperresponsiveness in dog model of asthma (O'Byrne et al., 1984a,b).

In dogs, after inhaled LTB_4, thromboxane B_2 (TxB_2) levels increase in BAL fluid at the time when airway hyperresponsiveness is present. Inhibition of thromboxane synthesis by sodium (E)-3-[4-(1-imidazolymethyl)phenyl]-2-propionate (OKY-046) prevents the rise in thromboxane levels in BAL fluid and airway hyperresponsiveness. In dogs, OKY-046 prevented increases in airway responsiveness after ozone (Aizawa et al., 1985), allergen (Chung et al., 1986), and PAF (Chung et al., 1986). These findings suggest that in dogs, thromboxanes are important mediators in the development of airway hyperresponsiveness after many stimuli (O'Byrne et al., 1987). In humans, the cyclooxygenase inhibitor

indomethacin prevented increases in airway responsiveness after allergen inhalation, but it did not prevent the early and late asthmatic responses (Kirby et al., 1987b). Thus, prostaglandins or thromboxanes are implicated in the pathogenesis of airway hyperresponsiveness.

There are species differences in the mediators causing airway hyperresponsiveness. In guinea pigs, (3-amino-1-3-trifluoromethyl-phenyl)-2-pyrazoline hydrochloride (BW 755C), an inhibitor of both cyclooxygenase and lipoxygenase, but not indomethacin prevents airway hyperresponsiveness after inhaled toluene diisocyanate (Lee & Murlas, 1985).

Recently, in normal subjects, it has been shown, in vivo, that inhaled LTD_4 induces a higher maximal response than methacholine and increases the maximal response to methacholine. Inhaled corticosteroids reduce maximal responses to LTD_4 and protect against the LTD_4-induced increases in maximal response to methacholine (Bel et al., 1987; Bel et al., 1989). This strongly suggests that the pro-inflammatory actions of LTD_4, such as increased vascular permeability and mucus hypersecretion, could account for part of the airway narrowing observed in asthma.

Chemotactic factors such as ECF-A, LTB_4, 5-HETE, and MW-NCF, acting alone or together, might play a role in the recruitment of eosinophils and neutrophils to bronchial and peribronchial tissue, probably leading to late, sustained reactions and nonspecific hyperresponsiveness (Kay, 1987). HMW-NCF and LTB_4 enhance the expression of neutrophil, eosinophil, and monocyte membrane receptors for complement (C3b) and IgG-(Fc) (Kay, 1987). 5-HETE is

much less potent than LTB_4. LTB_4 is produced by a number of different cell types including human neutrophils, alveolar macrophages, and monocytes (Kay, 1983; Holgate, 1983; Peters et al., 1987; Barnes, 1987).

Platelet activating factor (PAF)-acether, one of the membrane-derived mediators, is known to be present in a variety of cell types in various species including man (Benveniste, 1987). In man, large amounts of PAF are synthesized and released from platelets, endothelial cells, and eosinophils. PAF has a wide range of inflammatory effects, including bronchoconstriction, increased capillary permeability, and chemotactic activity for eosinophils.

Recently, it has been shown that PAF-acether can induce airway hyperresponsiveness in guinea pigs (Mazzoni et al., 1985), rhesus monkeys (Patterson et al., 1984), dogs (Chung et al., 1986), and in humans (Cuss et al., 1986). This may be the most interesting effect of PAF as no other putative inflammatory mediators have been shown to produce such effects (Barnes, 1987). According to Barnes (1987), PAF-acether may act via platelet receptors to release an unknown factor that is chemotactic for eosinophils (PAF itself is an active eosinophil chemotactic agent [Wardlaw et al., 1986]). Eosinophil products, such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) could then damage epithelium, which could lead to airway hyperresponsiveness. The precise role of PAF-acether in asthma awaits the use of specific inhibitors (Schellenberg, 1987; Benveniste, 1987). PAF may also induce hyperresponsiveness by other mechanisms as there is a relationship between the route of administration of PAF and its

activation of cyclooxygenase-dependent or lipoxygenase-dependent pathways (Benveniste, 1987).

The infiltration of the airways of asthmatics by eosinophils and neutrophils and other inflammatory cells, coupled with their ability to generate preformed and newly-generated inflammatory mediators, supports the involvement of airway inflammation in the development of bronchial hyperresponsiveness characteristic of the asthma.

Abnormality of the autonomic control of airways has been suggested as a possible mechanism for airway hyperresponsiveness because of the rapid changes in bronchomotor tone in asthma and the lack of a correlation between responses in vivo and in vitro in asthmatic and nonasthmatic humans. The autonomic innervation of airways is complex (Section 1.1.), and imbalance between the excitatory and inhibitory nervous systems could contribute to airway hyperresponsiveness (Casale, 1988; Barnes et al., 1988b). The individual components of neural control of airways and their defects in asthma are discussed in detail in Sections 1.3. & 1.4.. The recognition that inflammation plays a key role in asthma has suggested that there may be some interaction between neural and inflammatory mechanisms (Barnes, 1986). Thus, several inflammatory mediators may have effects on the release of neurotransmitters from airway nerves, or may act on autonomic receptors. Also, there might be direct association between nerves and inflammatory cells, especially mast cells, that are involved in many different acute and chronic inflammatory processes. In rat intestine, mast cells are found to interdigitate with substance P-containing nerves and appear to surround these nerves (Bishop et al., 1982). These findings

suggest a mechanism whereby mast cells may amplify inflammatory responses and act as a communication switchboard between the central nervous system and other local and migrating cell types (Bienenstock et al., 1987). Such nerve-mast cell communication may mediate mast cell degranulation resulting in major changes in epithelial ion flux and glucose and water transport (Perdue et al., 1984).

In the airways, cigarette smoke and mechanical and chemical irritants induce mucosal edema by increasing vascular permeability (Lundberg & Saria, 1983). This edema can be prevented by capsaicin pretreatment, suggesting a possible role for SP. SP could be released from SP-containing sensory nerves via antidromic stimulation and stimulate mast cells to release mediators responsible for the vascular permeability (Sertl & Kaliner, 1988). Thus, the link between nonspecific irritants and the release of SP and nonimmunologic activation of mast cells could contribute to inflammatory mechanisms in the lungs.

1.3. Airway Innervation

Airway innervation has been the subject of many recent reviews (Laitinen, 1988; Richardson, 1979, 1983, 1988; Barnes, 1986c; Andersson & Grundstrom, 1987; Burnstock, 1988). Briefly, the mammalian lung has at least three afferent (sensory) and three efferent neural pathways (Richardson, 1988). Efferent pathways comprise cholinergic, adrenergic, and nonadrenergic, noncholinergic (NANC) nervous systems.

1.3.1. Afferent Innervation

Afferent nerve fibers innervating lungs are of a sympathetic

(spinal) or vagal (medulla) origin.

1.3.1.1. Sympathetic Afferents

Afferent nerve fibers from the lower airways travel to the spinal cord over sympathetic nerve branches that traverse the stellate ganglia. Many fibers have endings in the pleura surrounding the lung roots, or in the structures in the lung roots, and some supply the trachea, but so far few endings have been identified within the lung itself (Holmes & Torrance, 1959; Kostreva et al., 1975). In dogs, pulmonary afferents traverse the right and left upper thoracic white rami communicantes (Kostreva et al., 1975); they respond to lung inflation, pinching of the lung parenchyma, and mechanical probing of the pulmonary arteries and veins. One of the roles of these sympathetic afferents may be reflex regulation of respiration in dogs (Kostreva et al., 1975) and vagotomized cats (Torrance & Whitteridge, 1947; Widdicombe, 1954a,c). In guinea-pig lungs, about 50% of the SP-immunoreactive fibers - presumed to be afferent fibers - have a nonvagal origin (Lundberg & Saria, 1987). Functional, histochemical, and biochemical evidence suggests that tachykinin-containing sensory ganglion cells of the upper thoracic spinal ganglia project to the lower airways via sympathetic pathways (Dalsgaard & Lundberg, 1984; Lundberg et al., 1983a; Saria et al., 1985). Observations in human patients indicate that the sympathetic afferent pathway from the tracheobronchial tree and lungs is not involved in pain sensation. Sensations from the lower airways, including pain, are transmitted via the vagi (Guz, 1977; Morton et al., 1951).

1.3.1.2. Vagal Afferents

Four types of vagal afferent end-organs have been identified in the lower airway walls and they have been the subject of recent reviews (Widdicombe, 1988; Coleridge & Coleridge, 1986; Lundberg & Saria, 1987; Sant'Ambrogio, 1987). They include slowly adapting pulmonary stretch receptors (SARs), rapidly adapting stretch (irritant) receptors (RARs), and pulmonary and bronchial C-fibers.

SARs are probably the best studied of the airway afferent endings. They are supplied by myelinated axons as determined by conduction velocity studies (Paintal, 1966). SARs produce a long-lasting discharge in response to a maintained lung inflation. Typically, they respond to stimuli with a rapid increase in activity that declines rapidly immediately after the inflation and slows progressively to a sustained firing (Sant'Ambrogio, 1987). SARs are located along the tracheobronchial tree down to the terminal bronchioles (Miserocchi & Sant'Ambrogio, 1974); higher concentrations of SAR are found in the larger, more proximal airways with a progressive declines toward the periphery (see Sant'Ambrogio, 1987). SARs of the trachea, main stem bronchi, and lobar bronchi are found only within the smooth muscle of the membraneous posterior wall (Bartlett et al., 1976; Bradley & Scheurmier, 1977). SARs located in the intrathoracic airways increase their rate of discharge in the course of inspiration. SARs located in the extrathoracic trachea increase their activity during expiratory flow (Sant'Ambrogio & Mortola, 1977). A particular category of SARs described in the intrathoracic airways (Luck, 1970) of rabbits, cats, monkeys, and rats (Luck, 1970; Tsubone, 1986; Wei & Shen,

1985) discharges during expiration whether the animal breathes spontaneously or is artificially ventilated. The responses of SARs are closely associated with changes in transpulmonary pressure and with circumferential tension (Bartlett et al., 1976).

SARs are responsible for the Hering-Breuer inflation (inhibitory) and deflation (excitatory) reflexes. Their discharge in inspiration provides an inspiratory off-switch, and their continuing discharge in expiration lengthens the respiratory pause. The respiratory pause involves reciprocal inhibition; inspiratory neurons are inhibited while expiratory neurons are excited (Bradley, 1977). Excitatory input is thought to be engaged only when lung volume is reduced below functional residual capacity (FRC). Thus, in general, the role of SAR is to signal the degree of stretch of the lungs and to modify breathing reflexly, the pattern of which is primarily set by the respiratory rhythm generator in the brainstem. There is little indication that SAR have an important role in disease, and these receptors do not appear to be involved significantly in asthma.

RARs are stimulated by mechanical events such as inflation or deflation of the lungs. They differ from SARs in having a higher volume threshold, a more rapid rate of adaptation, and a more irregular pattern of discharge (Coleridge & Coleridge, 1986). RAR are less numerous than SAR. The ratio of RARs to SARs is 1:4 in the rabbit (Roumy & Leitner, 1980) and 1:10 in the cat (Widdicombe, 1954a,b,c). RAR are not evenly distributed along the tracheobronchial tree, but are concentrated in the more proximal airways. In the extrapulmonary airways, the concentration of RAR

increases from the upper trachea to the main stem bronchi (Sant'Ambrogio et al., 1978) where they are distributed all around the circumference. This is unlike SAR that are confined to the posterior membraneous wall (Sant'Ambrogio et al., 1978/3). The hilar airways are accessible to chemical and mechanical irritants, and RAR in these airways could be activated easily in order to evoke protective reflex actions (Sant'Ambrogio, 1987). RAR are believed to correspond to the epithelial nerve endings identified in the intra- and extra-pulmonary airways of several mammalian species (Elftman, 1943; Fillenz & Woods, 1970; Fisher, 1964). These epithelial endings are the terminal arborizations of myelinated fibers that ramify in the tracheobronchial submucosa, frequently at points of bronchial branching. Different layers of the airway wall appear to be supplied with endings, as removal of the mucosa sensitive to local probing can leave the response to inflation and deflation intact (Coleridge & Coleridge, 1986).

Receptors concentrated at the carina and in the primary bronchi are believed to be cough receptors (Fillenz & Widdicombe, 1972; Widdicombe, 1954a,b,c, 1977); those in the intrapulmonary airways are thought not to cause cough (Fillenz & Widdicombe, 1972; Widdicombe, 1977, 1988). RARs are believed to be responsible for the occasional respiratory "sighs" described in many mammals (Davies & Roumy, 1982). The most important function of RAR may be to signal the onset of pathophysiological changes in the airways. The concept of a nociceptive function began with the observation that RAR in the intrapulmonary airways of rabbits are stimulated by acute pulmonary congestion, embolization, anaphylaxis, pneumothorax, and intravenous

injection of bronchoconstrictor chemicals (Mills et al., 1969, 1970; Sellick & Widdicombe, 1969, 1970, 1971). In addition to their sensitivity to mechanical stimuli and a number of lung pathological conditions, RAR can also be activated by a wide range of mediators including histamine and $PGF_{2\alpha}$, or by inhalation of various irritant gases such as cigarette smoke, sulfur dioxide, and ammonia (see Widdicombe, 1988). The findings that stimuli which activate the RAR also cause laryngeal constriction, bronchoconstriction, and tracheal mucus secretion (Widdicombe, 1963, 1964; Coleridge & Coleridge, 1986) suggest an important role for RAR in airway diseases including asthma.

Vagal C-fiber afferents supplying the tracheobronchial tree and the lung have been separated by both pharmacological and physiological criteria into two main categories: pulmonary C-fiber receptors (also called J receptors) and bronchial C-fiber receptors (Coleridge & Coleridge, 1977). The terms "pulmonary" and "bronchial" indicate the blood supply of the endings either via pulmonary or the bronchial circulation and thus their general locations. Pulmonary C-fibers are located in the lung parenchyma, outside the bronchial and bronchiolar walls, and the bronchial C-fibers are within the tracheobronchial walls.

In general, pulmonary C-fibers are sensitive to mechanical changes in the lungs but are relatively insensitive to chemicals such as lung autocoids; the reverse is true for bronchial C-fibers. The transpulmonary pressure gradient necessary to change activity was lowest for SARs, intermediate for RARs and pulmonary C-fiber receptors, and the highest for bronchial C-fiber endings (Kaufman et

al., 1982). Interstitial pressure increases such as those that occur in pulmonary congestion or edema are strong stimulators of pulmonary C-fiber activity (e.g., the strongest for J receptors [Paintal, 1969]), but have only weak effects on bronchial C-fiber endings (Coleridge & Coleridge, 1977). Bronchial C-fibers are stimulated by humoral mediators of inflammation, including histamine (Coleridge & Coleridge, 1977; Coleridge et al., 1978), PGs (Coleridge et al., 1976, 1978), serotonin (Coleridge & Coleridge, 1984), and bradykinin (Kaufman et al., 1980) after they are injected into the systemic circulation or inhaled in the form of an aerosol. By contrast, pulmonary C-fibers are stimulated by PGs, especially those of the E series (Coleridge et al., 1976, 1978), and remain virtually unaffected by histamine, serotonin, or bradykinin (Coleridge & Coleridge, 1984, Karczewski & Widdicombe, 1969; Kaufman et al., 1980).

Most of the reflex actions attributed to bronchial and pulmonary C-fibers are similar (Coleridge & Coleridge, 1984). They include rapid, and shallow breathing (often preceded by apnea), bronchoconstriction, mucous secretion, bradycardia, and hypotension; coughing has never been reported. It has been suggested that pulmonary C-fiber receptors provide excitatory input that opposes the inhibitory input from SARs and help control respiratory rate (Pisarri et al., 1986).

Although both C-fiber receptors and RARs are activated by similar stimuli (Coleridge & Coleridge, 1984, 1986) and mediate laryngeal constriction, bronchoconstriction, and mucous secretion, their effects can be differentiated by two types of experiment.

Minimal dosages of capsaicin or phenylbiguanide are thought to affect selectively C-fiber receptors (Coleridge & Coleridge, 1986). However, in guinea-pigs, iv capsaicin does not mediate bronchoconstriction via vagovagal reflexes (Biggs & Goel, 1985). Secondly, the vagi can be blocked by cooling or anodal current in such a way that reflexes mediated via only one type of receptors are left intact; vagal cooling to 6-8°C blocks myelinated afferent fibers subserving SARs and RARs, and further cooling to 0°C blocks unmyelinated vagal C-fiber receptors (Coleridge et al., 1982; Davis et al., 1982b). Vagal cooling has shown that Head's paradoxical reflex in rabbits, which had been ascribed to stimulation of RARs, is mediated via nonmyelinated vagal afferents (Widdicombe, 1967).

In the pulmonary vagal branches of cats, the unmyelinated afferents outnumber the myelinated by the ratio of 3:1, 4:1 (Agostoni et al., 1957) or 9:1 (Jammes et al., 1982). In mouse alveolar walls, unmyelinated vagal afferents, thought to be Paintal's J receptors, were found in close association with type I pneumocytes (Hung et al., 1973a). Similar terminals were also found between the epithelial cells of the mouse intrapulmonary bronchial mucosa (Hung et al., 1973b) and human tracheal mucosa (Rhodin, 1966). The terminal axon enlargements found in mouse bronchi (Hung et al., 1973b) are often associated with epithelial cells resembling the type I cells of the aortic and carotid bodies and containing many dense-cored vesicles. Such epithelial cells, found in the airway mucosa of many species, are associated with unmyelinated nerve terminals of afferent and efferent appearance (Lauweryns & Cokelaere, 1973). These complexes of epithelial cells and nerve

endings are termed "neuroepithelial bodies" and have been allocated a chemoreceptor sensory role (Lauweryns & Peuskens, 1972; Lauweryns et al., 1985). They probably respond to changes in pO_2 (Lauweryns & Cocelaere, 1973).

Sensory afferents described above, especially RARs and C-fiber endings, appear to play a significant role in the control of airway tone, both via axonal reflexes within the airway walls, and via central nervous reflexes.

1.3.2. Efferent Innervation

1.3.2.1. Cholinergic Efferents

The parasympathetic nervous system is the dominant neural bronchoconstrictor mechanism in all animals, including humans, and plays an important role in the regulation of airway tone (Barnes, 1986c). Parasympathetic efferent nerves arise in the vagal nuclei of the brain stem and pass down the vagus nerve to synapse in ganglia located within the airway wall; from these ganglia, relatively short postganglionic fibers pass to smooth muscle, the submucosal glands, and the blood vessels (Richardson, 1979). In humans, ganglia are scattered along the peribronchial nerve plexus down to the level of smaller bronchi; they are most abundant in large airways (Larsell, 1922; Larsell & Dow, 1933). In the trachea, they are found in chains extended along its length near the lateral borders of the posterior membrane (Fischer, 1964; Coburn, 1984). Electrical stimulation of the vagi in animals causes a bronchoconstriction due to smooth muscle contraction, which is potentiated by cholinesterase inhibitors and blocked by the muscarinic receptor antagonist

atropine (Nadel, 1980; Nadel & Barnes, 1984). Cholinergic nerve fibers are found in human airway smooth muscle from the trachea to the terminal bronchioles, but their density decreases in the smaller airways (Barnes, 1987a). In guinea pigs, cholinergic innervation is present in the airways at least down to the level of the main bronchi (Grundström et al., 1981a), with some innervation in the hilar bronchi (Karlsson & Persson, 1983). The distribution of the cholinergic innervation along the airway is consistent with cholinergic nerve effects. Thus, in vitro, cholinergic nerve effects in bronchi are greater than those in bronchiole (Parmer et al., 1986) and, in vivo, anticholinergic drugs induce greater bronchodilation in large than in small airways (Hensley et al., 1978). In normal subjects, the bronchodilator response to atropine and other anticholinergic drugs suggests a degree of resting vagal tone in human airways (de Troyer et al., 1979). Similarly, inhalation of the cholinesterase inhibitor edrophonium causes bronchoconstriction in normal subjects, confirming tonic release of acetylcholine in the airways (Quigley et al., 1985).

Vagal efferent pathways are involved in reflex bronchoconstriction mediated via sensory receptors in the upper and lower airways (Widdicombe, 1988) and via chemoreceptors and baroreceptors in the cardiovascular system (Nadel & Widdicombe, 1962b). This reflex bronchoconstriction is blocked by vagotomy or anticholinergic drugs (Nadel, 1980). Thus, cholinergic antagonists block or decrease the bronchoconstrictor response to various stimuli including histamine, carbon dust, cold air, and citric acid (Simonsson et al., 1967), prostaglandins (Alanko and Poppius, 1974),

and aerosols of propellant (e.g., dichlor-difluorethane) and surfactant (e.g., sorbitol trioleate, soya lecithin) (Sterling and Batten, 1969). In some studies (Itkin & Anand, 1970; Casterline et al., 1976), they have been reported to be ineffective, probably due to low concentrations of the stimuli and antagonists, and inadequate delivery of antagonist (Casale, 1988).

Cholinergic receptors are divided into nicotinic and muscarinic receptors. Nicotinic receptors are located on nerve cell bodies in airway parasympathetic ganglia and activated by acetylcholine (ACh) released from preganglionic vagal fibers and nicotinic agonists such as dimethylphenylpiperazinium (DMPP). Nicotinic receptors can be blocked by the nicotinic antagonist hexamethonium (Skoogh, 1983).

Muscarinic receptors are located on the target cells in the airway and activated by ACh released from postganglionic nerves; they are blocked by atropine and related drugs such as ipratropium bromide (Barnes, 1987). Activation of muscarinic receptors in airway smooth muscle causes contraction by stimulating the breakdown of membrane phosphoinositides, which results in the release of calcium ions from intracellular stores (Grandordy et al., 1986), and by inhibiting adenylate cyclase, which leads to a reduction in the concentration of cyclic AMP (Madison et al., 1985). Direct binding studies in bovine airways (Cheng & Townley, 1982) and autoradiographic mapping (Barnes at al., 1983) have shown that the density of muscarinic receptors is high in the smooth muscle of large airways and decreases in smaller airways, such that terminal bronchioles are almost devoid of receptors. The distribution of muscarinic receptors in the airway smooth muscle is consistent with

the pattern of vagally mediated airway contraction. Studies with cholinergic agonists suggested that the distribution of muscarinic receptors in human airway smooth muscle is similar to that in other species (Goldie et al., 1982).

Recently, it has been proposed that muscarinic receptors can be classified into three subtypes based on the selectivity of antagonists (Birdsall & Hulme, 1985; Hammer et al., 1986; Gross & Barnes, 1988): M_1, M_2, and M_3 receptors - which have high affinity for pirenzepine, gallamine and AF-DX 116, and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), and hexahydrosiladifenidol, respectively. In airways, M_1-receptors are located presumably at parasympathetic ganglia and facilitate parasympathetic ganglionic transmission in rabbits (Bloom et al., 1987), dogs (Bech et al., 1987), and humans (Lammers et al., 1989). M_2-receptors are located at postganglionic parasympathetic fibers and inhibit postganglionic prejunctional transmission in cats (Blaber et al., 1985), guinea pigs (Fryer & Maclagan, 1984), and humans (Minette & Barnes, 1988). M_3-receptors are located at smooth muscle and stimulate smooth muscle contraction.

1.3.2.2. Sympathetic Efferents

Adrenergic control of airway function has been reviewed recently (Zaagsma et al., 1987; Barnes, 1988). This section will discuss airway control by sympathetic innervation and circulating catecholamines.

The sympathetic nerve supply to the lung originates from the upper six thoracic segments of spinal cord. Preganglionic fibers

synapse in the middle and inferior cervical ganglia and the upper four thoracic ganglia. From these, postganglionic fibers run to the lung and enter at the hilum to intermingle with the cholinergic nerves and form a dense plexus around airways and vessels (Richardson, 1979; Barnes, 1986c). Unlike the dense parasympathetic nerve supply to airways in all species, sympathetic innervation is generally sparse, although there is considerable variation among species (Mann, 1971; Richardson, 1979; Doidge & Satchell, 1982). In human airways, a sparse sympathetic innervation has been demonstrated by fluorescence histochemistry, ultrastructural studies, and immunohistochemistry using an antibody to dopamine β-hydroxylase. Adrenergic nerve fibers have been found in close association with submucosal glands (Partanen et al., 1982; Pack and Richardson, 1984; Meyrick & Reid, 1970; Sheppard et al., 1983), airway ganglia (Richardson & Ferguson, 1979), and bronchial vessels (Doidge & Satchell, 1982; Partanen et al., 1982; Sheppard et al., 1983); however, very few, if any, adrenergic fibers have been demonstrated in the smooth muscle of the intrapulmonary airways (Doidge & Satchell, 1982; Laitinen et al., 1985; Partanen et al., 1982; Richardson & Beland, 1976; Sheppard et al., 1983).

Field stimulation (FS) of human airways obtained post mortem or during surgery induces cholinergic contraction followed by a relaxation. The relaxation is inhibited by tetrodotoxin but unaffected by propranolol (Davis et al., 1982a; Taylor et al., 1984; Palmer et al., 1986). The uptake-1 blocker cocaine has no effect on responses of human bronchi or lung strips to norepinephrine (Zaagsma et al., 1983). Thus, these studies do not indicate any functional

role of sympathetic innervation in human airway smooth muscle, consistent with the anatomical findings. However, these findings do not exclude the possibility that adrenergic nerves may influence bronchomotor tone indirectly in human airways (see section 1.4.1. for the details). In guinea-pig airways, functional studies with FS (Doidge & Satchell, 1982; Grundström et al., 1981a) or cocaine (Zaagsma et al., 1983) and histochemistry (O'Donnell et al., 1978) have demonstrated sympathetic innervation to tracheal but not bronchial smooth muscle.

Circulating catecholamines include dopamine, norepinephrine (NE), and epinephrine. Dopamine is present in very low concentrations, and infusion of dopamine at supraphysiological concentrations has no effect on bronchomotor tone (Michoud et al., 1984). NE is present in the highest concentrations: it is derived almost entirely from overspill of sympathetic nerve activity with the remainder from the adrenal medulla (Brown et al., 1981). Infusion of NE in normal subjects at concentrations within the physiological range has no significant effects on metabolic, cardiovascular, or airway function (Silverberg et al., 1978; Larsson et al., 1986; Berkin et al., 1985), suggesting that it functions as a neurotransmitter rather than as a hormone. By contrast, epinephrine, secreted by the adrenal medulla, functions as a circulating hormone, and is a potent bronchodilator in normal and asthmatic subjects, when infused at the levels found in severe excercise in normal subjects (Berkin et al., 1985; Warren & Dalton, 1983; Warren et al., 1984; Larssen et al., 1985; Berkin et al., 1986; Barnes et al., 1982b).

Despite the fact that β-blocker-induced asthma (McNeill, 1964; McNeill & Ingram, 1966; van Herwaarden, 1983) has been recognized for over 20 years, its mechanism is unknown. Normal subjects do not develop any deterioration in lung function (Richardson & Sterling, 1969; Tattersfield et al., 1973; Zaid & Beall, 1966) after β-blockers and do not show an increase in sensitivity to bronchoconstrictor agents such as histamine or methacholine (Townley et al., 1976). This suggests that β-blockers presumably antagonize some tonic β-adrenergic bronchodilator tone which is present in asthmatic patients but not in normal subjects. Since human airway smooth muscle has no demonstrable adrenergic innervation (Barnes, 1986c), this suggests that circulating catecholamines provide this drive (Barnes, 1988). However, circulating catecholamines are not elevated in asthmatic subjects (Barnes et al., 1982c), even in those subjects who develop bronchoconstriction after propranolol (Ind et al., 1984), and the concentrations of adrenaline in plasma are too low to have a direct effect on human airway smooth muscle tone.

This suggests that β-blockers may inhibit the action of catecholamines on some other target cells, such as mast cells or cholinergic nerves. Mediator release from human lung mast cells is potently inhibited by β-agonists (Peters et al., 1982) via β_2-receptors (Butchers et al., 1980). The effect of β-blockers may therefore be an increase in mediator release, which may be more marked in the "leaky" mast cells of asthmatics. This idea is supported by the observation that sodium cromoglycate, a mast cell stabilizer, prevents bronchoconstriction produced by inhaled propranolol (Koeter et al., 1982). Another possibility is that

catechoamines modulate cholinergic neurotransmission at parasympathetic ganglia or at neuromuscular junctions (see section 1.4.1.2. for the detailed discussion). In human airways, in vitro, prejunctional β_2-receptors on cholinergic nerves are able to influence airway smooth muscle tone, and much lower concentrations of β-agonist are required to bronchodilate than would be needed for a direct effect on airway smooth muscle (Rhoden et al., 1988). Blockade of these receptors may thus lead to an increase in vagal tone, which would cause more bronchoconstriction in asthmatic subjects, since airway smooth muscle would be more sensitive to the acetylcholine released from cholinergic nerves. Evidence in support of this idea is the observation that bronchoconstriction due to propranolol can be prevented by atropine (Grieco & Pierson, 1971; Ind et al., 1986).

The adrenergic receptors that mediate the effects of adrenergic agonists on airway cells are divided into α- and β-receptors, and are subdivided into α_1- and α_2-receptors, and β_1- and β_2-receptors.

Radioligand binding studies (Rugg et al., 1978; Barnes et al., 1980; Engel, 1981) and autoradiography (Barnes et al., 1982a; Carstairs et al., 1984) revealed a high density of β-receptors in many different cell types of lungs from many species including humans (Rugg et al., 1978; Barnes et al., 1980; Engel, 1981). In airway smooth muscle, β-receptors are found from trachea to terminal bronchioles, consistent with the in-vitro functional studies with β-agonists (Zaagsma et al., 1983; Davis et al., 1982; Goldie et al., 1982). The density of β-receptors increases with decreasing size of

Table 1-2. Airway adrenoceptor function (modified from Barnes, 1988)

Cell type	β-Receptor	α-Receptor
Smooth muscle	Relaxation	Contraction
Mast cell	Decrease in secretion	Increase in secretion
Submucosal gland	Increase in mucous secretion	Increase in serous secretion
Epithelium	Increase in ion transport	?
Cholinergic nerves	Inhibition	Inhibition
Clara cell	Increase in secretion	?
Type 2 pneumocyte	Increase in secretion	No effect
Bronchial vessels	Dilation Decrease in permeability	Constriction

airways in animals and humans (Barnes & Basbaum, 1983; Carstairs et al., 1985). β-Agonists modulate cholinergic neurotransmission, either at the level of cholinergic ganglia or at postjunctional nerve terminals (see section 1.4.1. for more discussion). Table 2 shows β-adrenoceptor-mediated effects on different cell types in airways. The coexistence of β_1- and β_2-receptors has been confirmed in animal and human lung (Rugg et al., 1978; Engel, 1981) by direct receptor-binding techniques with selective β-antagonists. In human lung, the ratio of β_1- to β_2-receptors is approximately 1:3. In guinea-pig tracheal smooth muscle, β_2-receptors predominate (Zaagsma et al., 1979).

It has been postulated that β_1-receptors are regulated by sympathetic nerves ("innervated" β-receptors) whereas β_2-receptors are regulated by circulating epinephrine ("hormonal" β-receptors) (Ariens & Simonis, 1983). This hypothesis has been supported by functional studies of airway smooth muscle preparations from cats, dogs, and guinea pigs (see Zaagsma et al., 1987). It confirms the relationship between the density of adrenergic innervation and β_1-receptors. Thus, in human airway smooth muscle which lacks significant sympathetic innervation (Richardson & Ferguson, 1979; Richardson, 1981), relaxation of central and peripheral airways was mediated only by β_2-receptors (Zaagsma et al., 1983; Goldie et al., 1984). This finding concurs with recent autoradiographic studies of human lung confirming that the β-receptors of human airway smooth muscle from bronchi to terminal bronchioles are entirely of the β_2-receptor subtype (Carstairs et al., 1985). Human submucosal glands, which receive a sparse sympathetic nerve supply, have a small population (10%) of β_1-receptors, when determined by autoradiography (Carstairs et al., 1985; Phipps et al,. 1982). Epithelial cells and mast cells, which are not adrenergically innervated, have only β_2-receptors (Butchers et al., 1980).

Other findings suggest that innervated β_2-receptors and noninnervated β_1-receptors are also present in the lung. Guinea-pig and human alveolar walls are devoid of direct noradrenergic innervation (Nahorski et al., 1985; Carstairs et al., 1985; Partanen et al., 1982; Pack & Richardson, 1984), but they have a significant number of β_1-receptors. Very occasionally,

guinea-pig tracheal smooth muscle has only β_2-receptors and the numbers appear to be similar to the β_1-receptors in tracheal smooth muscle of the vast majority of animals that has a heterogeneous population of β_1- and β_2-receptors (Zaagsma et al., 1979).

A fundamental defect in β-receptor function in asthma seems unlikely, since neither adrenalectomy nor chronic blockade of β-receptors produces asthma or bronchial hyperresponsiveness in normal subjects (Barnes, 1988). Furthermore, it is still controversial whether β-receptor function is impaired in asthma secondary to the disease. Earlier studies showed that the defect of cardiovascular, metabolic, and leukocyte β-receptor function in asthmatics was secondary to elevated circulating epinephrine levels or the presence of autoantibodies against β_2-receptors. However, these findings have not been confirmed and an increase in epinephrine levels has not been confirmed even in severe asthma. The autoantibodies that were detected in a small proportion of asthmatic patients also occur in normal subjects.

Airway responses to inhaled β-agonists are impaired in asthma and this impairment is related to the severity of asthma (Barnes & Pride, 1983). The impaired β-receptor function may be explained by a reduction in the delivery of drug to the airways as a result of airflow obstruction (Dolovich et al., 1976), or a reduced effect of β-agonist relaxation at high degrees of smooth muscle contraction (functional antagonism [Karlsson & Persson, 1981]). Failure of patients with acute asthma to respond to β-agonists may reflect luminal mucus plugging and bronchial edema. Some impairment in

airway β-receptor response may occur as a result of the inflammatory reaction in asthma because products of inflammation such as oxygen radicals (Engels et al., 1985) and PAF (Braquet et al., 1985) may impair airway β-receptor function or reduce β-receptor density. Studies of asthmatic airways in vitro are rare and their results appear to be conflicting (Ind & Barnes, 1988). None of these findings answer the question of whether β-receptor function is impaired in asthma, but such a defect may be of little clinical significance, since asthmatic patients bronchodilate so readily to β-agonists (Barnes, 1988).

In general, α_1-receptors (classic α-adrenoceptors) are located postjunctionally on airway cells including smooth muscle and mediate the effects of α-agonists, whereas α_2-receptors are presynaptic and modulate neurotransmitter release from nerve terminals.

α_1-Receptors have been demonstrated in total lung membranes from rats (Latifpour & Bylund, 1981), guinea pigs (Barnes et al., 1979), and humans (Barnes et al., 1980b) although they are considerably fewer than β-receptors. These receptors are localized to human and mammalian airway smooth muscle and they are sparse in the trachea but increase in density as the airways become smaller (Barnes et al., 1983a; Carstairs et al., 1984).

The exact role of α-receptors in regulating airway tone is controversial. In diseased human bronchi (chronic bronchitis or bronchopneumonia), norepinephrine induced marked contraction which does not occur in normal airways unless they are pretreated with endotoxin (Kneussl & Richardson, 1978; Simonsson et al., 1972). Similarly, in dog trachea and human bronchi, histamine or serotonin

pretreatment unmasks α-receptor-mediated contraction (Kneussl & Richardson, 1978; Barnes et al., 1983; Brown et al., 1983). Activation of airway α-adrenergic responses by histamine and serotonin has been shown in dogs, in vivo. In dog trachea, contractile responses to both exogenous NE and sympathetic nerve stimulation are mediated entirely by α_2-receptors, consistent with binding studies demonstrating few α_1-receptors and mostly α_2-receptors (Barnes et al., 1983f; Brown et al., 1983; Leff & Munoz, 1981). However, this was not observed in human bronchi, although α_2-receptor-mediated attenuation of excitatory nerve transmission was documented (Grundström & Andersson, 1985). α-Receptors mediate NE-induced contraction of guinea-pig and human lung strips (See Zaagsma et al., 1987; Black et al., 1981). However, the relative importance of α-receptor subtypes and their location is unknown. Although they may be present on small airways, other contractile elements such as Kapanci cells or small blood vessels could contribute. In guinea-pig lung strips, NE's effect is insensitive to cocaine which argues against involvement of blood vessels (densely innervated) in the contraction (Zaagsma et al., 1987).

The findings that α-adrenoceptors may be activated by inflammatory mediators (Kneussl & Richardson, 1978; Barnes et al., 1983b; Brown et al., 1983) suggest that α-adrenergic responses might be enhanced in asthmatic airways as proposed by Szentivanyi (1980). This idea is supported by the demonstration that α_1-agonists such as phenylephrine and methoxamine cause bronchoconstriction in asthmatic patients but not in normal subjects, even in the absence

of β-blockers (Black et al., 1982, 1984; Snashall et al., 1978; Patel & Kerr, 1973).

However, other studies do not support this theory. Although some authors observed enhanced α-receptor-mediated contraction in dog trachea probably due to a post-receptor mechanism involving activation of Ca^{2+} channels (Barnes et al., 1983b), other workers could not confirm this finding (Leff & Munoz, 1981). It has been shown that histamine failed to enhance contractions produced by NE in the presence of β-blocker in guinea-pig trachea and non-diseased human bronchi in vitro (Goldie et al., 1985). In the absence of propranolol, NE did not contract either guinea-pig trachea or normal human bronchi and this effect was still absent after histamine. In vivo, studies showing enhanced α_1-receptor-mediated bronchoconstriction in asthmatic subjects appear to be difficult to interpret. It has been found that phenylephrine produced marked bronchodilation (β-adrenoceptor-mediated) in asthmatics before β-blockade, but produced no significant improvement in lung function in non-asthmatic subjects (Patel & Kerr, 1973). This might be due to different levels of airway tone: the airways of asthmatics were expected to have greater airway tone (see section 1.2.3.). Such differences in baseline bronchial tone between asthmatic and nonasthmatic subjects could be partly responsible for enhanced α_1-receptor-mediated bronchoconstriction reported by some authors in asthmatics (Black et al., 1982, 1984; Snashall et al., 1978; Patel & Kerr, 1973). Enhanced α_1-receptor activity in asthmatics might be due to a pH effect as it has been shown that the pH of a solution could influence bronchial responses to inhaled histamine

(Cockcroft & Berscheid, 1982), and it is known that asthmatic airways tend to be more sensitive to irritant stimuli (Ind & Dollery, 1983). Thus, in contrast to studies demonstrating the bronchoconstricting effect of unbuffered solutions of phenylephrine in asthmatics (Snashall et al., 1978; Patel & Kerr, 1973), buffered solutions of phenylephrine has been shown to be as spasmogenic as buffered saline in asthmatics (Thomson et al., 1982), suggesting that bronchial smooth muscle α_1-receptor activity is insignificant in asthmatics who exhibited a wide range of nonspecific bronchial hyperresponsiveness. These findings further indicate that increased α_1-receptor activity is not the primary abnormality responsible for the variation among asthmatics in nonspecific bronchial hyperresponsiveness. Recently, Spina et al. (1989) have shown that phenylephrine failed to induce significant increases in tone in bronchi isolated from either non-diseased or asthmatic human lung even in the presence of β-blocker, consistent with low levels of α_1-adrenoceptors in bronchial smooth muscle. Thus, findings <u>in vitro</u> are consistent with the findings <u>in vivo</u> with buffered solutions of phenylephrine, suggesting that the enhanced α_1-receptor effect reported by some workers in asthmatics might be due to the effects of the pH of agonist solution.

If bronchoconstrictor α-receptors are activated in asthma, then α-blockers should dilate the airways of asthmatics and prevent the bronchoconstriction induced by nonspecific stimuli. However, the effects of α-blockers in asthma are controversial. Nonselective α-antagonists such as thymoxamine, phentolamine and indoramin can cause bronchodilation (Prime et al., 1972; Campbell, 1982) and

protect against bronchoconstriction induced by histamine (Kerr et al., 1970; Bianco et al., 1972), exercise (Walden et al., 1984), cold air (Walden et al., 1984), and inhaled antigen (Patel & Kerr, 1975). These effects may relate to the non-α-receptor blocking pharmacological actions of the drugs, which include direct effects on smooth muscle, release of catecholamines (Ind & Dollery, 1983; Svedmyr, 1984), and antihistaminic activity (Patel & Kerr, 1975; Bianco et al., 1972; Kerr et al., 1970). The selective α_1-antagonist, prazosin, which has no antihistamine effect and does not cause catecholamine release, has no bronchodilator effect when given by inhalation to asthmatic subjects (Barnes et al., 1981b; Baudouin et al., 1988) and does not protect against histamine challenge (Barnes et al., 1981c). Both prazosin and phentolamine give partial protection against exercise-induced asthma (Barnes et al., 1981c; Walden et al., 1984), but this might be explained by an effect on bronchial blood flow which might counteract any changes induced by the hyperventilation of exercise. Other studies have shown that prazosin, when given orally, has a bronchodilator effect (Marlin et al., 1982), but this might be explained by baroreflex changes consequent upon the fall in blood pressure.

In guinea-pig models of chronic asthma, the number of lung α_1-adrenoceptors significantly increased, although these receptors were not related specifically to airway smooth muscle in homogenates of whole lung containing many cell types (Barnes et al., 1980a; Mita et al., 1983). Despite twofold increase in the number of lung α_1-adrenoceptors, α-adrenoceptor-mediated contraction in response to NE was unchanged in the lung strips compared with strips from

control animals (Turner et al., 1983). These findings raise questions about the functional significance of the very marked increase in α_1-receptors in lung membranes from patients with asthma (Szentivanyi et al., 1979) or chronic bronchitis (Barnes et al., 1980b).

Furthermore, it is difficult to understand how airway α_1-receptors could be activated endogenously, as neither circulating adrenaline nor NE causes bronchoconstriction (Berkin et al., 1985, 1986; Larsson et al., 1986). In the absence of adrenergic innervation of human airways, NE could overflow from sympathetic nerves on blood vessels as in the guinea pig (Ainsworth et al., 1982) but the absence of any effects of tyramine is against this (Ind et al., 1983). Inhaled NE which would lead to high airway concentrations of NE, produces bronchodilation rather than bronchoconstriction, presumably by β_2-receptor stimulation (Pichurko et al., 1986). In the absence of β-blocker, even the specific α_1-agonist phenylephrine induces β-receptor-mediated relaxation of bronchi isolated from healthy and asthmatic subjects (Spina et al., 1989). Currently available data suggest that the role of α_1-adrenoceptors in asthma is controversial (see Goldie et al., 1985; Barnes, 1988; Zaagsma, 1987).

α_2-Adrenoceptors, located presynaptically at parasympathetic ganglia and/or at neuromuscular junction in the airways of several species, could modulate excitatory neurotransmission. Their possible role in asthma is discussed in detail in section 1.4.1.1..

1.3.3. Nonadrenergic Noncholinergic (NANC) Innervation

1.3.3.1. Inhibitory NANC (i-NANC) System

Airway i-NANC system has been the subject of several reviews and editorials (Richardson, 1981; Diamond & Gillespie, 1982; Diamond & Richardson, 1982; Barnes, 1986; Diamond & Altiere, 1988). Since i-NANC nerves were described in toad lungs by Campbell (1971), i-NANC nerves have been demonstrated in vitro by electrical field stimulation after adrenergic and cholinergic blockade in the airways of several species, including guinea pig (Coburn & Tomita, 1973), primates (Middendorf & Russell, 1980), and humans (Richardson & Beland, 1976; Barnes, 1986b). i-NANC responses to field stimulation are abolished by tetrodotoxin (TTX), implying a (postganglionic) neural origin. i-NANC nerves have also been demonstrated in vivo in cats and guinea pigs by electrical stimulation of the peripheral end of the cut vagus nerve after adrenergic and cholinergic blockade (Chesrown et al., 1980; Irvin et al., 1980; Yip et al., 1981). Stimulation of this pathway produces pronounced and long-lasting bronchodilation which is inhibited by ganglionic blockade, suggesting that the preganglionic fibers are carried in the vagi. This pathway can be activated reflexly by mechanical or chemical stimulation of the larynx in feline airways (Szarek et al., 1986; Inoue et al., 1989). Although it has been difficult to study this pathway in humans in vivo, preliminary studies of mechanical and chemical laryngeal stimulation indicate that reflex i-NANC bronchodilatation may occur (Michoud et al., 1985).

The pattern and degree of i-NANC innervation varies among species and along the tracheobronchial tree. In human airways, the i-NANC system is the only bronchodilator pathway, since there is no

functional sympathetic innervation (Barnes, 1986). i-NANC nerves were demonstrated from the trachea to the distal bronchi (Richardson & Béland, 1976; Hutas et al., 1981; Davis et al., 1982a; Taylor et al., 1984). This system is absent in several species including dog, rat, and pig. In guinea-pig airways, i-NANC relaxation was demonstrated in trachea, and occasionally in the main bronchi (Grundstrom et al., 1981a; Chesrown et al., 1980; Diamond & Altiere, 1988). In guinea-pig trachea, the contribution of i-NANC responses to the total inhibitory responses varies among studies: about 40% in strips from all segments (cervical, middle, thoracic) and about 20% in tube preparations (Kalenberg & Satchell, 1979); 20-40% in tube preparations in situ (Yip et al., 1981); and predominantly in chain preparations (Taylor et al., 1984).

This system is difficult to study because the neurotransmitter is unknown, no specific blockers are available, and the neural pathways are uncertain (Nadel et al., 1986). Although there is evidence in favor of purines as neurotransmitters of i-NANC nerves in several systems including gut, they do not appear to be involved in the airways. Exogenous ATP relaxes airway smooth muscle via P_2 receptors (Ito & Takeda, 1982), however, the P_2-antagonist quinidine failed to block NANC relaxation *in vitro* or *in vivo*, and a purine uptake inhibitor dipyridamole does not enhance NANC relaxation (Ito & Takeda, 1982; Irvin et al., 1982). Similarly, adenosine fails to mimic NANC relaxation, and P_1-receptor antagonists, such as theophylline, have no blocking action in guinea-pig (Coleman, 1980; Grundström et al., 1981a) and bovine trachea (Cameron et al., 1983), or in human airways (Davis et al.,

1982a). Desensitization to purines does not reduce NANC relaxations (Ito & Takeda, 1982). NANC relaxations are still present after maximal adenosine relaxations (Karlsson & Persson, 1984).

A more likely neurotransmitter candidate for i-NANC system is vasoactive intestinal peptide (VIP). VIP produces prolonged relaxation of airway smooth muscle which is unaffected by adrenergic or cholinergic blockade, and mimics the time-course of i-NANC responses both *in vitro* and *in vivo*. VIP-immunoreactive nerves have been found in airway smooth muscle of several species, including dogs and humans (Dey et al., 1981; Ghatei et al., 1982; Said & Dey, 1988). VIP also mimics the electrophysiological changes in airway smooth muscle produced by NANC nerve stimulation (Ito & Takeda, 1982; Cameron et al., 1983). Electrical field stimulation of tracheobronchial preparations releases VIP into the bathing medium, an effect blocked by TTX (Cameron et al., 1983; Matsuzaki et al., 1980). Furthermore, the amount of VIP released is related to the magnitude of nerve stimulation. Preincubation of guinea-pig trachea with a specific antibody to VIP reduces responses to exogenous VIP and to NANC stimulation (Matsuzaki et al., 1980). The close association between responses to VIP and NANC relaxation in different sizes of human and bovine airways also points to the role of VIP as a neurotransmitter. Although evidence in favor of VIP is persuasive, its neurotransmitter role cannot be proven until the development of a specific receptor antagonist. Indeed, there is some evidence against it as a neurotransmitter in airways. After pretreatment of guinea-pig trachea with a maximally effective concentration of VIP, there is no diminution of NANC relaxation,

which would be expected if all VIP receptors were occupied (Karlsson & Persson, 1984).

VIP appears to coexist with ACh in airways, and it is possible that (excessive) stimulation of cholinergic nerves results in release of VIP (Barnes, 1988b). In bovine tracheal smooth muscle, VIP has an inhibitory effect on cholinergic nerve-induced contraction only at high stimulation rates. Also, it reduces the contractile effect of exogenous ACh (Palmer et al., 1985), probably due to functional antagonism. This indicates that VIP counteracts the bronchoconstrictor effect of cholinergic bronchoconstriction and may function as a "braking" mechanism for airway cholinergic nerves (Barnes, 1987c). In asthma, the degradation of VIP may be increased by a variety of peptidases released by the inflammatory cells present in the asthmatic airways, resulting in airway hyperresponsiveness. In asthmatics, there could be congenital or acquired lack of i-NANC system in the airways as seen in Hirschprung's disease (Frigo et al., 1973), reversible blockade of the this system at the level of the ganglia or nerve endings (Richardson, 1985), or deficiency of airway VIP receptors. However, until now, no definite abnormalities in the i-NANC system have been demonstrated in a large series of patients with pulmonary diseases (Richardson, 1988).

i-NANC responses discussed above are abolished by TTX. However, i-NANC responses evoked with FS at strong stimulation parameters (frequency >10 Hz; duration >0.5 msec in human airways) are partly abolished by TTX, suggesting that a substantial part of i-NANC responses are nonneural (Davis et al., 1982a; Taylor et al., 1984).

The mechanism underlying TTX-insensitive relaxation is uncertain but several possible explanations have been proposed. Coburn & Tomita (1973) attributed TTX-insensitive relaxations to a direct effect of electrical FS on smooth muscle membranes. The findings that TTX is more efficacious in blocking FS-induced relaxation in human airways denuded of connective tissue have suggested that impaired diffusion of TTX to intrinsic nerves is involved (Richardson & Beland, 1976). However, the differential sensitivity of cholinergic and NANC responses to the inhibitory effects of TTX make these explanations appear untenable. More likely, other nonneural mechanisms may be involved, such as release of mediators from secondary cell types insensitive to the effects of TTX (Middendorf & Russell, 1980). It is also possible that in NANC nerves the process of depolarization is inherently less sensitive to TTX than it is in other autonomic nerves (Fukuda & Kameyama, 1980). Whatever the mechanism, it is clear that TTX-resistant i-NANC responses are a substantial part of the inhibitory responses to FS, particularly in primate airways (Diamond & Altiere, 1988). It will be important to determine whether the same or different mediators are involved in TTX-sensitive and TTX-resistant i-NANC responses and whether TTX-resistant i-NANC responses play any physiologically important role in regulating airway tone.

1.3.3.2. Excitatory Nonadrenergic Noncholinergic (e-NANC) System

In vitro, electrical field stimulation of guinea-pig bronchi produces a component of contraction which is not inhibited by atropine (Grundström et al., 1981a; Lundberg & Saria, 1982; Karlsson & Persson, 1983; Lundberg et al., 1983b,c; Anderson & Grundström,

1983) but which is blocked by an antagonist to substance P (SP) (Karlsson et al., 1985; Leander et al., 1984; Lundberg et al., 1983d). In man, however, no consistent atropine-resistant bronchial contraction has been obtained upon electrical field stimulation in vitro (Davis et al., 1982a; Richardson & Beland, 1976; Taylor et al., 1984). This observation does not exclude a role for e-NANC system in the control of bronchial smooth muscle tone in man, as bronchodilatory mechanisms may dominate during FS in atropinized human bronchial preparations (Richardson & Beland, 1976; Taylor et al., 1984). e-NANC responses are abolished by TTX, implying a nervous origin.

SP causes bronchoconstriction in the airways of several species including man, and capsaicin, which releases SP from sensory nerves, induce a similar contraction (Lundberg et al., 1983b,c; Lundberg & Saria, 1982; Szolcsanyi & Bartho, 1982). In the airways, SP-immunoreactive nerves are located superficially beneath and within airway epithelium, around blood vessels and, to a lesser extent, in airway smooth muscle (Barnes, 1988b). Capsaicin-sensitive sensory nerves in the lung also contain neurokinin A (NKA)-, and neuropeptide K (NPK)-like immunoreactivities (-LI) as well as calcitonin gene-related peptide (CGRP) and an eledoisin (ELE)-related peptide (Hua et al., 1985b; Lundberg et al., 1985a,b). Tachykinin-LI are localized to cell bodies in the jugular and nodose ganglia as well as axons in the vagi. Tachykinins are also contained in sensory cell bodies in thoracic spinal ganglia, which project to the lower airways via sympathetic pathways (Dalsgaard & Lundberg, 1984; Lundberg et al., 1983a; Saria et al., 1985).

Substance P and/or other tachykinins released from capsaicin-sensitive sensory C-fibers appear to serve as potential neurotransmitters for the airway e-NANC system. Tachykinins released from sensory C-fibers mediate their effects via at least three types of tachykinin receptors which may be differentiated by their different responses to a series of tachykinins (Lee et al., 1986). In some tissues, SP is more potent than neurokinins (SP-P or NK_1-receptor), whereas in others the order of potency is either NKA > NKB > SP (SP-E or NK_2-receptor), or NKB > NKA > SP (SP-N or NK_3-receptor).

SP is a well-known bronchoconstrictor *in vivo* and *in vitro* (Andersson & Persson, 1977; Lundberg & Saria, 1982; Nilsson et al., 1977) in a variety of species including man (Finney et al., 1985; Lundberg et al., 1983c). *In vivo*, SP infusion causes bronchoconstriction [illegible] which is partly blocked by atropine, suggesting that SP-induced release of acetylcholine is responsible for part of its bronchoconstrictor action in vivo (Tanaka & Grunstein, 1984). In human subjects, infusion of SP has marked cardiovascular effects, but little effect on airway function: a small bronchoconstrictor response is followed by bronchodilation at higher doses (Fuller et al., 1987). Even when given by inhalation, SP has no significant effect on airway function in mild asthmatic subjects who are hyperresponsive to histamine given in the same way (Fuller et al., 1987). This may be due to enzymatic dagradation of SP in the airways or its inability to cross the epithelium (Barnes, 1988b). *In vitro*, SP contracts the airway smooth muscle of several species, including man (Lundberg et al., 1983c; Karlsson et al.,

1984). In vitro, the contractile effect of SP on airway smooth muscle are inhibited by SP antagonists, suggesting a direct effect on smooth muscle cells. However, the specificity of these antagonists has been questioned (Karlsson & Persson, 1984).

In guinea pigs (Hua et al., 1985a) or in isolated human bronchi (Karlsson & Persson, 1985; Lundberg et al., 1985b; Martling et al., 1987), NKA and ELE are much more potent bronchoconstrictors than SP, suggesting that the SP-E receptor is dominant in airway smooth muscle. Since NKA-LI has been detected in lung, NKA, rather than SP, is probably the endogenous stimulant of SP receptors, at least in airway smooth muscle. Infusion of NKA in human subjects causes bronchoconstriction and relatively few cardiovascular effects, suggesting that NK_2-receptors are important in regulation of bronchial tone in vivo (Theodorsson-Nerheim et al., 1985). CGRP is the most potent constrictor of human airways so far described (Palmer et al., 1985a). Contraction induced by CGRP is unaffected by cholinergic, histamine, or leukotriene antagonists and probably acts via specific receptors (Barnes, 1986,1987).

SP, neurokinins, and CGRP are capable of producing many of the pathologic features of asthma, including contraction of airway smooth muscle, edema, plasma extravasation, and mucous hypersecretion (Barnes, 1986a; Lundberg & Saria, 1987), so it is possible that they contribute to the pathology of asthma. Stimulation of C-fiber endings that are exposed due to epithelial damage in asthmatics (see section 1.2.3.), can also result in axonal reflexes, with the release of sensory neuropeptides from collaterals in the airway.

It has been discovered that a membrane-bound enzyme called neutral endopeptidase (NEP; also called enkephalinase), localized on specific cells that contain tachykinin receptors (epithelium, smooth muscle, glands, blood vessels, neutrophils, sensory nerves, vagus nerves), modulates the actions of tachykinins by cleaving and inactivating them. Thus, it is possible that down-regulation of NEP activity in airways may enhance the effects of sensory neuropeptides released by local axonal reflexes and contribute to the pathophysiology of asthma (Nadel, 1988).

1.4. Modulation of Cholinergic Neurotransmission

As discussed in sections 1.2.3. & 1.3., imbalances in the excitatory and inhibitory autonomic nervous systems could lead to bronchial hyperresponsiveness (Casale, 1988; Barnes et al., 1988). In many species, including man (Richardson, 1979), the cholinergic nervous system is the dominant bronchoconstrictor and has been shown to mediate the rapid changes in airway caliber provoked by injection or inhalation of irritants such as histamine (Boushey, 1984). Thus, it may be possible that overactivity of cholinergic mechanisms leads to bronchial hyperresponsiveness in asthma (Nadel et al., 1986). This possibility was first suggested by the findings that muscarinic antagonists are often effective bronchodilators (Cavanaugh & Cooper, 1976; Chamberlain et al., 1962; Herxheimer, 1959) and muscarinic agonists are potent bronchoconstrictors (Curry, 1947) in asthmatics. This view has been further supported by the finding that many of the stimuli that induce bronchospasm in asthma (e.g., sulfur dioxide, prostaglandins and histamine) also stimulate airway vagal afferents

(irritant and C-fiber) and induce an increase in vagal reflex activity (Sant'Ambrogio, 1982; Widdicombe, 1982).

An increase in activity in vagal reflex pathways offers an attractive explanation for airway hyperresponsiveness. Several possible sites in the sensory and motor pathways may be involved: vagal sensory endings, nodose or sensory ganglia, central nervous system, vagal motor ganglia, release of acetylcholine from the postganglionic nerve terminals, and the smooth muscle muscarinic receptor sites (Holtzman et al., 1980; Boushey, 1984; Nadel et al., 1986). There could be an increase in airway afferent receptor discharge via the effects of inflammatory mediators and exposure of afferent nerve endings by the airway epithelial damage found in asthma. This hypothesis has been supported by studies of responses of the respiratory system that are known to be influenced by afferent vagal activity, such as the pattern of breathing (Lee et al., 1980) and the threshould for cough (Empey et al., 1976). However, it is unclear whether the sensory endings responsible for cough are also responsible for bronchoconstriction. Asthmatics have been shown to respond to psychological stimuli with bronchoconstriction that is inhibited by atropine (McFadden et al., 1969). Thus, neural output from the central nervous system could contribute to increased vagal efferent activity. Holtzman et al. (1980) have shown in atopic subjects that hexamethonium did not alter the bronchoconstriction induced by inhaled methacholine but prevented that induced by inhaled histamine, indicating that ganglionic transmission is not necessary for the response to methacholine. These findings suggest that inhaled methacholine acts

directly on the smooth muscle muscarinic receptors to cause bronchoconstriction, and that the increased bronchomotor responses result from acetylcholine-mediated cholinergic nervous activity. An increase in the number or the binding affinity of the muscarinic receptors on airway smooth muscle could lead to airway hyperresponsiveness. In this case, bronchomotor responses to muscarinic agonists or other stimuli that cause, through whatever mechanism, an increase in efferent traffic to the postganglionic nerve ending, may be enhanced (Boushey, 1984). This may account partly for nonspecificity of airway hyperresponsiveness in asthma. Mita et al. (1983) showed that numbers of muscarinic receptors in lung homogenates were increased in a guinea-pig model of asthma, supporting the possible role of increased numbers of muscarinic receptors on smooth muscle in asthma.

It is also possible that cholinergic neurotransmission may be facilitated at cholinergic ganglia and neuromuscular junctions by inflammatory mediators or other neurotransmitters. The following sections will discuss in detail "braking" and "facilitatory" mechanisms in the cholinergic motor pathway.

1.4.1. Modulation by Neurohumoral Mediators

Airway cholinergic neurotransmission is under the influence of various inflammatory mediators and neurotransmitters. In isolated canine trachea, prostaglandin E_2 (PGE_2) has inhibitory effects on cholinergic neurotransmission at a prejunctional site (Walters, 1984). By contrast, in intact dogs, $PGF_{2\alpha}$ increases airway responsiveness to inhaled ACh, and the increase is blocked by hexamethonium, indicating the involvement of neural mechanisms

(O'Byrne et al., 1984a). Serotonin appears to potentiate the release of ACh from cholinergic nerves in dog airways (Hahn et al., 1978; Sheller et al., 1982).

In cats, guinea pigs, and rabbits, SP induces bronchoconstriction which is partially blocked by atropine (Andersson & Persson, 1977; Tanaka & Grunstein, 1984), suggesting that the release of ACh mediates part of its bronchoconstrictor actions (Tanaka & Grunstein, 1984). In several species, including human, both immunohistochemical (Lundberg et al., 1984b) and electrophysiological studies (Barnes, 1986c) have indicated SP-containing afferent input into airway ganglia and into bronchial smooth muscle. Thus, the release of SP from sensory afferents could modulate ganglionic neurotransmission (Barnes, 1986c).

In animal and human lungs, VIP-containing nerve fibers are found in close association with the airway smooth muscle layers from trachea to small bronchiole (Said & Dey, 1988). Many of the nerve cell bodies in airway ganglia contain VIP (Uddman et al., 1978; Uddman & Sundler, 1979; Dey et al., 1981), and VIP is present in nerve terminals that contact the nerve cell bodies within the ganglia. Ultrastructural studies suggest that VIP coexists in the same nerve terminals as ACh (Laitinen, 1985). These morphological findings may indicate that VIP-ergic nerves could modulate cholinergic neurotransmission in airway ganglia or affect smooth muscle. In ferret isolated trachea, VIP potentiates responses to field stimulation at low concentrations (up to 10^{-9} M), but reduces responses at higher concentrations via presynaptic mechanisms (Sekizawa et al., 1988).

In guinea-pig airways, neuropeptide Y (NPY) reduces the cholinergic component of FS-induced contraction via a prejunctional mechanism by acting directly on receptors on cholinergic nerve terminals (Stretton & Barnes, 1988). These findings suggest that NPY released by adrenergic nerves modulates cholinergic neurotransmission in guinea-pig airways.

Mast cells are closely associated with airway ganglia, and this suggests that various inflammatory mediators (see section 1.2.3. for mediators) released from these cells also influence ganglionic neurotransmission.

1.4.2. Sympathetic Modulation

Although there appears to be no direct functional sympathetic innervation of human airway smooth muscle as discussed above (see section 1.3.2.2.), it is possible that adrenergic nerves may influence bronchomotor tone indirectly (Barnes, 1988a). Histochemistry has demonstrated catecholamine-containing nerve fibers in the parasympathetic ganglia of calf (Mann, 1971; Jacobowitz et al., 1973), pig, rabbit and foetal sheep lung (Mann, 1971), and in human airway ganglia (Richardson & Ferguson, 1979; Partanen et al., 1982). In guinea-pig airways, sympathetic fibers were not noted in parasympathetic ganglia (Baluk et al., 1985), but sympathetic and cholinergic varicosities were in close apposition near airway smooth muscle (Jones et al., 1980). These anatomical findings suggest that the sympathetic nervous system could modulate neurotransmission at the airway parasympathetic ganglia or at the cholinergic neuromuscular junction. The evidence for this will be

discussed below.

In intact dogs, electrical stimulation of the thoracic sympathetic nerves inhibits the bronchoconstrictor effect of vagal nerve stimulation (Cabezas et al., 1971). In isolated canine bronchi, NE and isoproterenol reduce contractile responses to cholinergic stimulation but have little effect on responses to exogenous ACh, suggesting that their inhibitory effects are on the release of ACh from postganglionic nerve terminals via prejunctional β_1-receptors (Vermeire & Vanhoutte, 1977, 1979; Vanhoutte & Flavahan, 1988). In preparations with the epithelium removed, electrical field stimulation evokes a rapid increase in tension which is not sustained and the "spontaneous" decrease in the size of the response is prevented by propranolol (Vermiere & Vanhoutte, 1979). These observations suggest that NE released from adrenergic nerve terminals reduces the release of ACh via prejunctional β-adrenoceptors. In the canine bronchi, adrenergic and cholinergic varicosities were found in close apposition (Knight et al., 1981), supporting a role for sympathetic modulation of cholineric neurotransmission *in vitro* and *in vivo*.

In ferret trachea, NE inhibits cholinergic neurotransmission at parasympathetic ganglia via presynaptic α-adrenoceptors, which mediate the inhibition of the firing of ganglion cells (Baker et al., 1983). These findings have been confirmed in nerve-muscle preparations of ferret trachea, but only in about half of the preparations tested (Skoogh, 1986, 1988). Skoogh (1986, 1988) showed that isoproterenol prevented cholinergic neurotransmission at parasympathetic ganglia probably via presynaptic β_2-adrenoceptors

that inhibit ACh release (Skoogh, 1986, 1988). Ferret tracheal ganglia are known to contain at least two different cell types (Baker et al., 1986) and two different types of nerve terminals (Coburn, 1984). However, the adrenergic innervation of these ganglia has not been demonstrated in ferret tracheal ganglia.

In guinea pigs, stimulation of the sympathetic outflow to the lungs induces bronchodilation (Ainsworth et al., 1982). However, there is no evidence for direct sympathetic innervation of intrapulmonary airways, which suggests that effect of sympathetic stimulation on the airways is indirect. In isolated guinea-pig trachea, the sympathomimetics NE, epinephrine, and salbutamol inhibited responses to FS at low frequency, and this inhibition was blocked by β-antagonists (Jones et al., 1980). However, others (Grundström et al., 1981b) reported that NE inhibited responses to FS in isolated trachea and main bronchi during β-adrenoceptor blockade, an effect blocked by the selective α_2-adrenoceptor antagonist yohimbine. In anesthetized guinea-pigs, the α_2-agonist clonidine significantly inhibited atropine-sensitive bronchoconstriction induced by electrical vagal stimulation. Again, this effect was reduced by yohimbine (Andersson et al., 1986). These findings indicate that both prejunctional α_2- and β-adrenoceptors mediate the inhibitory effects of adrenergic agonists on cholinergic transmission in guinea-pig airways. In guinea-pig trachea with the vagi intact, McCaig (1987) showed that inhibition of vagal responses by isoproterenol was blocked by propranolol, but that inhibition by NE was blocked only by a combination of propranolol and phentolamine, indicating the involvement of both prejunctional α-

and β-adrenoceptors in the neuromodulatory effects. Also, she showed that sympathetic nerve stimulation inhibited vagal responses in half of the preparations tested.

In isolated human bronchi, sympathomimetics inhibited the atropine-sensitive contraction induced by FS probably via prejunctional α_2- (Grundström & Andersson, 1985) and β_2-adrenoceptors (Rhoden et al., 1987) located on postganglionic parasympathetic nerves.

In sensitized guinea-pigs (Andersson et al., 1986) and in asthmatic patients (Lindgren et al., 1986), clonidine inhibited bronchospasm induced by antigen challenge. This might be due in part to inhibition of vagal reflex bronchoconstriction via presynaptic α_2-adrenoceptors on cholinergic efferents as vagal reflex bronchoconstriction occurs in airway anaphylaxis (Widdicombe, 1977, 1982). β-Blockers may precipitate bronchoconstriction in asthmatic subjects (McNeill, 1964; McNeill & Ingram, 1966; van Herwaarden, 1983) by an unknown mechanism. One possibility is that, in asthmatics, catecholamines exert a tonic braking effect on cholinergic neurotransmission at parasympathetic ganglia or at neuromuscular junctions and the release of this braking effect by β-blockers results in bronchoconstriction (see section 1.3.2.2.).

The above evidence indicates that cholinergic neurotransmission can be inhibited by the sympathetic nervous system and sympathomimetics in the airways of mammalian species including humans. Therefore, it is possible that a decrease in such neuromodulatory functions could lead to airway hyperresponsiveness in asthma.

1.5. Preamble

It is clear from the literature review that nonspecific airway hyperresponsiveness is a fundamental characteristic of asthma. Thus, it is essential to understand the mechanism of airway hyperresponsiveness in order to develop treatments for asthma. Out of the many potential mechanisms proposed for airway hyperresponsiveness, abnormality of autonomic control of the airways has received much attention. This is because of the often rapid changes in bronchomotor tone in asthma, and the absence of a correlation between airway responses in vivo and in vitro. Imbalance between the excitatory and inhibitory nervous systems could [illegible]te to airway hyperresponsiveness (Barnes et al., 1988; [illegible]988). In many species, and in man (Richardson, 1979), the [illegible] nervous system is the dominant bronchoconstrictor and [illegible]ty of cholinergic mechanisms could lead to the bronchial [illegible]veness in asthma (Nadel et al., 1986). Sites in the [illegible] and motor pathways responsible for increased vagal efferent activity could include: vagal sensory endings, the nodose ganglia, the central nervous system, the vagal motor ganglia, postganglionic nerve terminals, and smooth muscle muscarinic receptor sites. Minette et al. (1988) claimed that inhibitory muscarinic autoreceptor function is decreased in asthmatic subjects. This suggests an important role for inhibitory presynaptic receptors in airway hyperresponsiveness. Prejunctional α_2-adrenoceptors inhibit cholinergic neurotransmission in the airways of guinea pigs and humans (Grundström et al., 1981; Grundström & Andersson, 1985). In

sensitized guinea pigs (Andersson et al., 1986) and in asthmatic patients (Lindgren et al., 1986), the α_2-agonist clonidine inhibits bronchospasm induced by antigen challenge, probably by inhibiting vagal reflex bronchoconstriction by actions on presynaptic α_2-adrenoceptors located on cholinergic efferents. However, whether inhibitory α_2-adrenoceptor function is altered in asthma is unknown. Despite many studies, evidence of a role for α_1-adrenoceptors in airway hyperresponsiveness is conflicting. We examined whether α_1- and α_2-adrenoceptors are involved in airway hyperresponsiveness in guinea pigs.

1.6. Hypothesis

Up-regulation of excitatory α_1-adrenoceptors and down-regulation of acetylcholine-release-modulating presynaptic α_2-adrenoceptors contribute to airway hyperresponsiveness in asthma.

1.7. Objectives

Overall: Determine the role of α-adrenoceptors in experimental asthma, with special emphasis on their neuromodulatory function.

1. Compare the inhibitory effects of adrenergic agonists on tracheal responses to pre- (NS) and post-ganglionic (FS) vagal stimulation, to determine whether α_2-adrenoceptors modulate neurotransmission at parasympathetic ganglia in tracheas from control guinea pigs.
2. Study the effects of uptake 1 and 2 blockers, and chemical

sympathectomy with 6-hydroxydopamine, on neuromodulatory function of NE in tracheas from control guinea pigs.

3. Characterize the effects of sympathetic ganglionic stimulation on tracheal responses to vagal stimulation, to study the neuromodulatory function of endogenous NE in tracheas from control guinea pigs.

4. Characterize the inhibitory effects of NE on tracheal responses to pre- (NS) and post-ganglionic (FS) vagal stimulation in tracheas from ovalbumin-sensitized and hypersensitized guinea pigs, and compare the results with those from 1 above.

1.8. Animal Model

The guinea pig was used as the animal model in these experiments because:

1. The pattern of the sympathetic, parasympathetic, and NANC innervation is similar in guinea pigs and in man (Richardson, 1979).

2. Modulatory α_2-adrenoceptors have been described in the airways of guinea pigs and man (Andersson & Grundström, 1987).

3. Models of asthma are available in guinea pigs (Barnes, 1980; Mita et al., 1983).

4. The smooth muscle in guinea-pig airways is abundant (Miller, 1947), making the airways especially sensitive to broncho-constrictors.

5. The guinea-pig tracheal tube is one of the few preparations in which it is possible to stimulate parasympathetic nerves pre- and post-ganglionically (McCaig & Blackman, 1983).

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2. ALPHA$_2$-ADRENOCEPTORS DO NOT MODULATE NEUROTRANSMISSION THROUGH PARASYMPATHETIC GANGLIA IN GUINEA-PIG TRACHEA*

2.1. Introduction

Involvement of the sympathetic nervous system and adrenoceptors in the control of airway smooth muscle has been the subject of several recent reviews (Barnes, 1986,1988; Goldie et al., 1985; Leff, 1988). Barnes (Barnes, 1986) claimed that the sympathetic nervous system is minimally involved in autonomic regulation of airway tone in humans and that the non-adrenergic, non-cholinergic (NANC) system is the major bronchodilator system in the airways. Nonetheless, adrenergic nerves have been demonstrated in ganglia in human airways (Richardson & Ferguson, 1979), and Skoogh (Skoogh, 1986,1988) has proposed that sympathetic effects on transmission through airway ganglia modulate airway tone. In guinea pigs, the sympathetic supply to the trachea is rich in the upper part but sparser toward the bronchi (Doidge & Satchell, 1982; Jones et al., 1980; Smith & Satchell, 1985) and α_2-adrenoceptor agonists reduce bronchospastic responses to vagal stimulation in vivo (Andersson et al., 1985,1986). In vitro, norepinephrine (NE) inhibits transmission through parasympathetic ganglia in the ferret trachea (Baker et al., 1983), and α_2-adrenoceptors modulate neurotransmission in guinea-pig trachea by a presynaptic action on postganglionic fibers (Grundström et al., 1981a,b).

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It seemed possible that α-adrenoceptors and the sympathetic nervous system affect ganglionic transmission in the airways; however, nothing was known of the effects of adrenoceptor agonists on neurotransmission through parasympathetic ganglia in guinea-pig trachea. Therefore, we compared the inhibitory effects of NE on pre- and post-ganglionic nerve stimulation in guinea-pig tracheal tube preparations (Blackman & McCaig, 1983; Widmark & Waldeck, 1986).

2.2. Method

Tissue preparation. Female Hartley-strain (350–550 g, n=64) guinea pigs were anesthetized with pentobarbital sodium given ip, 35–40 mg/kg body weight, or killed by a blow to the head; 10–15 ml of blood was removed by cardiac puncture to ensure a clear field for dissection. The extrathoracic trachea and vagi were dissected free in the neck and the thorax was opened. The lungs, intrathoracic trachea, and heart were removed en bloc, and placed in oxygenated (95% O_2/5% CO_2) Krebs–Henseleit solution (composition, in mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2.2H_2O$ 2.5, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, and glucose 11.7) containing propranolol (5 μM) and indomethacin (10 μM), at room temperature. The trachea, with its vagi and recurrent laryngeal nerves intact on both sides, was dissected free from surrounding tissues, cannulated at both ends (PE 240), and filled with oxygenated Krebs' solution. It was mounted vertically in a 45-ml organ bath containing oxygenated Krebs' solution at 37°C, in a tissue holder equipped with two parallel platinum wire electrodes for transmural electrical field

stimulation (FS). The cannula at the bottom of the trachea was connected to a peristaltic pump (Masterflex, Cole-Parmer) via a stopcock; the other cannula was connected to a pressure transducer (Statham P23D or Gould P50; Hewlett-Packard 8805A or Buxco preamplifier; Hewlett Packard 680M or 7708 recorder) via another 3-way stopcock. Between recordings, both stopcocks were opened and the tracheal lumen was perfused at 1 ml/min with oxygenated Krebs' solution at 37°C; the stopcocks were closed when intraluminal pressure was recorded. The recording system was meticulously checked for leaks just before the start of each experiment; its compliance was kept as low as possible. The starting pressure of tracheal tubes was standardized at zero transmural pressure. Maximal FS of preparations using this system yielded intratracheal pressures of 40–50 cm H_2O.

Stimulation. After equilibration of the tissues for 1 h, both vagi were drawn into a single shielded platinum tunnel electrode for nerve stimulation (NS) with rectangular pulses (0.5 or 1 ms; 40-50 V) applied via a Phipps & Bird 611 stimulator. For FS, bipolar rectangular pulses (0.5 or 1 ms; 80 V) were applied from a Grass SD9 stimulator. NS and FS were applied at 2, 8, and 32 Hz at 3-min intervals with an automated timer, using trains of 64 pulses or for 10 s. Before the preparation was used, alternate NS and FS were applied for 0.5 h or until pressure increases were constant.

Norepinephrine pD_2. As preliminary experiments showed maximal inhibitory effect of NE on cholinergic transmission 15–18 min after addition to the bath, all data points were taken during this period. For each block of 4 experiments, NE was added immediately

after control responses to NS in 2 experiments and after FS in the other 2. The NE concentration (M) inhibiting control responses by 50% (ED_{50}) was determined from linear regression of 3 or 4 concentrations inducing 15–85% inhibition, and pD_2 values were calculated as the negative logarithm of this.

Yohimbine pA_2. Values were calculated from a Schild plot (5) by comparing the inhibitory effects of NE without and with at least 2 concentrations of the blocker. Slopes of the lines were obtained from linear regression or conventionally.

Chemical sympathectomy. Animals were given 6-hydroxydopamine (50 mg/kg body weight, ip) 24 h before experiments, or the isolated tracheas were incubated for 1 h at 37^0 with 6-hydroxydopamine (100 µM) then washed with Krebs' solution for 30 min (40).

Dose–response curves. Desipramine (0.1 uM) was added, and 30 min later cumulative dose-response curves were obtained to acetylcholine without and with NE (3 µM).

Experiments. pD_2 for NE, and pA_2 for yohimbine against it, were measured in 6 groups of 4 tissues using NS and FS: 3 groups were stimulated at 2, 8, and 32 Hz, respectively, for 10 s, and 3 groups received trains of 64 stimuli at the same frequencies. Similarly, pD_2 and pA_2 values were determined in 4 groups of 4 tissues in the presence of blockers of NE uptake: uptake 1: cocaine (10 µM) and desipramine (0.1 µM]); and uptake 2: corticosterone (1 µM). NE pD_2 and yohimbine pA_2 were determined in 3 groups of 4 tissues (tracheas chemically sympathectomized in vivo or in vitro): in 4 tissues, the effects of NE with and without prazosin (0.3 µM)

were compared; in 8 tissues, we determined pD_2 values for the selective α_2-adrenoceptor agonists xylazine (n=4) and clonidine (n=4) and pA_2 values for yohimbine against them. Drugs except NE, xylazine, and clonidine were added to the bath at least 30 min before the experiment.

Statistical analyses. Data were expressed as mean ± SEM. At least 4 replicates were obtained in each series of experiments, and significance was assumed at the 5% level. pD_2 and pA_2 values were compared by 2- and 3-way analysis of variance, with repeated measures for subjects measured more than once, using SPSS-X and UANOVA (Taerum, 1989). Scheffe's test was used to rank differences. Individual comparisons were made with unpaired Student's *t* tests. Least-squares regression analysis was performed with unweighted values, using STATISTIX (version 1.0).

Drugs. The following drugs were used: L-ascorbic acid (BDH Chemicals, Toronto, Ont.); desipramine HCl, 6-hydroxydopamine HCl, indomethacin, propranolol HCl, and yohimbine HCl (Sigma, St.Louis, MO); L-noradrenaline-D-bitartrate (Fluka, Ronkonkoma, NY); pentobarbital sodium injection (Euthanyl, MTC Pharmaceuticals, Mississauga, Ont.); and prazosin HCl (gift from Pfizer Canada Inc., Kirkland, Que.).

Drug solutions were freshly prepared each day. 6-Hydroxydopamine was dissolved in distilled water containing 0.02% ascorbic acid, indomethacin was dissolved in 0.2% sodium carbonate, and prazosin was suspended in 5 ml glycerine and further dissolved in 5 ml 5% dextrose. All other drugs were made up as stock

solutions in distilled water. NE solutions were stored on ice in the dark.

2.3. Results

Characterization of responses to nerve and field stimulation. Hexamethonium abolished responses to NS but left responses to FS unchanged; atropine (0.5 μM) then abolished the latter. Atropine (0.5 μM) eliminated responses to both NS and FS in preparations not previously treated with hexamethonium.

Effect of pentobarbital anesthesia. Comparison of pressure increases to NS and FS with 0.5-ms stimuli at 2, 8, and 32 Hz with 64 pulses revealed no significant differences in the magnitude of responses in tracheas from animals anesthetized with pentobarbital or killed by a blow to the head.

Effects of frequency of stimulation. The size of tracheal responses to both NS and FS at 2, 8, and 32 Hz for 10 s correlated positively with frequency of stimulation but, for trains of 64 pulses, correlated negatively with frequency.

Norepinephrine pD_2 and yohimbine pA_2 against NS and FS at 2, 8, and 32 Hz (Table 1). For both NS and FS, with stimulation for 10 s or 64 pulses, at all frequencies the NE pD_2 values correlated negatively with frequency of stimulation; i.e., less NE was required at low frequencies to inhibit pressure increases (Fig. 1). Comparison of pD_2 values for NS and FS at the same frequencies revealed no significant differences whether tissues were stimulated for 10 s or with trains of 64 stimuli. With one exception, yohimbine (0.3 or 3 uM) did not affect control pressure increases to NS and

Table 2-1. Norepinephrine pD_2 and yohimbine pA_2 against nerve (NS) and field stimulation (FS) at 2, 8, and 32 Hz.

Stimulation Parameters	Stimulation	Norepinephrine pD_2 Frequency (Hz) 2	8	32	Yohimbine pA_2 Frequency (Hz) 2	8	32
10 s, 1 ms	NS	5.53±0.06	5.11±0.06	4.74±0.08	6.89±0.17	6.41±0.18	6.21±0.17*
	FS	5.37±0.05	5.00±0.08	4.40±0.05	6.58±0.11	6.28±0.14	5.62±0.07*
10 s, 0.5 ms	NS	5.87±0.07	5.52±0.07	5.15±0.16			
	FS	5.78±0.10	5.51±0.09	5.07±0.08			
64 pulses, 0.5 ms	NS	5.57±0.12	5.20±0.04	4.89±0.10	7.18±0.21	7.40±0.18	6.55±0.32
	FS	5.61±0.10	5.25±0.05	5.04±0.08	7.22±0.15	6.96±0.10	6.70±0.03

* Significantly different, NS vs FS.

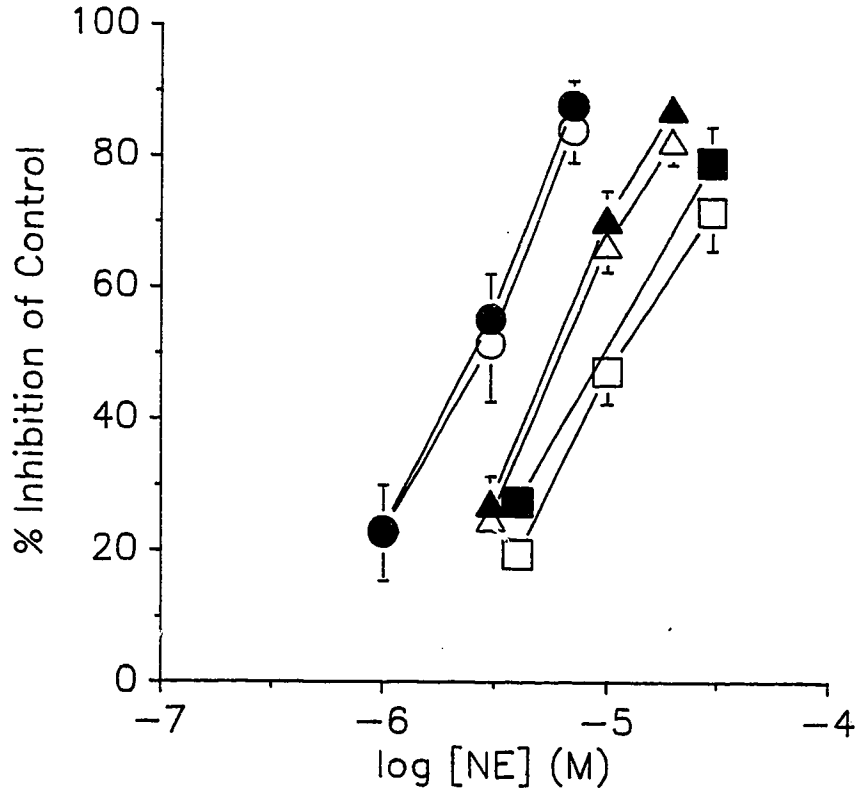

Fig. 2-1. Inhibitory effects (as % control) of norepinephrine on increases in intratracheal pressure induced by electrical nerve (open symbols) or field (filled symbols) stimulation at 2 Hz (circles), 8 Hz (triangles), and 32 Hz (squares).

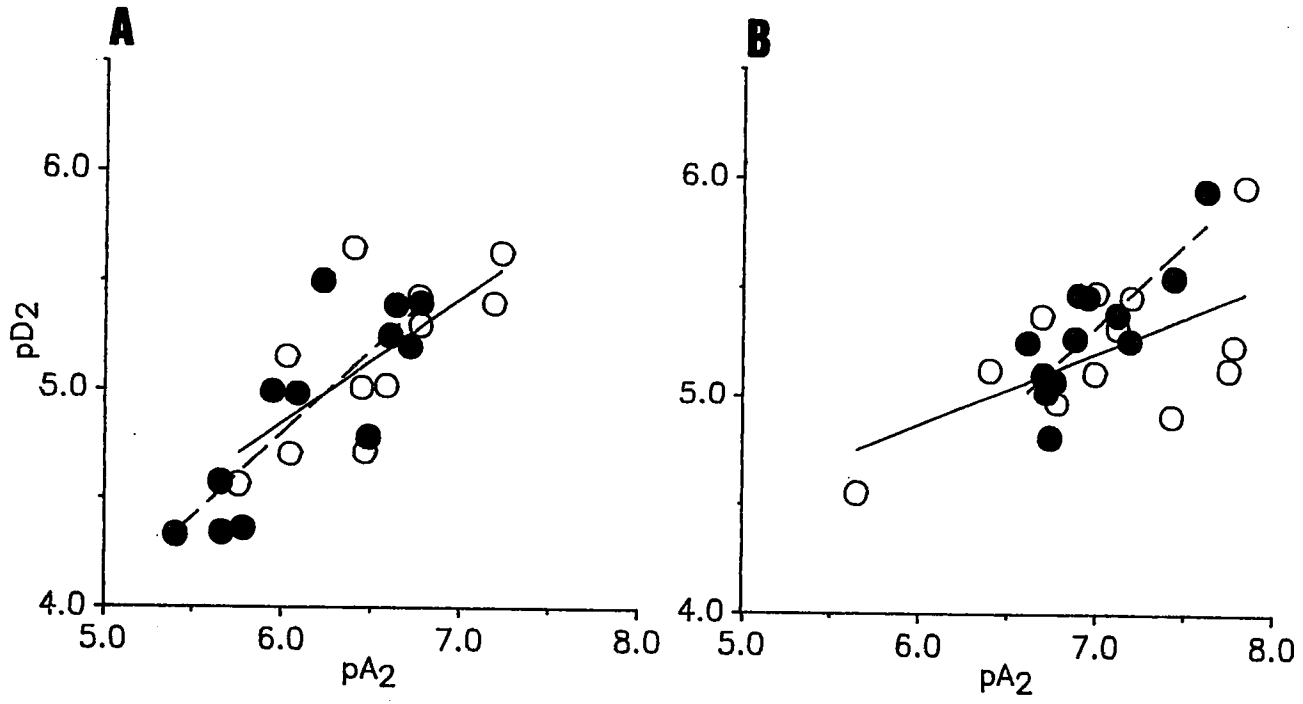

Fig. 2-2. Plots showing the correlation between norepinephrine pD_2 and yohimbine pA_2 values, with nerve stimulation (NS, open circles, broken lines) and field stimulation (FS, filled symbols, solid lines). A: 10-s stimulation for 1 ms at 2, 8, and 32 Hz. B: Trains of 64 0.5-ms pulses at 2, 8, and 32 Hz.

FS; a small reduction was noted when 64 pulses were delivered at 32 Hz. Yohimbine pA_2 against the inhibitory effects of NE on responses to stimulation correlated negatively with frequency for FS but not NS. Plotting NE pD_2 vs yohimbine pA_2 (Fig. 2a,b) at the 3 frequencies with both modes of stimulation yielded a straight line, ($p<0.05$).

Effects of chemical sympathectomy (Table 2). In response to NS, stimulation of the tissues for 10 s at 2 Hz or 64 pulses at 8 Hz, induced pressure increases similar to control in tracheas chemically sympathectomized in vitro but significantly greater in tracheas from animals chemically sympathectomized in vivo. All responses to FS were similar to control. In tissues treated with 6-hydroxydopamine in vitro, NE induced greater inhibition than in controls (Fig. 3) and NE pD_2 values were significantly greater, but yohimbine pA_2 was unchanged. In tracheas from animals treated with 6-hydroxydopamine in vivo, NE pD_2 and yohimbine pA_2 were similar to control. For both NS and FS, pD_2 and pA_2 values were not significantly different at the same frequencies.

Effects of desipramine, cocaine, and corticosterone (Table 3). Desipramine in concentrations of 0.5 and 1 uM depressed tracheal responses and therefore were not used. At a concentration of 0.1 uM it had no effect on control responses to NS and FS at the 3 frequencies and two modes of stimulation. Except with 2 Hz for 10 s, desipramine significantly increased NE pD_2 for both NS and FS, values correlating negatively with frequency of stimulation for NS but not FS. There were no significant differences among pD_2 values for NS and FS in the presence of desipramine. Yohimbine

Table 2-2. Effect of chemical sympathectomy with 6-hydroxydopamine (6HD) *in vivo* and *in vitro* on norepinephrine pD_2 and yohimbine pA_2 against nerve (NS) and field (FS) stimulation.

Stimulation Parameters	6HD	Stimulation	Norepinephrine pD_2	Yohimbine pA_2
2 Hz, 10 s	*in vivo*	NS	6.18±0.16	7.62±0.16
		FS	6.11±0.15	7.66±0.12
2 Hz, 10 s	*in vitro*	NS	6.27±0.11	7.50±0.13
		FS	6.12±0.08	7.61±0.12
8 Hz, 64 stimuli	*in vitro*	NS	5.49±0.04	7.67±0.07
		FS	5.61±0.04	7.64±0.06

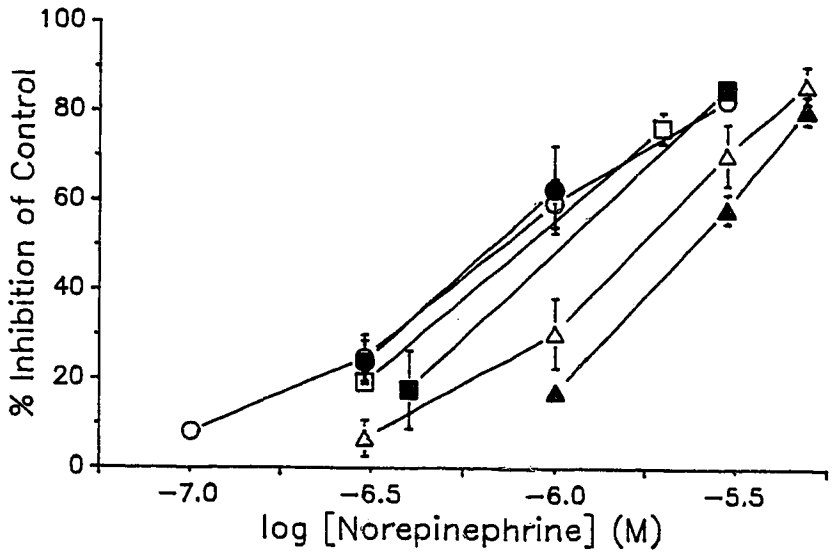

Fig. 2-3. The effects of 6-hydroxydopamine applied in vitro (100 μM; open circles) or in vivo (50 mg/kg, 24-h beforehand; closed circles), desipramine (0.1 μM; filled squares), cocaine (10 μM; open squares), and corticosterone (1 μM; filled triangles) on the inhibitory effects of norepinephrine (NE) on tracheal responses to field stimulation. 6-Hydroxydopamine treatments, desipramine, and cocaine significantly enhanced NE's potency compared with control (open triangles), whereas corticosterone slightly decreased it.

Table 2-3. Effects of cocaine (10 μM), corticosterone (1 μM), or desipramine (0.1 μM) on norepinephrine pD_2 and yohimbine pA_2 against nerve (NS) and field (FS) stimulation.

Treatment	Stimulation Parameter	Stimulation	Norepinephrine pD_2	Yohimbine pA_2
Cocaine (10 μM)	2 Hz, 10 s	NS	6.29±0.04	7.84±0.08
		FS	6.08±0.04	7.94±0.16
Corticosterone (1 μM)	2 Hz, 10 s	NS	5.62±0.07	7.28±0.04
		FS	5.61±0.03	7.40±0.06
Desipramine (0.1 μM)	2 Hz, 10 s	NS	6.02±0.10	7.93±0.18
		FS	5.97±0.06	8.00±0.18
	2 Hz, 64 stimuli	NS	6.36±0.02	
		FS	6.30±0.04	
	8 Hz, 64 stimuli	NS	5.96±0.06	
		FS	5.98±0.10	
	32 Hz, 64 stimuli	NS	5.85±0.09	
		FS	6.07±0.12	

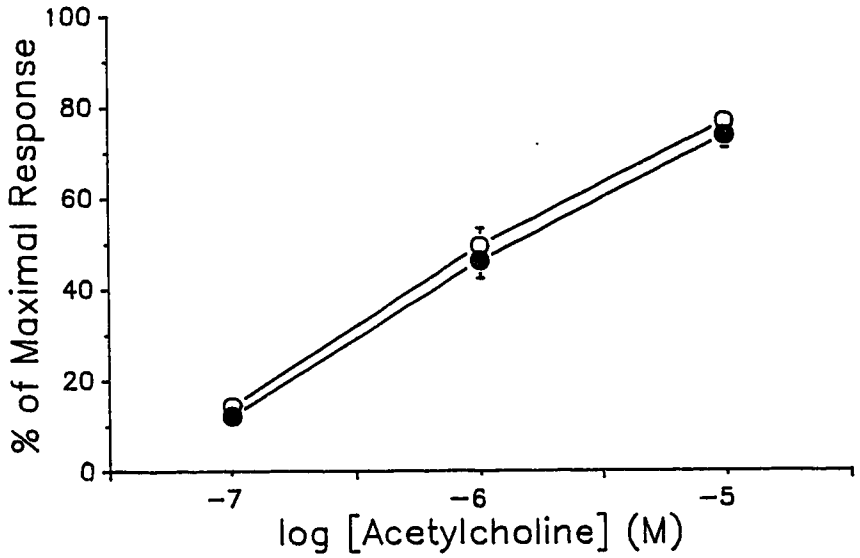

Fig. 2-4. Dose–response curves to acetylcholine without norepinephrine (open circles) and with it (NE; 3 μM). Desipramine (0.1 μM) was present in all experiments.

pA_2 values at 2 Hz for 10s were similar for NS and FS; with FS, values were significantly greater than in controls. Cocaine (10 μM) increased responses to NS but not to FS; at 50 uM it depressed responses to both; 10 uM significantly increased NE's inhibitory effects (Fig. 3) and yohimbine pA_2. As corticosterone was insoluble in concentrations >1 μM, only this was used. Corticosterone increased responses to NS without affecting those to FS, but it had no effect on NE pD_2 and yohimbine pA_2 values at 2 Hz (the only frequency tested).

Effect of NE on responses to exogenous acetylcholine (Fig. 4). NE (1–100 μM) induced increases in intratracheal pressure not exceeding 2 cm H_2O; these were abolished by prazosin (0.3 μM). NE (3 μM) had no effect on the dose–response curve to acetylcholine (0.1–10 μM).

Effects of xylazine and clonidine (Table 4). With 10-s stimulation at 2 Hz, xylazine and clonidine pD_2 values were similar and were significantly greater than for NE. pA_2 values for yohimbine against xylazine were greater than against clonidine, and both were greater than corresponding values against NE for FS and NS. Variance was large and differences did not attain significance.

Schild plot slopes (Table 5). All values for the slopes were ≤1.0, even when values were measured in the presence of U1 and U2 blockers or in tracheas chemically sympathectomized with 6-hydroxydopamine.

Table 2-4. Clonidine and xylazine pD_2 and yohimbine pA_2 against nerve (NS) and field (FS) stimulation.

Stimulation Parameter	Agonist	Stimulation	Agonist pD_2	Yohimbine pA_2
2 Hz, 10 s	Clonidine	NS	6.75±0.16	7.43±0.24
		FS	6.53±0.17	7.11±0.15
	Xylazine	NS	6.38±0.09	7.95±0.18
		FS	6.32±0.10	7.81±0.12

Table 2-5. Schild plot slopes

Treatment	Stimulation parameter	Stimulation	Schild slope
-	2 Hz, 10 s	NS	0.85±0.14
	1 ms	FS	1.07±0.10
-	8 Hz, 10 s	NS	0.72±0.08
	1 ms	FS	0.82±0.09
-	32 Hz,10 s	NS	0.58±0.05
	1 ms	FS	1.19±0.26
-	2 Hz, 64 stimuli	NS	0.60±0.03
		FS	0.60±0.02
-	8 Hz, 64 stimuli	NS	0.47±0.04
		FS	0.57±0.01
-	32 Hz,64 stimuli	NS	0.55±0.08
		FS	0.62±0.06
-	2 Hz, 10 s	NS	0.72±0.06*
		FS	0.85±0.04*
Cocaine (10 μM)	2 Hz, 10 s	NS	0.74±0.04
		FS	0.69±0.06
Desipramine (0.1 μM)	2 Hz, 10 s	NS	0.61±0.04
		FS	0.59±0.03
Cortico-sterone (0.1 μM)	2 Hz, 10 s	NS	0.57±0.10
		FS	0.52±0.02
6-Hydroxy-dopamine (bath)	2 Hz, 10 s	NS	0.81±0.05
		FS	0.69±0.03
	8 Hz, 64 stim.	NS	0.55±0.00
		FS	0.64±0.01
6-Hydroxy-dopamine (ip)	2 Hz, 10 s	NS	0.67±0.05
		FS	0.67±0.05

Agonist: norepinephrine except for * (Agonist: xylazine)
Pulse duration is 0.5 ms unless otherwise specified.

2.4. Discussion

The guinea-pig tracheal tube is one of the few airway preparations in which it is possible to stimulate parasympathetic nerves pre- or post-ganglionically. The guinea-pig trachea contains parasympathetic ganglia (Baluk et al., 1985), and hexamethonium (100 μM), which blocks ganglionic transmission, abolished responses to stimulation of the vagal nerves but did not affect responses to FS. Atropine (0.5 μM) abolished pressure increases induced by both. These findings, which confirm others' (Bloom et al., 1988; Faulkner et al., 1986; Skoogh et al., 1982), show that mediation of responses to NS is via ganglia and to FS is via post-ganglionic parasympathetic nerves.

The ease of differentiation of responses to NS and FS with selective antagonists is surprising in view of their complexity: both NS and FS stimulate many types of nerves - parasympathetic, sympathetic (Blackman & McCaig, 1983; Kalenberg & Satchell, 1979; Yip et al., 1981), nonadrenergic noncholinergic (Kalenberg & Satchell, 1979, Yip et al., 1981), and afferent nerve fibers (Grundström, 1986; Trenchard, 1983). The results of our experiments reveal the relative contributions by some of these nerve types, and the receptors they activate, to the responses. Addition of the non-specific β-blocker propranolol precluded participation by β-receptors in the responses to NS or FS, and the indomethacin (10 μM) in the bathing solution excluded any neuromuscular effects of prostaglandins (Hardy et al., 1984; Ito & Tajima, 1981; Walters et al., 1984). Pressure increases in response to exogenous NE mediated via α_1-adrenoceptors were small (<2 cm H_2O), and any

contribution from the pressor effects of neurally released NE to the responses to NS and FS was insignificant. Inhibitory, sympathetically mediated α_2-adrenoceptor effects on parasympathetic responses may have occurred as Jones et al. (Jones et al., 1980) reported close proximity of both sympathetic and parasympathetic nerves in guinea-pig airways; however, Baluk et al. (1985) saw no sympathetic endings in guinea-pig tracheal ganglia. As in Grundström et al.'s (Grundström et al., 1981b) experiments, the α_2-adrenoceptor blocker yohimbine did not increase pressor effects of NS or FS; also, the uptake 1 blockers desipramine (0.1 μM) and cocaine (10 μM) did not reduce responses to NS or FS, which were similar in normal tracheas and in those chemically sympathectomized *in vivo* or *in vitro* with 6-hydroxydopamine. These findings, coupled with the inhibitory effects of exogenous NE on transmitter release from adrenergic nerves (Langer, 1981), indicate that any actions of endogenous NE mediated via α_2-adrenoceptors on postganglionic parasympathetic nerves - even if they exist - cannot be distinguished in this preparation using these methods of stimulation. In control tissues, the sympathetic nervous system may slightly inhibit cholinergic transmission via α_2-adrenoceptors; this would be minimized by high concentrations of exogenous NE, which reduce the release of NE from sympathetic nerves and thus diminish or even eliminate neural release of NE. Theoretically, inhibition by low concentrations of NE, clonidine, and xylazine would be slightly increased.

Nonadrenergic noncholinergic (NANC) responses in this tracheal preparation are small (Blackman & McCaig, 1983; Zaagsma et al.,

1987). As indomethacin was present in all our experiments, our preparations had low inherent tone and atropine had minimal relaxant effects (<1 cm H_2O); this absence of tone would hamper detection of relaxant responses (Venugopalan et al., 1986). Moreover, atropine abolished pressure increases in response to NS, suggesting that antidromic stimulation of vagal afferent nerves to the trachea and orthodromic stimulation of vagal sympathetic efferents (Smith & Satchell, 1985) have no (detectable) effect on intratracheal pressure. Furthermore, under these experimental conditions, exogenous NE should reduce any NANC excitation via its actions on α_2-adrenoceptors (Grundström et al., 1985). When atropine was present, FS-induced NANC responses were small (<2 cm H_2O) delayed decreases in intratracheal pressure and their slow onset precluded any effect on the more rapid cholinergic pressure increases.

Co-transmission in the parasympathetic and sympathetic systems (Blackman & McCaig, 1983; Smith & Satchell, 1985) suggests that the release of multiple transmitters by single nerve impulses may confound the analyses. Nevertheless, any contribution from other (co)transmitters was small and could not have contributed significantly to the responses to NS and FS. As the responses to NS or FS represent the net sum of contractile and relaxant effects, there is no way of knowing whether they are summed arithmetically or via more complex functions; thus, although the analysis that follows is broadly correct, other possibilities cannot be eliminated.

Exogenous NE was equally effective at blocking NS and FS; i.e., pD_2 values for NE against NS and FS were not significantly different at any of the frequencies tested, with either method of

stimulation. NE pD_2 was inversely dependent upon frequency - all values were significantly greater at low frequencies - and results were similar for yohimbine pA_2. These findings were independent of the size of the responses - after 10-s stimulation the responses were invariably greater at 32 Hz than at 8 or 2 Hz, and vice versa for trains of 64 stimuli, yet the pD_2 or pA_2 values were similar. Also, onset time for maximal effect of NE was similar at all frequencies; despite differences between the numbers of pulses applied by 10-s stimulation and trains. The pD_2 values in normal tracheas in the absence of uptake 1 and 2 blockers were lower than those reported by Grundström (1986), 5.3 [interpolated for frequency] vs 6.7; similarly, yohimbine pA_2 values measured with uptake blockers absent were an order of magnitude lower (6.0-6.5 [interpolated for frequency] vs 7.8) than those obtained by Grundström et al. (Grundström et al., 1981a) with blockers present. Nevertheless, α_2-adrenoceptors mediated NE's inhibitory effects on the NS- and FS-induced pressure increases. NE pD_2 was similar for NS and FS in all our experiments. This is contrary to the results of experiments with different preparations by others (Bloom et al., 1988; Brown & Quilliam, 1964; Skoogh, 1983; Skoogh et al., 1982) who have used them to support a theory of selectivity at ganglia. With our preparation, the findings indicate that α_2-adrenoceptors modulate cholinergic neurotransmission only via presynaptic receptors on post-ganglionic neurons; we excluded any direct effect of NE on tracheal smooth muscle because dose-response curves to exogenous acetylcholine were similar with and without NE.

Theoretical problems that can confound measurements of pD_2 and

pA_2 arise when uptake mechanisms for the agonist are present (see reviews by Kenakin [1982, 1985]), reducing the absolute pD_2 and pA_2 values and resulting in Schild plot slopes <1.0. All of our values for slopes were ≤1.0, even when uptake 1 and 2 blockers were present or the tracheas had been chemically sympathectomized with 6-hydroxydopamine, suggesting that neither uptake 1 nor uptake 2 was fully blocked by the sympathectomy or by desipramine, cocaine, or corticosterone in the concentrations used. Metabolic degradation of NE by catechol-O-methyltransferase and monoamine oxidase could be another contributing factor for Schild plot slope ≤ 1. Also, the pD_2 and pA_2 values cannot be 'true', as the theoretical basis for the measurements cannot be applied - although inhibition of responses to NS and FS measured simultaneously can be used to compare a drug's effects qualitatively. Interestingly, pD_2 correlated with pA_2, indicating a similar degree of effect by intact uptake mechanisms. Although xylazine and clonidine were more potent than NE and should not be subject to uptake, neither gave Schild plot slopes of unity; both may act, in part, indirectly via NE release. It is possible that 'breakthrough' of β-blockade could occur at high concentrations of NE and that this resulted in slopes <1.0, but the findings with xylazine and clonidine indicate that this was not a factor.

The similarity of NE pD_2 against FS at 2 Hz, with cocaine or desipramine present, or after treatment with 6-hydroxydopamine strongly suggests that NE release from sympathetic nerves does not contribute to the effect mediated by α_2-adrenoceptors, but a different mechanism is required to explain larger pD_2s in tissues

stimulated at 8 Hz with desipramine present than after 6-hydroxydopamine treatment. McCaig (1987) noted that stimulating the sympathetic nerves to the trachea reduced its cholinergic responses. However, the demonstration of sympathetic-nerve-mediated inhibition of parasympathetic responses requires stimulation of these nerves for much longer and at higher frequencies than we used (Blackman & McCaig, 1983; McCaig, 1987).

In regard to NE uptake, Grundström et al.'s (1981a) original experiments were performed on tracheas from reserpine-treated animals with cocaine and corticosterone present to inhibit uptake 1 and 2 respectively. They claimed validation of their pA_2 values and uptake inhibition from the slopes of their Schild plots even though the differences between the ranges of the slopes they reported were 0.74–1.18. In our experiments, both desipramine and cocaine increased NE pD_2 values 7- to 10-fold, indicating significant neural uptake of NE by uptake 1 (Zaagsma et al., 1987) and thus indicating, indirectly, significant numbers of sympathetic nerves. Experiments on tracheas from animals treated with 6-hydroxydopamine <u>in vivo</u> and <u>in vitro</u> (Stretton & Barnes, 1988) gave pD_2 values similar to those with cocaine or desipramine present, confirming a significant degree of uptake 1 and the presence of sympathetic nerves. Uptake 2 appears to play a lesser role in this tissue as NE pD_2 values with NS and FS at 2 Hz with corticosterone present were similar to those in normal tracheas. Our results may also have been influenced by the concentrations of corticosterone (Grundström et al., 1981a) and propylene glycol (Black et al., 1984; Starke et al., 1975). It was difficult to

dissolve corticosterone in Krebs-Henseleit solution and we used one tenth the concentration used by Grundström et al. (10 uM, [Grundström et al., 1981a]), and the propylene glycol in the Krebs' solution was 4.1 µM compared with 7.1 µM used by them.

Our findings with NS and FS may not be strictly comparable. Even if of similar size, as in our study, pressure responses to the different forms of stimulation may be mediated via different nerve fibers or patterns of neurotransmitter release (Coburn, 1987) and parasympathetic ganglia probably 'filter' responses through them (Barnes, 1988; Coburn, 1987; Skoogh, 1986). Normally, a response arriving via a preganglionic neuron at a ganglionic synapse is amplified through the contact of axonal terminals with dendrites on more than one postganglionic neuron, perhaps rendering ganglia susceptible to modulation by transmitters and drugs. Skoogh (1986) reported preliminary findings that α-adrenoceptors modulated cholinergic neurotransmission in ferret paratracheal ganglia, whereas we found no evidence of such an effect in the guinea-pig preparation. If transmission occurs through similar (but not identical) pathways, with inhibition at both ganglionic and postganglionic presynaptic sites, one would expect the arithmetic sum of the inhibition at both sites (Ariens & Simonis, 1964) and greater inhibition of responses to NS than FS. But this was not so; therefore, it is unlikely that α_2-adrenoceptors have significant modulatory effects at ganglia in this preparation. This conclusion concurs with the failure to observe adrenergic terminals in morphologic studies of guinea-pig tracheal ganglia (Baluk et al., 1985), but it does not unequivocally exclude the possibility that

they do exist and exert an effect on ganglionic transmission too small to be detected under these experimental conditions.

De Groat (1967) reported modulation by β-adrenoceptors in studies of sympathetic ganglia, and Suzuki & Volle (1979) stated that NE (10^{-4} M) either depolarized or hyperpolarized cells in hamster submandibular ganglia - effects blocked by dihydroergotamine a non-specific α-adrenoceptor antagonist. Suzuki and Volle (1979) concluded that α-adrenoceptors were present on the ganglion cells and that some might be α_2-adrenoceptors, possibly those mediating hyperpolarization. In studies of ferret paratracheal ganglia, Coburn (1987) and Baker and others (1983) reported evidence of modulatory α-receptors but only at high NE concentrations (10 μM). Therefore, modulation of cholinergic neurotransmission in our preparation via actions on α_2-adrenoceptors at ganglia is not a significant factor at the stimulation parameters and concentrations of NE used in this study.

Although the amount of acetylcholine released per impulse is greater at low than at high frequencies of stimulation, greater additive effects were expected from responses invading the nerve terminal at higher frequencies of stimulation. As this was not the case, it seems likely that the increased resistance of cholinergic transmission to the inhibitory effects of α_2-adrenoceptor stimulation at higher frequencies of stimulation could result from the increased summation of nerve-terminal responses.

It is not known how α_2-adrenoceptors mediate these inhibitory responses. In other tissues the effects are mediated through a receptor/G-protein coupled action on potassium channels resulting in

efflux of potassium, and hyperpolarization of the membrane (Aghajanian & Wang, 1986; Lujan et al., 1984; Tucker, 1984), a mechanism unlikely in this preparation in view of the slow onset and recovery of the response. Actions involving cAMP and cGMP are also possible but were not studied.

α_1-adrenoceptor-mediated contractions of tracheal smooth muscle have been observed in vivo and in vitro (see Goldie et al., 1985) but these responses were noticeably absent or very small in our preparations. The small increases in intratracheal pressure induced by NE (5–100 μM) were abolished by prazosin (0.3 μM), indicating mediation via α_1-adrenoceptors. Because of the smallness of these responses compared with the responses to NS and FS, we did not include prazosin in the bathing solution, which already contained propranolol and indomethacin.

In summary, our findings indicate that, in guinea-pig trachea, α-adrenoceptor agonists inhibit cholinergic transmission via α_2-adrenoceptors located on postganglionic parasympathetic nerves, and α_2-adrenoceptors do not modulate cholinergic transmission through parasympathetic ganglia.

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3. ALPHA-ADRENOCEPTOR FUNCTION IN GUINEA-PIG MODELS OF ASTHMA*

3.1. Introduction

Both α_1- (Simonsson et al., 1972; Kneussl & Richardson, 1978) and α_2- (Grundström et al., 1981; Grundström & Andersson, 1985) adrenoceptors have been described in the airways of humans and animals. However, their precise role in asthma remains controversial (Barnes, 1988; Zaagsma et al., 1987; Goldie et al., 1985; Andersson et al., 1986a). Reviewing neural contribution to airway hyperresponsiveness in asthma, Casale (1988) has recently proposed that an increase in α_1-adrenoceptors and a decrease in inhibitory prejunctional α_2-adrenoceptors are contributing factors. α_1-Adrenoceptors are greatly increased in the lung homogenates from guinea-pig models of asthma (Barnes et al., 1980; Mita et al., 1983). Nevertheless, α-adrenoceptor-mediated contraction of the lung strips is unchanged compared with lung strips from control animals (Turner et al., 1983). α_2-Adrenoceptors inhibit cholinergic transmission in guinea-pig trachea and human bronchi on postganglionic nerves (Grundström & Andersson, 1985; Grundström et al., 1981) but not at parasympathetic ganglia (Biggs et al., 1988). The selective α_2-agonist clonidine inhibits bronchospasm induced by antigen challenge in sensitized guinea-pigs (Andersson et al., 1986b) and in asthmatic patients (Lindgren et al., 1986), probably

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in part via its effect on α_2-adrenoceptors on cholinergic efferent pathway. However, nothing was known as to whether modulatory role of α_2-adrenoceptors is altered in asthma. To establish whether α-adrenoceptor function is altered in asthma, we assessed pressor responses to norepinephrine (NE) and compared the inhibitory effects of NE on pre- and post-ganglionic nerve stimulation in tracheal tube preparations (Blackman & McCaig, 1983; Widmark & Waldeck, 1986; Biggs et al., 1988) obtained from control, sensitized, and hypersensitized guinea pigs.

3.2. Methods

Preparation of Animals. Three groups of female Hartley-strain (350-550 g) guinea pigs were sensitized or hypersensitized according to the methods of Mita et al. (1983) or Biggs & Ladenius (1989). Briefly, Group 1 (sensitized) were given ovalbumin (OA) intraperitoneally (5 mg on day 1, and 10 mg on day 4) and maintained for 21 days before use. Group 2 (asthma model A) inhaled OA-aerosols (2% in 0.9% saline, Vix Acorn nebulizer, compressed air @ 10 psi) in a Plexiglass chamber for up to 8 min on 10 consecutive days. Group 3 (asthma model B) were sensitized by intraperitoneal injection of OA (20 mg/kg), and 21 days later breathed an OA aerosol for up to 8 min daily for 10 consecutive days. A similar group of animals which inhaled saline (0.9%) aerosols for 8 min on 10 consecutive days served as controls for Groups 1, 2, and 3. All the animals in Group 2 developed asthma-like signs such as panting, cyanosis, respiratory distress after 8 days of exposure and 1/10 animals died. All of the animals in Group 3 developed these signs upon the first exposure,

and showed decreasing signs with each day of exposure. All the animals in asthma model A and B recovered from these signs within 30 min after exposure spontaneously or with the help of adrenaline (1 mg/kg, subcutaneously). The guinea pigs were used in experiments within one week after 10 days of exposure.

Tissue Preparation. The tracheal tube preparation, with its vagi and recurrent laryngeal nerves intact on both sides, was dissected out and mounted in an organ bath containing oxygenated Krebs' solution at 37°C to record intraluminal pressure, as previously described in detail (see chapter 2). The bathing solution contained propranolol (5 μM) and indomethacin (10 μM) throughout the experiment.

Both vagi were drawn into a single shielded platinum tunnel electrode for nerve stimulation (NS) with rectangular pulses (0.5 ms; 50 V) applied via a Phipps & Bird 611 stimulator. For transmural electrical field stimulation (FS), bipolar rectangular pulses (0.5 ms; 80 V) were applied from a Grass SD9 stimulator via two parallel platinum wire electrodes, one of which was placed inside and the other outside the trachea.

Experimental Protocols. For measuring pD_2 for NE against NS and FS, three sets of 4 tissues from Groups 1, 2, and control were stimulated for 10 s at 2, 8, 32 Hz, and another three sets of 4 tissues from Groups 2, 3, and control stimulated for trains of 64 pulses at the same frequencies. In the experiments with trains of 64 pulses, prazosin (0.3 μM) and desipramine (0.1 μM) were present in the bath throughout the experiment. Tracheal responses to NE and

phenylephrine, and tracheal responses to exogenous acetylcholine in the absence and presence of NE were also determined in these tissues.

After tissues were equilibrated for 1 h, they were stimulated alternately via NS and FS (2 Hz, 10 s) for 0.5 h or until responses were constant. Then cumulative dose-response curves to NE and phenylephrine were obtained. After washing with Krebs' solution and reestablishing responses to NS and FS over 0.5 h at stimulation parameters to be used, pD_2 values for NE's inhibition of tracheal responses to NS and FS were determined non-cumulatively. Following these experiments, tissues were washed repeatedly for 0.5 h with Krebs' solution, and then cumulative dose-response curves to acetylcholine were constructed and 30 min after washing with Krebs' solution repeated in the presence of NE (10^{-6} or 3×10^{-6} M). Dose-response curves to ACh were reproducible.

Statistical analyses. The pD_2 values for NE in inhibiting cholinergic transmission were calculated as the negative logarithm of the molar concentration of NE inhibiting control responses by 50% (ED_{50}). All data points were taken 15-18 min after adding NE to the bath, as described previously (see chapter 2). Similarly, pD_2 values for ACh were calculated as the negative logarithm of the molar concentration of ACh inducing 50% of maximum responses to ACh (10^{-3} M). Data were expressed as mean $\pm$ SEM. At least 4 replicates were obtained in each series of experiments and significance was assumed at the 5% level. Mean values were compared by 3-way analysis of variance, with repeated measures for subjects measured more than

once, using SPSS-X and UANOVA (Taerum, 1989). Scheffe's test was used to rank differences. Individual comparisons were made with unpaired Student's *t* tests. Least-squares regression analysis was performed with unweighted values, using STATISTIX (version 1.0).

Drugs. The following drugs were used: propranolol hydrochloride, indomethacin, desipramine hydrochloride, albumin (chicken egg, ovalbumin, Grade V) (Sigma, St.Louis, MO); L-noradrenaline-D-bitartrate (Fluka, Ronkonkoma, NY); L-ascorbic acid (BDH Chemicals, Toronto, Canada); prazosin hydrochloride (gift of Pfizer, Kirkland, Quebec); and pentobarbital sodium injection (Euthanyl, MTC Pharmaceuticals, Mississauga, Canada). Most drugs were dissolved in distilled water; indomethacin was dissolved in 0.2% sodium carbonate, and prazosin was dissolved in a mixture of glycerine/5% dextrose solution (1:1).

3.3. Results

α_1-Adrenoceptor-Mediated Pressure Responses. The effects of norepinephrine (0.1 - 30 μM) and phenylephrine (0.1 - 30 μM) were compared among tracheas from Groups 1, 2, and 3 (Fig. 1). In tracheas from all Groups, NE induced small (<4 cm H_2O) increases in intratracheal pressure which were dose-dependent within a preparation but varied greatly among tissues. Pressure increases to NE were similar in tracheas from control and all three sensitized groups. Prazosin (0.3 μM) abolished pressure increases induced by NE in all tissues. In Groups 2 and 3, phenylephrine was approximately as potent as NE in inducing increases in pressure, and followed a

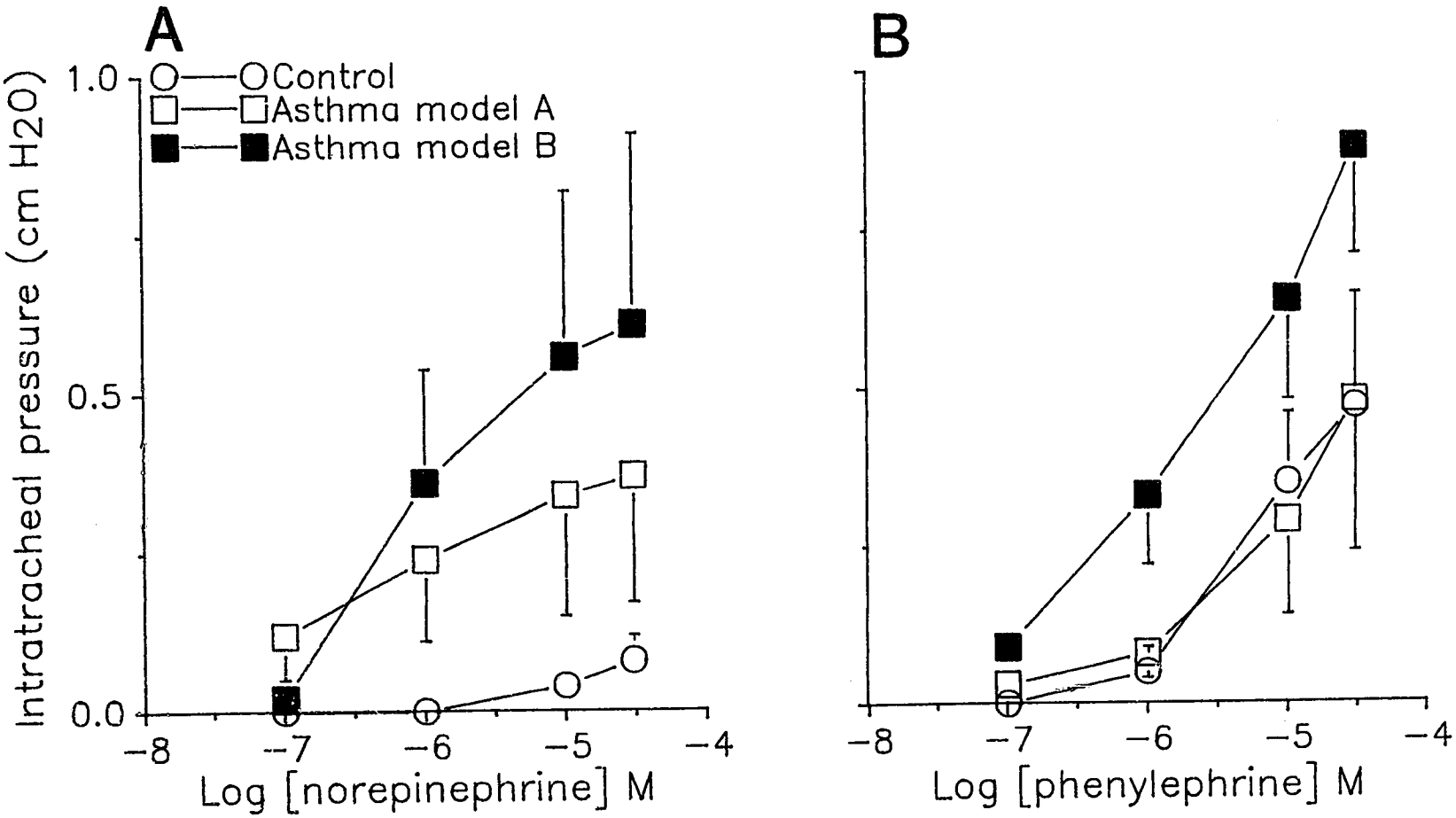

Fig. 3-1. Pressor effects of norepinephrine (A) and phenylephrine (B) on tracheas from controls and asthma model A and B.

similar pattern; pressure increases were similar to control.

α_2-Adrenoceptor-Mediated Inhibition of Cholinergic Transmission (Table 1). In control tissues, with stimulation for 10 s at 2, 8, and 32 Hz, the NE pD_2 values for NS and FS correlated negatively with frequency; i.e., less NE was required at low frequencies to inhibit pressure increases. However, with trains of 64 stimuli, the NE pD_2 values correlated negatively with frequency for NS but not FS.

Sensitization did not significantly alter the NE pD_2 for both NS and FS, values correlating negatively with frequency for both NS and FS with 10 s stimulation (Fig. 2). In tracheas from hypersensitized animals (asthma model A), the NE pD_2 values were not significantly different from controls with both modes of stimulation; values for both NS and FS correlated negatively with frequency for 10 s stimulation but not for trains of 64 pulses (Fig. 2, 3). Stimulation of the tissues for trains of 64 pulses at all frequencies produced similar NE pD_2 values for NS and FS in tracheas from control and hypersensitized (asthma model B) animals; values for tracheas from this asthma model correlated negatively with frequency for both NS and FS (Fig. 3). For both NS and FS, the NE pD_2 values were not significantly different at the same frequencies in tracheas from all groups.

Responses to Nerve and Field Stimulation (Table 2). In tracheas from sensitized animals, responses to NS and FS for 10 s at 2, 8, and 32 Hz were similar to control (Fig. 4). Comparison of pressure

Table 3-1. Norepinephrine pD_2 against nerve (NS) and field stimulation (FS) for trains of 64 pulses or for 10 s at 2, 8, and 32 Hz in tracheas from control, sensitized, and hypersensitized guinea pigs.

		64 pulses			10 s stim		
		Frequency (Hz)			Frequency (Hz)		
Treatment	Type	2	8	32	2	8	32
Control	NS	6.36±0.02	5.96±0.06	5.85±0.09	5.87±0.07	5.52±0.07	5.15±0.16
	FS	6.30±0.04	5.98±0.10	6.07±0.12	5.78±0.10	5.51±0.09	5.07±0.08
Model A	NS	6.34±0.08	6.14±0.07	6.00±0.15	5.62±0.08	5.27±0.10	4.96±0.13
	FS	6.35±0.03	6.13±0.05	6.27±0.12	5.58±0.10	5.33±0.09	5.15±0.09
Model B	NS	6.27±0.09	5.85±0.03	5.70±0.08	-	-	-
	FS	6.25±0.04	5.98±0.06	5.58±0.06	-	-	-
Sensitized	NS	-	-	-	5.66±0.07	5.29±0.09	4.87±0.14
	FS	-	-	-	5.71±0.09	5.37±0.07	5.21 0.13

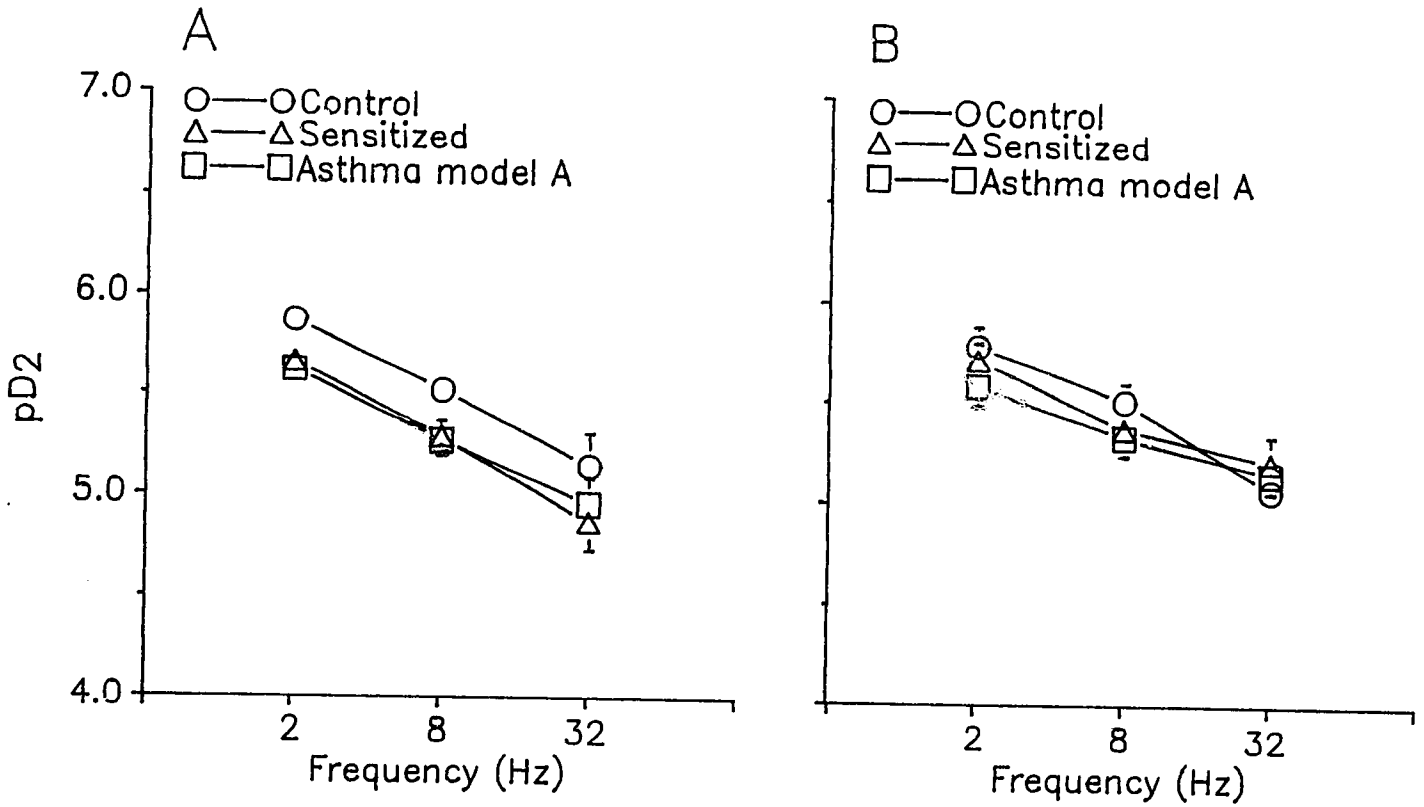

Fig. 3-2. NE pD_2 against NS (A) and FS (B) at 2, 8, and 32 Hz for 10 s in tracheas from control, sensitized, and hypersensitized (asthma model A)

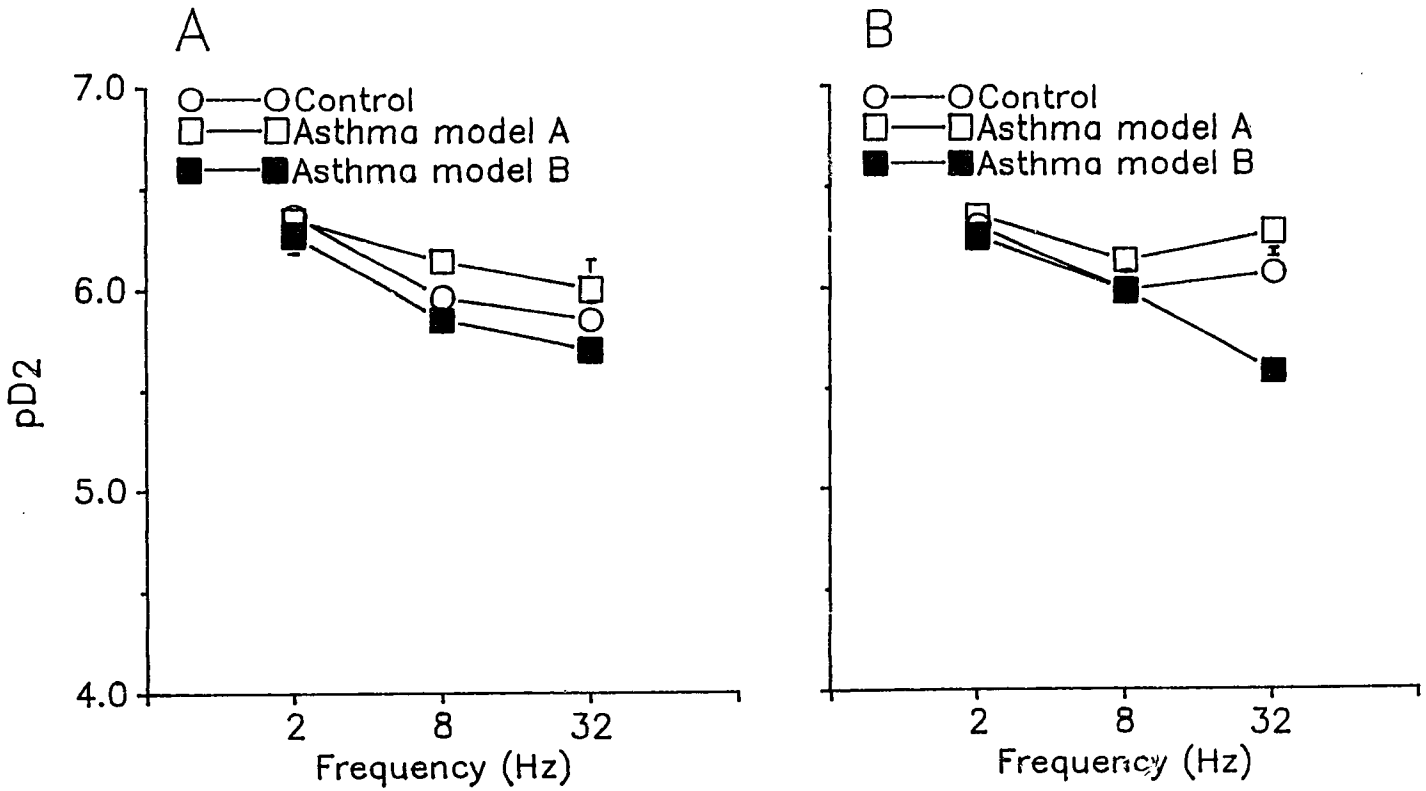

Fig. 3-3. NE pD_2 against NS (A) and FS (B) at 2, 8, and 32 Hz for trains of 64 pulses in tracheas from control, sensitized, and hypersensitized (asthma model A and B). Desipramine (0.1 uM) were present throughout the experiment.

Table 3-2. Pressure responses (cmH_2O) to nerve (NS) and field stimulation (FS) for trains of 64 pulses or for 10 s at 2, 8, and 32 Hz in tracheas from control, sensitized, and hypersensitized guinea pigs.

		64 pulses			10 s stim		
		Frequency (Hz)			Frequency (Hz)		
Treatment	Type	2	8	32	2	8	32
Control	NS	42.6±1.0	40.4±1.5	30.2±0.9	22.0±1.1	37.4±1.2	40.8±1.1
	FS	43.3±1.0	39.8±2.1	29.5±1.7	30.2±2.1	42.2±1.4	49.5±2.1
Model A	NS	41.8±2.9	36.8±2.2	27.5±3.2	29.6±1.1	38.2±1.9	40.3±1.8
	FS	41.9±1.1	36.8±1.7	24.6±3.0	32.3±1.1	39.0±1.7	42.9±1.6
Model B	NS	39.4±1.2	36.3±0.5	30.0±0.9	-	-	-
	FS	37.6±1.3	34.5±2.0	30.8±1.3	-	-	-
Sensitized	NS	-	-	-	32.4±1.0	39.9±1.3	43.8±0.9
	FS	-	-	-	33.3±1.3	39.7±1.3	44.0±1.8

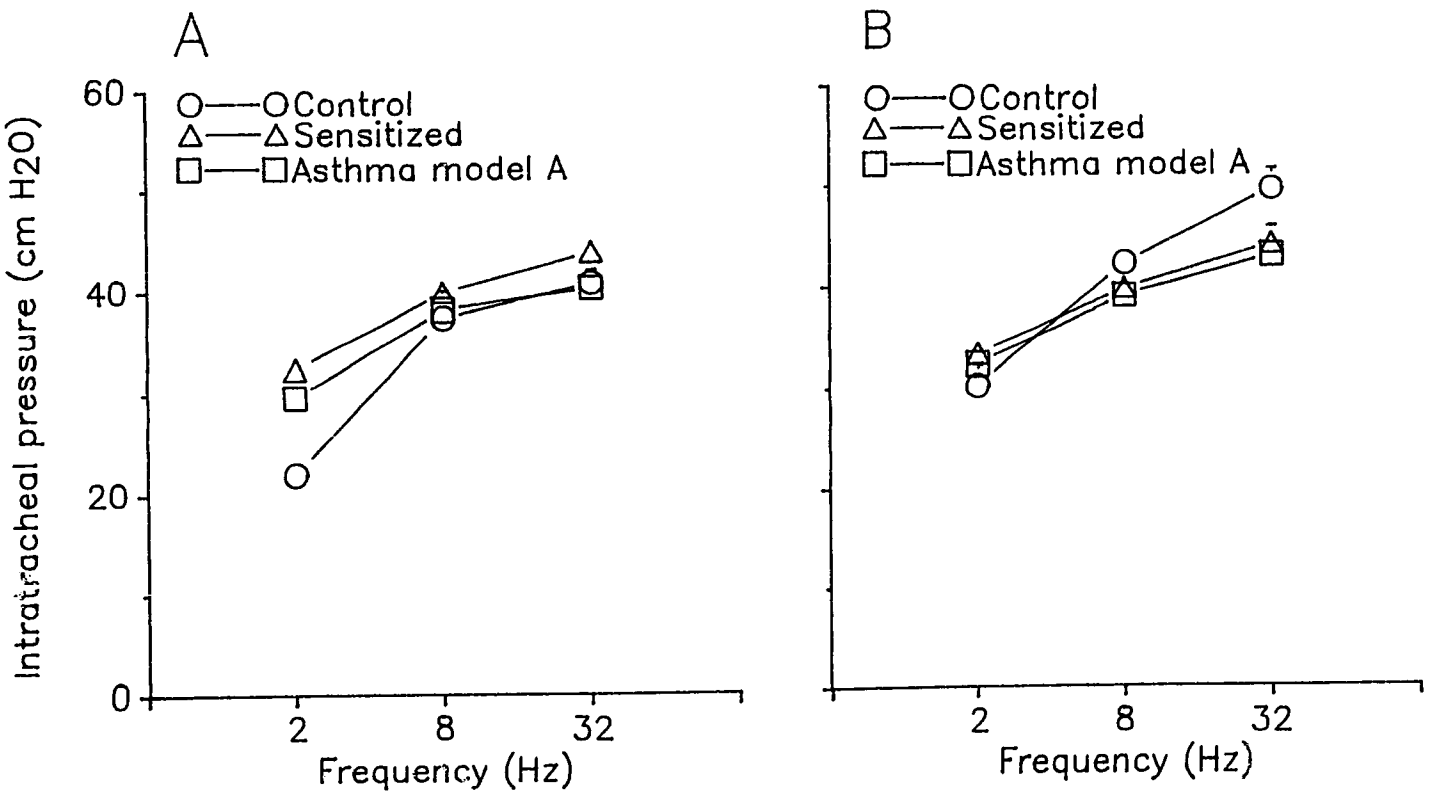

Fig. 3-4. **Pressor responses to NS (A) and FS (B) at 2, 8, and 32 Hz for 10 s in tracheas from control, sensitized, and hypersensitized (asthma model A).**

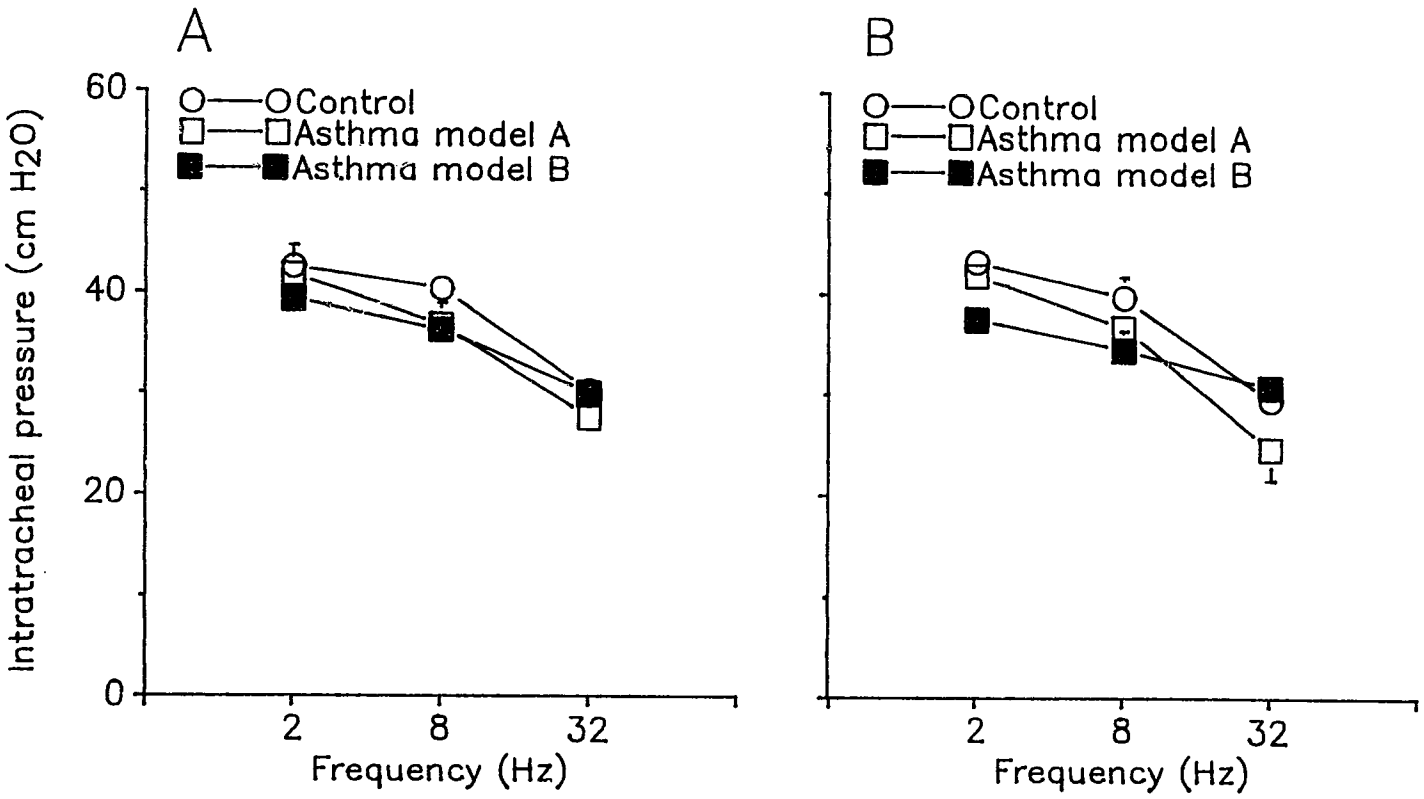

Fig. 3-5. Pressor responses to NS (A) and FS (B) at 2, 8, and 32 Hz for trains of 64 pulses in tracheas from control, sensitized, and hypersensitized (asthma model A and B). Desipramine (0.1 uM) were present throughout the experiment.

increases to NS and FS, with stimulation for 10 s or trains of 64 pulses, at all frequencies, revealed no significant differences in the size of responses in tracheas from control and hypersensitized (asthma model A) animals (Fig. 4, 5). In tissues from hypersensitized animals (asthma model B), responses to NS and FS for trains of 64 pulses at all frequencies were also similar to control (Fig. 5).

In tracheas from all groups, the size of responses to both NS and FS at all frequencies correlated positively with frequency for 10 s stimulation, but negatively for trains of 64 pulses; responses to NS and FS were not significantly different at the same frequencies with both modes of stimulation.

Responses to Exogenous Acetylcholine. Desipramine (0.1 μM) did not alter responses to acetylcholine (ACh) in tracheas from control and asthma model A. In the presence of desipramine (0.1 μM), ACh induced dose-dependent increases in pressure in tracheas from control and two asthma models A and B. In tracheas from asthma model A, pressure responses to ACh were similar to control. However, pressure responses to ACh were significantly smaller in asthma model B than in tracheas from control and asthma model A as demonstrated by a decrease in the mean pD_2 value for ACh (Table 3). There was no significant difference in maximum pressure responses to acetylcholine (10^{-3} M) among tracheas from these three groups (Table 3). NE (1 or 3 μM) did not alter dose-response curves to added acetylcholine in tracheas from all these groups (Fig. 6).

Table 3-3. Maximal pressor responses to and pD_2 values for acetylcholine in tracheas from control and two asthma models.

Treatment	Control	Model A	Model B
Maximum pressure (cmH_2O)	69.7±2.8	79.6±4.9	72.6±3.0
Mean pD_2	5.92±0.10	5.71±0.08	5.29±0.07*
n	4	4	3

* Significantly different from control ($p<0.01$)

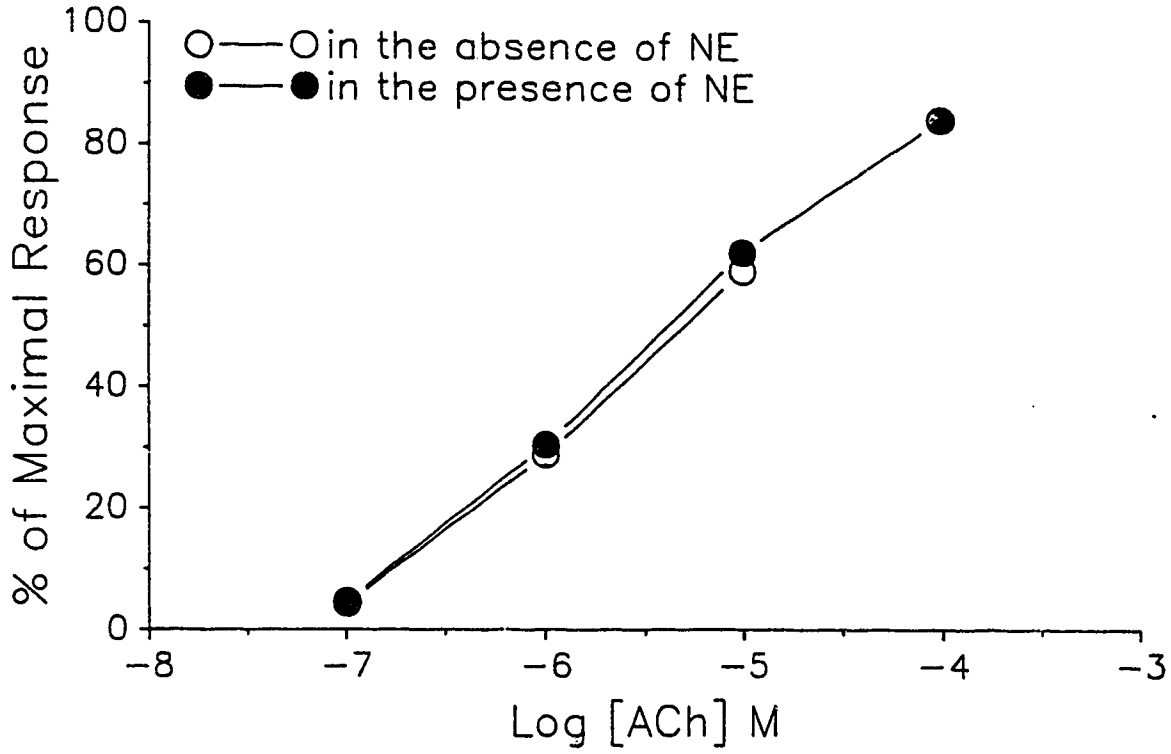

Fig. 3-6. Dose-response curves to ACh in the absence and in the presence of NE (3 μM) in tracheas from asthma model B. Responses were expressed as % of maximal responses induced by ACh.

3.4. Discussion

Our two asthma models are different from each other as shown by different onset time for asthma-like symptoms during 10-day exposure to OA aerosol, and these models have shown changes in α-adrenoceptors in lung homogenates (Mita et al., 1983) or hyperresponsiveness to various pharmacological stimuli (Biggs & Ladenius, 1989), making these models suitable for the study of airway α-adrenoceptor function in asthma.

The finding that NE and phenylephrine did not increase intraluminal pressure in tracheal tubes from two guinea-pig models of human asthma compared with those from controls indicate that postjunctional α_1-adrenoceptor-induced contraction of airway smooth muscle is of no significance in causing or contributing to bronchoconstriction in asthma. Our findings in trachea are particularly interesting because we extended to the level of trachea, in a similar model of chronic allergic asthma, the finding by others in lung strips that NE concentrations for threshold, EC_{50}, or maximal responses for α-adrenoceptor-mediated contraction of the lung strips were unchanged in sensitized guinea pigs challenged daily by an antigen aerosol for 4 wk, compared with nonsensitized animals (Turner et al., 1983), despite two-fold increase in lung α_1-adrenoceptor sites (Barnes et al., 1980). Our findings of minimal increases in pressure in response to NE and phenylephrine in tracheas from control animals concur with the in vivo results in this species by Biggs and D'Souza (1987), which indicate that α_1-adrenoceptor-mediated airway contraction is minimal, as judged by pulmonary flow resistance and dynamic

pulmonary elastance.

This observation raises the possibility that, in two models of human asthma, the increase in α_1-adrenoceptor binding sites in tracheobronchial tree may not have been accompanied by any functional change, reflected in contractile response to NE. However, no radioligand binding studies were done in tracheas from these asthma models. α_1-Adrenoceptors mediate contraction of human peripheral lung strips (Black et al., 1981) and markedly increase in lung membranes from humans with chronic bronchitis (Barnes et al., 1980). However, in these peripheral lung preparations, the contractile response is more likely to be due to α-adrenoceptors on other contractile elements such as pulmonary vessels or interstitial Kapanci cells (Barnes, 1988), and the functional significance of the marked increase in α_1-sites is questioned.

In guinea-pig lung strip preparations, NE-induced contraction was completely insensitive to cocaine, which argues against a significant role of α_1-adrenoceptors located in (densely innervated) blood vessels in the contraction. Similarly, in our experiments, desipramine (0.1 μM) did not increase NE-induced contraction of tracheas from control and hypersensitized animals although, in this species, the tracheal smooth muscle is more richly innervated by sympathetic nerves than the peripheral airways (O'Donnell et al., 1978). These findings may further indicate that tracheal contraction mediated via smooth muscle α_1-adrenoceptors is not sensitive to uptake-1 blockers, presumably a reflection of a minor role of these receptors in airway contraction.

Our results are consistent with recent findings by Goldie et al. (1985), who did not find any evidence for significant bronchial α-adrenoceptor-mediated contraction in a large number of *in vitro* experiments with asthmatic and nondiseased human bronchi. Our findings further support the observation that inhaled buffered phenylephrine failed to induce significant bronchospasm in asthmatics (Thomson et al., 1982) and α-adrenoceptor antagonists had no useful part to play in the treatment of asthma (Svedmyr, 1984).

α_1-Adrenoceptor-mediated contraction of tracheal smooth muscle were noticeably absent or very small in our preparations. This may have been due to low inherent tone resulting from exclusion of muscular effects of prostaglandins by indomethacin (Hardy et al., 1984; Ito & Tajima, 1981), since pretreatment with histamine, serotonin, or potassium were found to enhance the contractions produced by NE in canine trachea in vitro (Barnes et al., 1983 a,b) and in vivo (Barnes et al., 1983a; Brown et al., 1983), and in nondiseased human tracheal and bronchial preparations in vitro (Kneussl & Richardson, 1978), suggesting that histamine and possibly other chemical mediators of asthma could facilitate or activate α-adrenoceptor function in airway smooth muscle.

The guinea-pig tracheal tube is one of the few airway preparations in which it is possible to stimulate parasympathetic nerves pre- or post-ganglionically (Blackman & McCaig, 1983; Widmark & Waldeck, 1986). Under the experimental conditions used in the present study, sympathetic, nonadrenergic noncholinergic, and afferent nervous systems, and (co)transmitters in these systems could not have contributed significantly to tracheal responses to NS

and FS, as previously discussed in detail (see chapter 2), making this preparation suitable for the study of modulation of cholinergic transmission through parasympathetic ganglia.

In tracheas from OA-sensitized or hypersensitized groups, the NE pD_2 values for NS and FS were not significantly different at any of the frequencies tested, with either method of stimulation, as in tissues from control (unsensitized) group, and they were also similar compared with those for control tissues. α_2-Adrenoceptors may have mediated NE's inhibitory effects on the NS- and FS-induced pressure increases as shown previously in tissues from control animals (Biggs et al., 1988; Grundström et al., 1981) and confirmed by experiments with propranolol and prazosin present in the bathing solution. Our findings indicate that α_2-adrenoceptors modulate cholinergic neurotransmission at neuromuscular junctions but not at ganglia and these modulatory functions are unaltered in two guinea-pig models of human asthma. A great deal of evidence has shown that sympathetic agonists could inhibit cholinergic transmission in airways of several species including humans, via different types of adrenoceptors located at neuromuscular junctions or at ganglia: α- in ferrets (Baker et al., 1983; Skoogh, 1986, 1987), and α_2- in guinea pigs and humans (Biggs et al., 1988; Grundström et al., 1981; Grundström & Andersson, 1985), β- in guinea pigs (McCaig, 1987), β_1- in dogs (Denser et al., 1987), and β_2-adrenoceptors in ferrets and humans (Rhoden et al., 1987; Skoogh, 1986, 1988). Our findings, which do not support the idea that the decrease in inhibitory prejunctional α_2-adrenoceptors may contribute to airway hyperresponsiveness in asthma (Casale, 1988)

are particularly interesting in the light of inhibitory effects of adrenergic agonists on cholinergic transmission and the suggestion that the release of the braking mechanism on airway cholinergic transmission might result in exaggerated bronchoconstrictor responses (Barnes, 1986). However, this does not exclude the possibility that α-adrenoceptor agonists could improve respiratory function in asthma via presynaptic α_2-adrenoceptors on postganglionic cholinergic fibers, as shown in our asthma models. This possibility is further supported by others who reported that clonidine inhibited bronchospasm induced by antigen challenge in sensitized guinea-pigs (Andersson et al., 1986b) and in asthmatic patients (Lindgren et al., 1986); a considerable part of the allergen-provoked airway obstruction can be accounted for by mechanisms involving vagal cholinergic innervation (Widdicombe, 1982).

The finding of the increase of α_2- rather than α_1-adrenoceptors in lymphocytes and adipocytes from asthmatics suggested the selective proliferation of α_2-adrenoceptors and their role in asthma (Szentivanyi et al., 1984). However, the studies of α-adrenoceptors in human airways did not identify α_2-adrenoceptors on the bronchial smooth muscle (Andersson et al., 1985; Grundstrom & Andersson, 1985), though prejunctional α_2-adrenoceptors, which mediate the inhibition of cholinergic neurotransmission, were thought to locate on postganglionic cholinergic fibers (Grundstrom & Andersson, 1985). Thus, although it is not clear whether prejunctional α_2-adrenoceptors are increased in airways of asthmatics, our results from two models of human

asthma appear to indicate an insignificant role of these receptors in asthma.

Pressure responses to exogenous ACh were significantly reduced in asthma model B compared with control and model A as shown by a decrease in the mean pD_2 values for ACh. These findings are interesting, because asthmatics are known to express a greater degree of bronchoconstriction in response to a number of nonspecific stimuli, including cholinergic agonists than normal subjects (Gross & Skorodin, 1984), and Biggs & Ladenius (1989) have shown large increases in airway responsiveness to iv injected methacholine in their in vivo asthma model, which is similar to ours. These authors further observed the greatly enhanced bronchospastic effect of vagal stimulation in their hypersensitized animals, the first demonstration of this in an asthma model. However, in our in vitro experiments, responses to vagal stimulation were smaller, though not significantly, in tracheas from two asthma models. These differences in responses to ACh and vagal stimulation may indicate no relationship between cholinergic responsiveness in vitro and in vivo, as shown in human airways (Roberts et al., 1984), suggesting that hyperresponsiveness results from an abnormality in the control mechanism for airway smooth muscle rather than the target organ itself (Barnes, 1986; Vincenc et al., 1983; Armour et al., 1984). However, our findings in tracheas are similar to those by Compton et al. (1987), who reported increased sensitivity to carbachol in lung parenchymal strips, but decreased sensitivity in tracheas from OA-challenged guinea pigs, compared with those from unchallenged (sensitized) animals. Thus, ours and these authors' findings may

indicate regional differences in airways' response to cholinergic agonists in hypersensitized animals. It is not clear why guinea-pig tracheas from asthma model B were less responsive to ACh and vagal stimulation. Down-regulation of postjunctional muscarinic receptors might have occurred as a result of ACh outpouring during antigen-induced vagal discharge (Widdicombe, 1982), as suggested by Compton et al. (1987). Similar responses to ACh in tracheas from control and asthma model A appear to be supported by no change in muscarinic receptors in lung homogenates from this model (Mita et al., 1983).

In summary, smooth muscle α_1-adrenoceptor-induced tracheal responses are not changed in two different models of asthma. An unaltered function of inhibitory α_2-adrenoceptors located on parasympathetic efferents in hypersensitized animals is the first demonstration of this in asthma models.

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4. PHYSIOLOGICAL AND PHARMACOLOGICAL CHARACTERIZATION OF TRACHEAL RESPONSES TO STIMULATION OF THE SYMPATHETIC NERVES*

4.1. Introduction

The guinea-pig tracheal tube is one of the few airway preparations, physiologically and pharmacologically well characterized (Blackman & McCaig, 1983; Widmark & Waldeck, 1986); it is possible to stimulate parasympathetic nerves pre- or post-ganglionically. Using these preparations from normal animals (Biggs et al., 1988) and two asthma models (Shin & Biggs, 1989), it has been shown that adrenoceptor agonists inhibit cholinergic neurotransmission only via α_2-adrenoceptors on post-ganglionic neurons and have no modulatory actions at parasympathetic ganglia. In this preparation, sympathetic and parasympathetic nerves may also be stimulated separately, making it possible to study interaction between these two nervous systems; electrical and mechanical responses to sympathetic (ganglionic) stimulation were characterized in some detail (Blackman & McCaig, 1983; McCaig, 1986a). Thus, McCaig (1987) has recently shown that stimulation of sympathetic stellate ganglia reduced tracheal responses to vagal stimulation, presumably via presynaptic mechanism. However, it was not known whether sympathetic stimulation induced these inhibitory effects through its action on parasympathetic ganglia or postganglionic fibers.

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Nonadrenergic noncholinergic (NANC) inhibitory nerves were observed in the airways of many species including humans (Diamond & Altiere, 1988) and are considered to be a major inhibitory nervous system in human airway smooth muscle, lacking functional inhibitory adrenergic nerves (Richardson & Beland, 1976; Richardson, 1981). NANC inhibitory nerves are thought to receive preganglionic supply from the vagi (Hammarstrom & Sjostrand, 1979; Chesrown et al., 1980; Yip et al., 1981), and in guinea-pig airways, the proportion of adrenergic and NANC inhibitory responses are controversial (Kalenberg & Satchell, 1979; Yip et al., 1981; Taylor et al., 1984). Therefore, we examined and further characterized NANC inhibitory responses and the effects of sympathetic ganglionic stimulation on tracheal responses to vagal nerve and field stimulation in guinea-pig tracheal tube preparations.

4.2. Methods

Tracheal tube preparation. After female Hartley-strain (350-550 g) guinea pigs were killed by a blow to the head, tracheal tube, with its vagi and recurrent laryngeal nerves intact on both sides, was dissected out, as previously described in detail (see chapter 2). Cervical sympathetic trunks were traced to locate sympathetic ganglia at the level of the first and third rib, and these ganglia dissected free from the surrounding tissues, taking extreme care to keep their fine nerve branches intact. The trachea was mounted in a tissue holder equipped with two parallel platinum wire electrodes for transmural electrical field stimulation (FS), the ganglia placed on platinum hook electrodes connected to flexible fine wires for a

minimal stretch of these ganglia, and then the whole tissue placed vertically in a 45-ml organ bath containing oxygenated (95% O_2/5% CO_2) Krebs-Henseleit solution at 37°C. After 1 h equilibration, intraluminal pressure was recorded as before (see chapter 1).

Sympathetic ganglionic stimulation. (a) Effects on intratracheal pressures. Tracheal responses to sympathetic stimulation (n=4) were determined by stimulating ganglia at 3 min intervals with rectangular pulses applied from a Phipps & Bird 611 stimulator. Pulse duration, voltage setting, frequency, or duration of stimulation was varied while other parameters were kept constant. Responses were expressed as % of the maximal response to isoproterenol (10^{-6} M). In separate experiments (n=2) for characterization of responses, ganglia were stimulated for 10 s with rectangular pulses (1 ms; 50 V; 40 Hz) after adding each of the following drugs successively: atropine (10^{-6} M), atenolol (10^{-6} M), hexamethonium (5×10^{-4} M), and propranolol (5×10^{-6} M).

(b) Effects on responses to nerve and field stimulation. The inhibitory effects of sympathetic ganglionic stimulation on tracheal responses to vagal nerve (NS) and field stimulation (FS) were studied in a group of tissues (n=5). Both vagi were drawn into a single shielded platinum tunnel electrode for NS with rectangular pulses (0.5 ms; 25 V) applied via a Grass S 44 stimulator. For FS, bipolar rectangular pulses (0.5 ms; 80 V) were applied from a Grass SD9 stimulator. Before the preparation was used, alternate NS and FS were applied at 3 min intervals at 2, 8, and 32 Hz for 5 s until

pressure increases were constant. Rectangular pulses (1 ms; 50 V; 40 Hz) were applied to ganglia 30 s before control responses to NS or FS.

To characterize the effects of sympathetic stimulation on tracheal responses to NS and FS, these experiments (n=2) were done at 8 Hz after ganglionic blockade with hexamethonium (10^{-4} M, 30 min incubation) alone and followed by chemical sympathectomy with 6-hydroxydopamine (10^{-3} M, 1 h incubation)

Propranolol (5 x 10^{-6} M) and indomethacin (10^{-5} M) were present in the bath throughout the experiment. Some experiments were done in the presence of desipramine (10^{-6} M) and yohimbine (3 x 10^{-6} M). Percentage inhibition of control responses to NS and FS was calculated. Responses were compared using Student's t test for paired or unpaired samples, as appropriate, and differences were accepted as significant at $p<0.05$. All data are expressed as mean $\pm$ SEM.

Nonadrenergic noncholinergic responses. Tracheal responses to NS and FS were characterized by adding the following drugs successively: atropine, atenolol, hexamethonium, propranolol, phentolamine (5 X 10^{-6} M), and tetrodotoxin (3 X 10^{-6} M). Each drug was added to the bath at least 30 min before the experiment. Rectangular pulses of varying stimulation parameters were applied as given in the results section. Otherwise indicated, pulse duration and voltage setting were the same as above. At the end of each experiment, tracheal tone was confirmed by aminophylline (10^{-3} M).

Drugs. The following drugs were used: atenolol, atropine sulfate, desipramine hydrochloride, 6-hydroxydopamine hydrochloride, indomethacin, and propranolol hydrochloride (Sigma, St.Louis, MO); DL-isoproterenol sulfate (Fluka AG, Buchs SG, Switzerland); L-ascorbic acid (BDH Chemicals, Toronto, Canada); aminophylline injection, USP (Squibb Canada Inc., Canada); phentolamine mesylate for injection (Ciba Pharmaceutical Co., Div. Ciba-Geigy Corp., Summit, N.J.); hexamethonium bromide (K & K Laboratorie Inc. Plainview, New York); and tetrodotoxin crystalline 3X (Sankyo Co., Ltd. Tokyo). Most drugs were dissolved in distilled water; 6-hydroxydopamine was dissolved in 0.02% ascorbic acid, indomethacin was dissolved in 0.2% sodium carbonate, and prazosin was dissolved ure of glycerine/5% dextrose solution (1:1).

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with sympathetic ganglia. Tracheal responses to ganglionic stimulation. Stimulation of sympathetic anglia induced a decrease in intratracheal pressures; occasionally, this decrease was preceded by an increase in intratracheal pressure. The decrease in intratracheal pressure returned to the control level slowly after cessation of the stimulation. Atropine (10^{-6} M) abolished completely the evoked increase in intratracheal pressure, leaving the evoked decrease (9.5 cm H_2O) unchanged (n=2). In the presence of atropine, the evoked decrease in intratracheal pressure was slightly (10.9%) reduced by atenolol (10^{-6} M), a selective β_1-adrenoceptor antagonist; however, the responses were reduced significantly (63.6%) by subsequent addition of hexamethonium

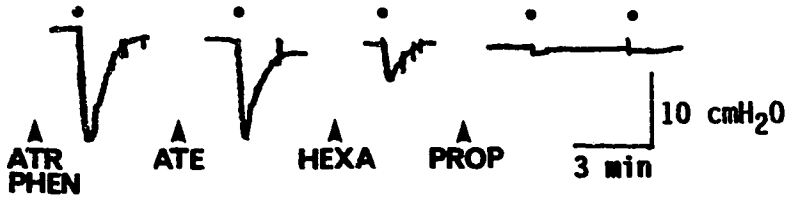

Fig. 4-1. Effects of selective β_1-adrenoceptor atenolol, ganglionic blocker hexamethonium, and nonselective β-blocker propranolol on tracheal responses to sympathetic (ganglionic) stimulation. Atropine (1 uM) and phentolamine (5 uM) were present throughout the experiment.

(5 X 10^{-4} M), an autonomic ganglionic blocker, and completely abolished by further addition of propranolol (5 X 10^{-6} M), a nonselective β-adrenoceptor antagonist (Fig. 1).

The effects of pulse duration, frequency, voltage, and duration of stimulation were shown in Fig. 2. Supramaximal voltage was determined by increasing voltage up to 50 V while a constant number of pulses (0.5 ms) were delivered at 20 Hz. The increase in the voltage from 1 to 5 V induced a rapid increase in the intratracheal pressure response from 9.8 ± 5.9 to 65.7 ± 16.0% of the maximal responses to isoproterenol, and responses reached the maximum at 50 V (89.5 ± 16.0%) (Fig. 2a). The effects of pulse duration on tracheal responses to sympathetic stimulation were determined while trains of 200 pulses were delivered at 20 Hz at the supramaximal voltage (50 V). Responses increased rapidly from 28.8 ± 8.8% to 83.3 ± 12.5% as pulse duration increased from 0.04 to 0.5 ms, and there was a small change in the response between 0.5 and 4 ms (Fig 2b).

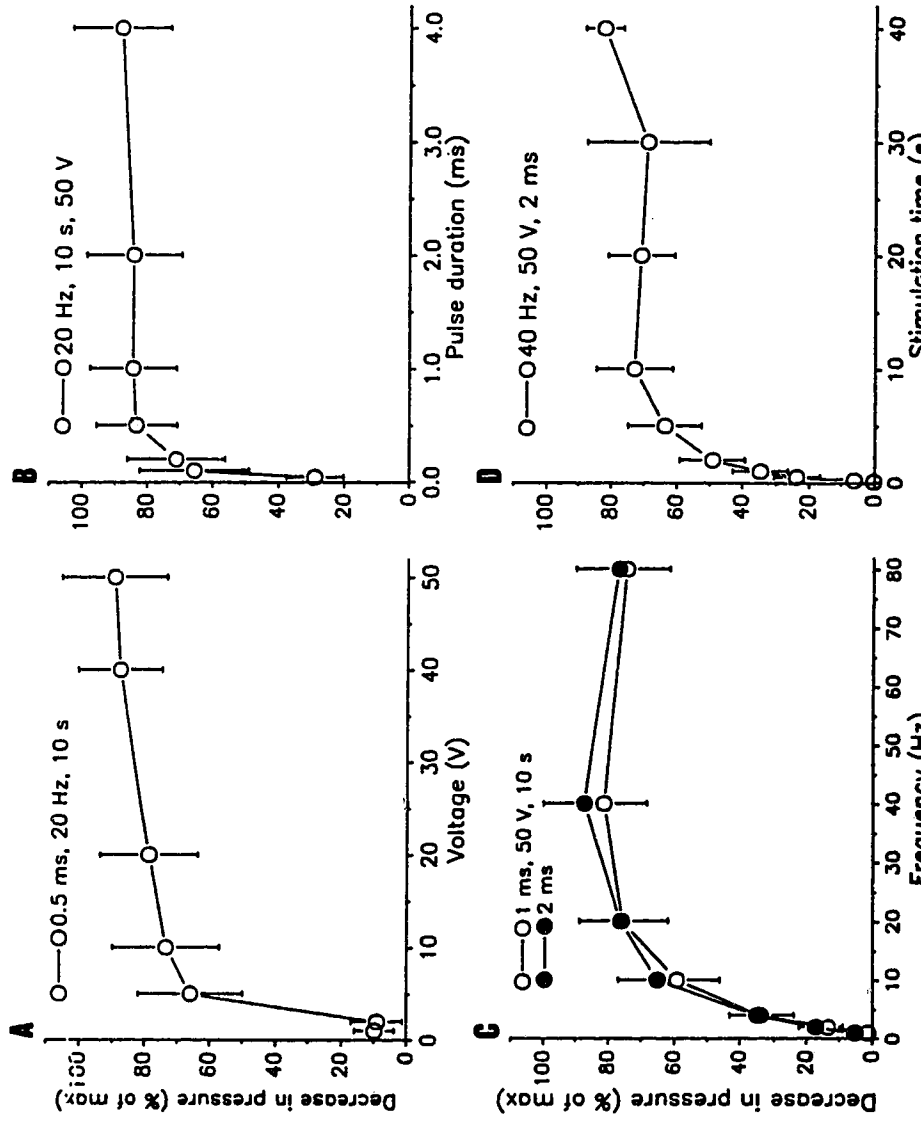

Fig. 4-2. Effects of sympathetic stimulation on intratracheal pressure. A, B, C, and D represent the effects of pulse duration, frequency, voltage, and duration of stimulation, respectively. Atropine (1 μM) and phentolamine (5 μM) were present throughout the experiment. Responses were expressed as % of the maximal response to isoproterenol (1 μM).

Tracheal responses to sympathetic stimulation increased with frequency until the maximal responses were reached at 40 Hz (81.4 ± 13.2% and 87.4 ± 12.8% with 1 and 2 ms pulses, respectively), when pulses of 1 and 2 ms were applied at the supramaximal voltage for 10 s (Fig 2c). Responses increased rapidly with the duration of stimulation up to 10 s, but small changes in response were seen for over 10 s stimulation (Fig 2d).

Effects on responses to nerve and field stimulation. Sympathetic stimulation reduced tracheal responses to NS and FS equi-effectively and in a frequency-dependent manner, i.e., sympathetic stimulation was more effective in inhibiting responses at low than high rates of stimulation (Fig. 3a). The inhibitory effects of sympathetic stimulation on tracheal responses to NS and FS at 2 Hz were slightly enhanced by desipramine (10^{-6} M) and significantly ($p<0.05$) reduced by yohimbine (3×10^{-6} M) (Fig. 3b).

In separate experiments (n=2) without propranolol and indomethacin, sympathetic ganglionic stimulation reduced tracheal responses (32.2 cm H_2O) to FS at 8 Hz by 60%, and this inhibitory effect was significantly reduced to 16% by hexamethonium (10^{-4} M) and further reduced to 2.5% after subsequent sympathectomy with 6-hydroxydopamine (10^{-3} M) (Fig. 4). These experiments were done only for FS, because hexamethonium significantly reduced tracheal responses (30.3 cm H_2O) to NS by 76.5%, but not to FS.

Nonadrenergic noncholinergic (NANC) responses. In the presence of atropine (10^{-6} M) and phentolamine (5×10^{-6} M),

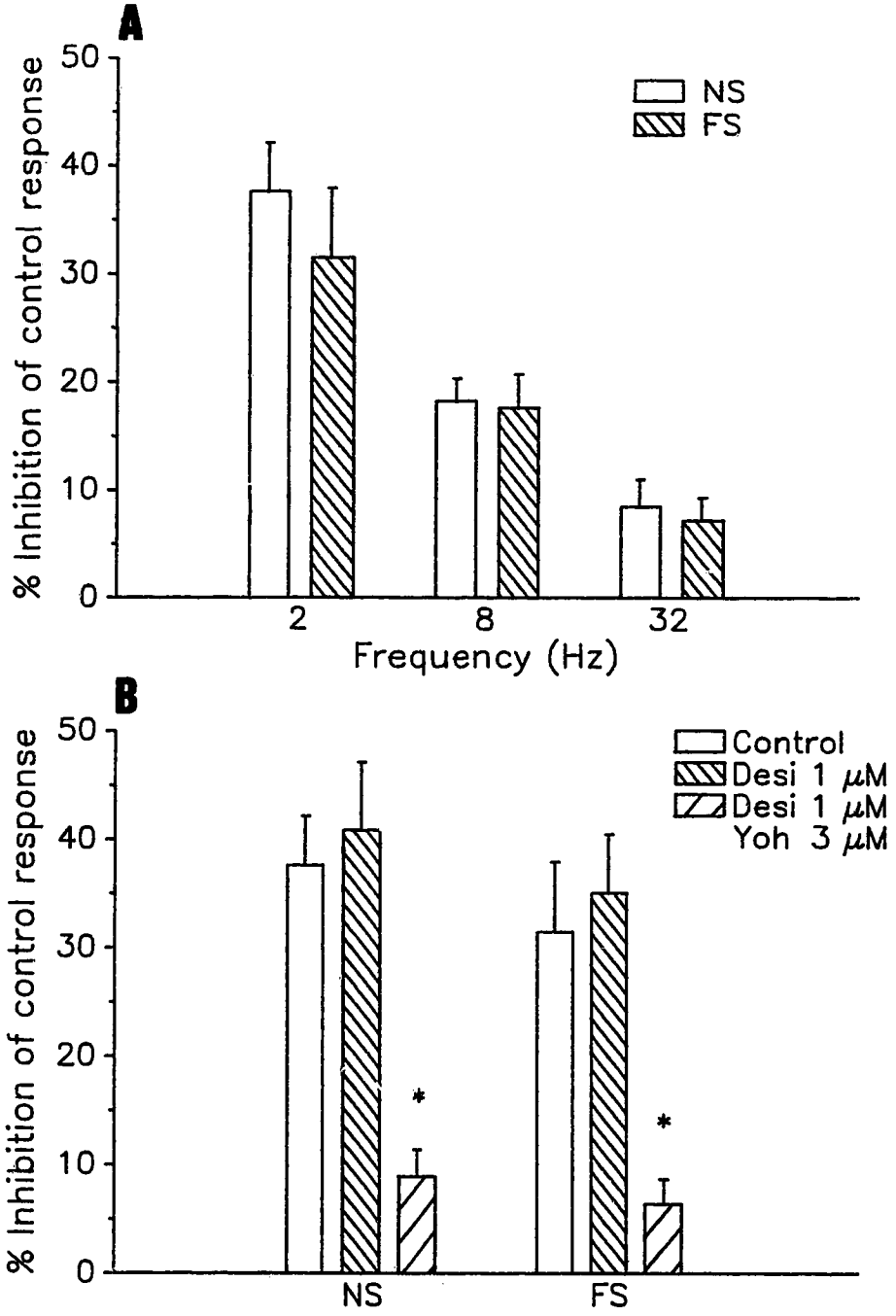

Fig. 4-3. **Inhibitory effects of sympathetic stimulation on tracheal responses to NS and FS. A. in the absence of desipramine (1 μM) and yohimbine (3 μM). B. in the presence of desipramine (1 μM) and yohimbine (3 μM); 2 Hz. Propranolol (5 μM) was present throughout the experiment.**

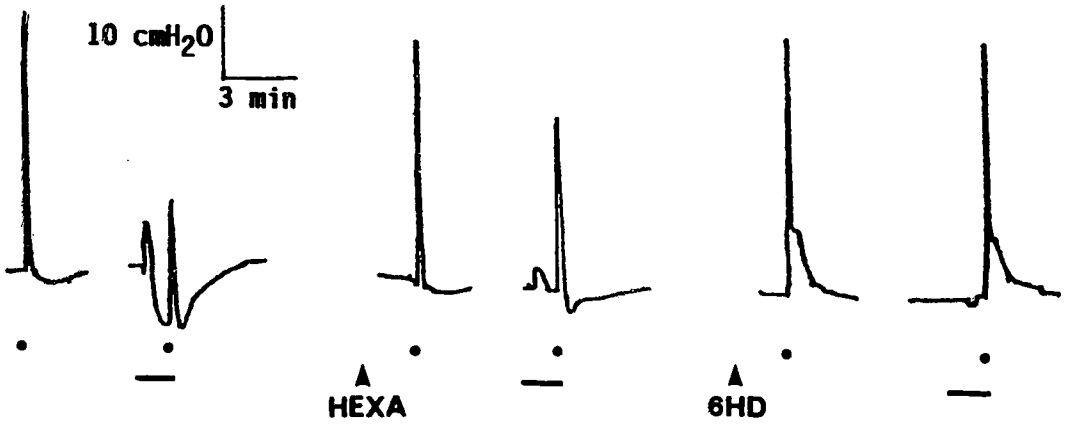

Fig. 4-4. Effects of hexamethonium (100 μM) and sympathectomy with 6-hydroxydopamine (1 mM) on the inhibitory effect of sympathetic stimulation (8 Hz) on tracheal responses to NS.

nerve and field stimulation for 10 s induced depressor responses in a frequency-dependent manner. Tracheal responses (n=2) to NS were smaller than those to FS at all frequencies examined (0.9, 5.0, and 7.4 cm H_2O for NS vs. 4.4, 8.6, and 8.1 cm H_2O for FS at 2, 8, and 32 Hz, respectively). Tracheal responses to FS at 8 Hz were slightly reduced from 5.4 to 4.2 cm H_2O after atenolol and not further reduced by subsequent addition of propranolol. After addition of atenolol, propranolol, and hexamethonium, tracheal responses to FS were slightly reduced to 3.6, 7.3, and 7.6 cm H_2O at 2, 8, and 32 Hz, respectively (Fig. 5a); responses to NS were completely abolished at these frequencies, but sustained NS for 1 min with pulses of strong stimulation parameters (4 ms; 40 or 120 Hz) induced a minimal decrease (< 1.5 cm H_2O) in intratracheal pressure (Fig. 5b). NANC inhibitory responses to FS seen in the presence of the above autonomic blocking agents were almost

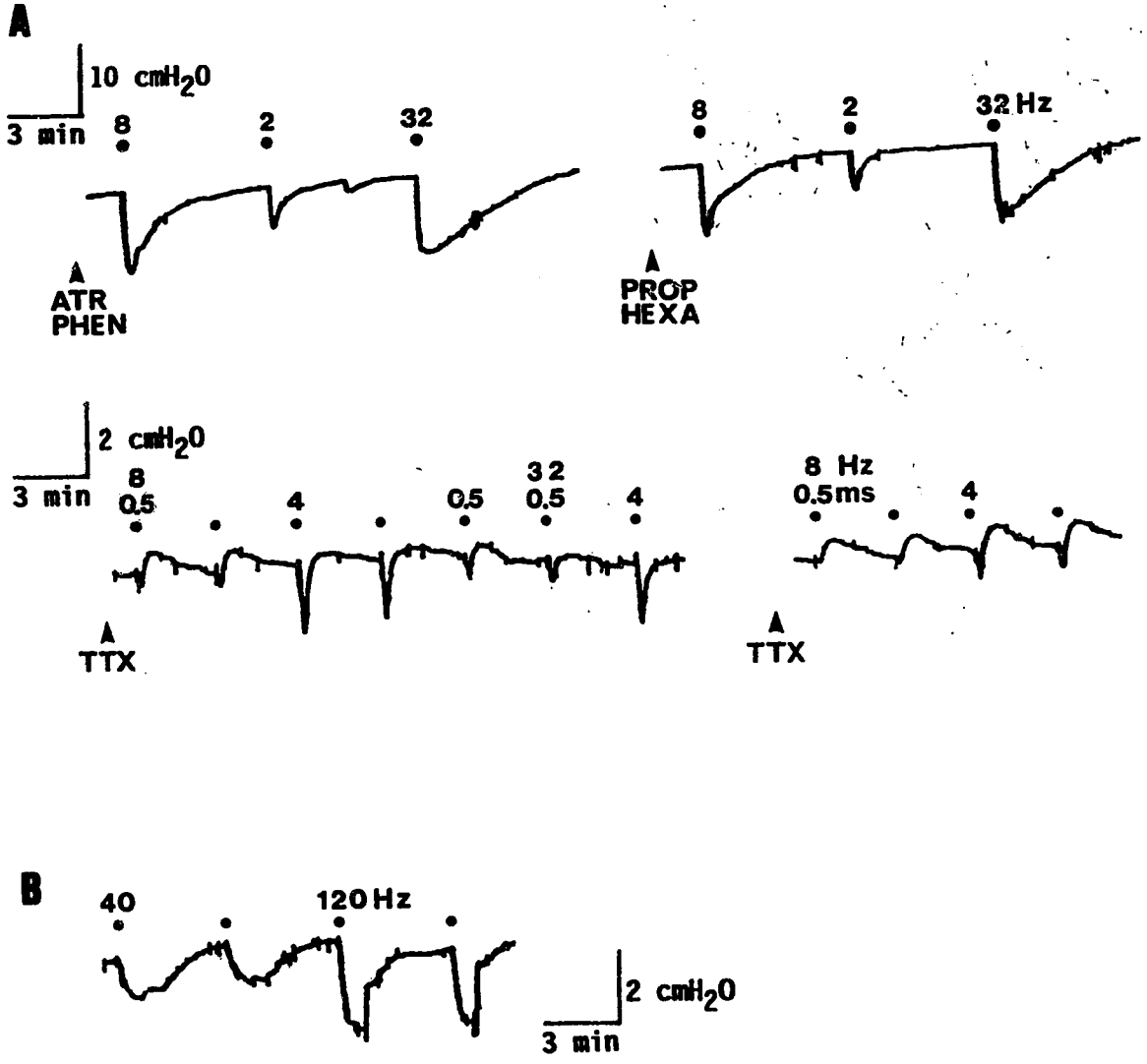

Fig. 4-5. Nonadrenergic noncholinergic responses. A. Field stimulation, the effect of tetrodotoxin (3 μM). B. Sustained nerve stimulation (1 min; 4 ms; 40 or 120 Hz). (.) indicates stimulation at a given stimulation parameters.

completely abolished by tetrodotoxin (TTX, 3×10^{-6} M). However, tetrodotoxin could not abolish all the evoked responses; depressor response turned into dual depressor/pressor response (Fig. 5a). Thus, a minimal decrease followed by a minimal increase in intratracheal pressure (n=2) was observed for FS with rectangular pulses (0.5 ms, 32 Hz; 4 ms, 8 and 32 Hz; 10 s); TTX-resistant NANC inhibitory responses were dependent on pulse duration (Fig. 5a). With pulses of a short duration (0.5 ms, 8 Hz, and 10 s), TTX-resistant NANC inhibitory responses were seen when NANC inhibitory responses were large (8.3 cm H_2O); however, only TTX-resistant excitatory responses were seen when small (4.0 cm H_2O). Subsequent addition of aminophylline (10^{-3} M) induced a decrease in pressure (11.8 cm H_2O), similar in size to the response to FS at 8 Hz.

4.4. Discussion

Guinea-pig tracheal muscle is densely innervated by adrenergic nerve fibers (Doidge & Satchell, 1982; O'Donnell & Saar, 1973; O'Donnell et al., 1978), derived from superior cervical ganglia (Smith & Satchell, 1985) and stellate ganglia (Hammarstrom & Sjostrand, 1979; Blackman & McCaig, 1983). The in vitro electrical stimulation of the stellate ganglia induced relaxation of trachea, which were blocked by propranolol (Blackman & McCaig, 1983) or by guanethidine (Hammarström & Sjostrand, 1979). In our experiments, stimulation of sympathetic ganglia at a variety of stimulation parameters induced depressor responses, which were slightly reduced by β_1-selective antagonist atenolol and completely abolished by

nonselective β-adrenoceptor antagonist propranolol. These findings confirm those of others (Blackman & McCaig, 1983) and provide further evidence for coexistence of β_1- and β_2-adrenoceptors and functional importance of β_2-adrenoceptor-mediated relaxation in guinea-pig trachea, as shown by others (Zaagsma et al., 1979; Carswell & Nahorski, 1983). Occasionally, depressor responses were preceded by atropine-sensitive pressor responses; these findings confirm those by others (Binger et al., 1931; Hebb, 1940; Hammarström & Sjostrand, 1979) and indicate that guinea-pig trachea receives cholinergic fibers via both the vagi and sympathetic ganglia.

Unlike others (Blackman & McCaig, 1983), depressor responses induced by sympathetic ganglionic stimulation were significantly reduced by hexamethonium, suggesting that in our preparations ganglionic stimulation stimulated both pre- and post-ganglionic sympathetic nerves. This suggestion is further supported by our finding that hexamethonium also significantly reduced the inhibitory effects of sympathetic ganglionic stimulation on tracheal responses to FS.

Stimulation of sympathetic ganglia for 35 s at the maximal stimulation parameters (1 ms; 50 V; 40 Hz) for the tissue were equi-effective in inhibiting pressor responses to NS and FS at 2, 8, and 32 Hz, and this inhibitory effect was significantly reduced by yohimbine as seen at 2 Hz. It has been shown in tracheal tubes from normal (Biggs et al., 1988) and hypersensitized (Shin & Biggs, 1989) guinea pigs that exogenous norepinephrine were equally effective at blocking NS and FS at these frequencies whether for 10 s stimulation

or for trains of 64 pulses. Thus, our findings indicate that, as for adrenoceptor agonists, sympathetic ganglionic stimulation also modulate cholinergic neurotransmission via α_2-adrenoceptors located solely on post-ganglionic neurons and have no modulatory actions at parasympathetic ganglia; any direct effect of sympathetic stimulation on tracheal smooth muscle was excluded because sympathetic stimulation did not alter mechanical responses to exogenous acetylcholine (McCaig, 1987).

However, the inhibition of tracheal responses to NS and FS by sympathetic ganglia is significantly smaller than those by exogenous norepinephrine (Grundström et al., 1981; Biggs et al., 1988); stimulation of sympathetic ganglia at the maximal stimulation parameters inhibited control responses to NS at 2 Hz by only 37.6±4.5% compared with complete inhibition by exogenous norepinephrine of responses to NS even at a higher frequency (32 Hz). These findings appear to indicate that, under physiological conditions, norepinephrine released from sympathetic nerves may have a minor role in modulating cholinergic neurotransmission.

Using similar stimulation parameters, McCaig (1987) observed substantial inhibition of pressor responses to vagal stimulation in approximately half the preparations tested; in the remaining preparations, vagal responses were unaltered. This author suggested, based on these findings, that anatomical apposition between cholinergic and adrenergic nerve fibers may vary from animal to animal; thus, in preparations in which sympathetic stimulation is ineffective, neurally-derived norepinephrine does not reach adrenoceptors mediating neuromodulation. McCaig (1987) also showed

that both presynaptic α- and β-adrenoceptors may inhibit cholinergic neurotransmission. Unlike this author, we conducted all the experiments in the presence of propranolol to study only α-adrenoceptor-mediated modulation. Thus, smaller inhibition observed in our experiments might be due to the blockade of β-adrenoceptor-mediated inhibition and the possible variance among animals in anatomical apposition between cholinergic and adrenergic nerve fibers. Our finding that the inhibitory effect of sympathetic stimulation were almost completely abolished by sympathectomy with 6-hydroxydopamine indicate that neurally-derived norepinephrine is responsible for the neuromodulatory effect of sympathetic stimulation. Unlike the experiments with exogenous norepinephrine (see chapter 2), the neuromodulatory effects of sympathetic ganglia were not altered by the uptake 1 blocker desipramine, and this might have been attributed to the maximal effect exerted by sympathetic ganglia stimulated at the maximal parameters.

In the presence of atropine, FS induced depressor responses at 2, 8, and 32 Hz; these responses were slightly reduced by propranolol and almost completely blocked by tetrodotoxin. These findings indicate that NANC inhibitory responses of nerve origin are predominant in these preparations. The proportion of adrenergic and NANC inhibitory responses are controversial in guinea-pig trachea. It has been shown that NANC inhibitory responses make up about 20-40% of the total relaxation induced by FS of tracheal strips (Kalenberg & Satchell, 1979) or tubes (Kalenberg & Satchell, 1979; Yip et al., 1981). However, in more recent studies using guinea-pig tracheal chain preparations, Taylor et al. (1984) concluded that

NANC nerves are major inhibitory nerves in this tissue. Our results appear to support these authors' conclusion; the discrepancies among these studies are not clear, presumably indicating variation among individuals. Like others (McCaig, 1986b), sustained NS for 1 min with strong stimulation parameters (4 ms; 40 or 120 Hz) induced a minimal NANC inhibitory responses (even in the presence of hexamethonium [10^{-4} M]). The smallness of vagally-mediated NANC inhibitory responses compared with those by FS, and the need for sustained stimulation at much stronger stimulation parameters raise the question about how these NANC responses are excited under physiological conditions. TTX-resistant depressor responses were followed by pressor responses; by contrast, Coburn & Tomita (1973) observed contraction followed by relaxation in their tracheal strips. TTX-resistant depressor responses were dependent on pulse duration rather than frequency, the size at longer pulse duration being similar to TTX-sensitive NANC inhibitory responses induced by vagal stimulation at strong parameters. Thus, these findings might suggest that both TTX-sensitive and TTX-resistant NANC inhibitory responses are important in controlling the airway tone in guinea pigs, as in primate airways (Daimond & Altiere, 1988). The mechanism for TTX-resistant NANC pressor and depressor responses and their physiological significance remain to be further investigated.

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5. GENERAL DISCUSSION AND CONCLUSIONS

The literature review revealed that:

1. The sympathetic nervous system can modulate airway tone via its effects on cholinergic transmission through airway ganglia, a notion supported by the electrophysiological, morphologic, and pharmacological studies in several species.
2. Prejunctional α_2-adrenoceptors inhibit cholinergic neurotransmission by reducing the release of acetylcholine from postganglionic nerve endings in the airways of guinea pig and human.
3. Stimulation of postjunctional α_1-adrenoceptors in asthmatic may induce bronchospasm.

The above information led us to postulate that up-regulation of excitatory α_1-adrenoceptors and down-regulation of inhibitory presynaptic α_2-adrenoceptors could contribute to airway hyperresponsiveness in asthma. Therefore, we compared the inhibitory effects of NE (and selective α_2-adrenoceptor agonists clonidine and xylazine) on pre- and post-ganglionic nerve stimulation of guinea-pig tracheal tubes prepared from control, sensitized, and hypersensitized guinea pigs (asthma models). In tissues from control animals, the effects of sympathetic ganglionic stimulation on responses to pre- (NS) and post-ganglionic nerve stimulation (FS) were also compared. Also, we compared the effects of NE and selective α_1-adrenoceptor agonist phenylephrine in tissues from the different groups of animals.

The results of this study show that NE and sympathetic

stimulation inhibit tracheal responses to NS and FS equi-effectively at all frequencies. This suggests that, in guinea-pig trachea, adrenoceptor agonists mediate their inhibitory effects via α_2-adrenoceptors located solely on post-ganglionic neurons and have no modulatory actions at parasympathetic ganglia. These findings concur with morphological evidence confirming the absence of adrenergic fibers in airway parasympathetic ganglia of this species. Extrapolation of these data to human airways may be misleading as, unlike guinea-pig airways, morphological evidence indicates the presence of adrenergic fibers in parasympathetic ganglia of human airways. Also, we have not excluded the possibility that β-adrenoceptors exist on parasympathetic ganglia and modulate ganglionic transmission, as reported in other species (Skoogh, 1986).

The NE pD_2 values for NS and FS in tissues from asthma model A and B were not significantly different from those for control tissues. Thus, inhibitory α_2-adrenoceptor function is unaltered in these two guinea-pig models of human asthma. The lack of involvement of inhibitory α_2-adrenoceptors in airway hyperresponsiveness is supported by the finding that, in control tissues, the inhibition of cholinergic responses by sympathetic stimulation was significantly less than with exogenous NE, even though the supramaximal stimulation parameters were used. α_2-adrenoceptors have been reported to inhibit cholinergic neurotransmission at the neuromuscular junctions in isolated human bronchi. However, this study suggests that prejunctional α_2-adrenoceptors do not contribute to airway hyperresponsiveness in human asthma. Our study

indicates that α_2-adrenergic agonists would reduce the degree of airway hyperresponsiveness in asthma if it involved cholinergic transmission. The α_2-adrenergic agonist clonidine prevents bronchospasm induced by antigen provocation in asthmatics and sensitized guinea pigs, presumably by inhibition of vagal reflex bronchoconstriction via presynaptic α_2-adrenoceptors on cholinergic efferents.

Many types of receptors could modulate cholinergic neurotransmission at neuromuscular junction or at airway ganglia. However, it is not known whether their functions are altered in asthma. These data draw our attention to other presynaptic receptors for their possible role in airway hyperresponsiveness in asthma.

The second part of the present study revealed that α_1-adrenoceptor function was unchanged in two animal models of asthma. Several studies have demonstrated the enhanced α_1-adrenoceptor effects in asthmatics. However, this might be due to other effects not involving α_1-adrenoceptors. For example, apparent enhanced α_1-adrenoceptor effects in asthmatics may be due to a pH effect of the solution. Thus, unlike unbuffered solutions of phenylephrine, buffered phenylephrine has been shown to be no more spasmogenic than buffered saline in asthmatics. Our findings <u>in vitro</u> concur with those of others who showed that phenylephrine failed to induce significant increases in tone in bronchi isolated from either non-diseased or asthmatic human lungs. It was shown that nonselective α-antagonists (such as thymoxamine, phentolamine and indoramin) increase resting airway conductance in asthmatics and protect against bronchoconstriction induced by many stimuli.

However, these effects may relate to the non-α-receptor blocking pharmacological actions of the drugs, including direct relaxation of smooth muscle, catecholamine release, or antihistaminic activity. Also, the absence of any beneficial effects of the α_1-selective antagonist prazosin in asthma supports these findings. It is possible that α_1-adrenoceptor function is increased in the smaller airways in asthma - the number of α_1-adrenoceptors increases as the airways become smaller. These experiments were conducted in a large airway and it may not be possible to extrapolate the findings to small airways.

In summary, results of this study show that, in guinea-pig models of asthma, alterations in α_1- and α_2-adrenoceptors do not contribute to airway hyperresponsiveness.

6. INTRODUCTION AND PROPOSED RESEARCH

6.1. Cardiovascular Receptors

Work over the past 40 years has shown that the cardiovascular system and lungs contain many receptors mediating reflexes affecting the respiratory, cardiovascular, and other systems. Cardiovascular receptors comprise **systemic arterial baroreceptors, peripheral arterial chemoreceptors, pulmonary vascular receptors,** and **atrial and ventricular receptors** (Daly, 1986). This section will be limited to discussion of cardiovascular receptors (see Chapter 1. of Section I for receptors in lungs).

Systemic arterial baroreceptor areas derive from the aortic branchial arches (see Kirchheim, 1976). In man and mammals, the receptor endings are located in segments of the arterial system that have an elastic structure (Grigoreva, 1962; Muratori, 1967). Receptors are found in the following regions: 1) in the aortic arch at the origins of the brachiocephalic artery, the left subclavian artery, and Botallo's duct; 2) in the brachiocephalic artery at its bifurcation into the right subclavian and right common carotid artery; 3) in the carotid sinuses; 4) along both common carotid arteries; and 5) in both common carotid arteries at the origin of the superior thyroid artery (see review by Kirchheim, 1976). There is no experimental evidence that the latter two "minor areas" induce typical reflex changes in blood pressure in species other than in the cat (Green, 1967). Sensory innervation by the left aortic or depressor nerve reaches the aortic arch at the point where the left subclavian artery originates from the descending part of the aorta.

The principal contribution to the right aortic nerve comes from the subclavian-carotid angle and the neighboring part of the brachiocephalic artery. Recently, the pathway of aortic depressor nerves has been described in the guinea pig (Sun & Biggs, 1986). The carotid sinus region is supplied by the sinus nerve, a branch of the glossopharyngeal nerve (see next section for the details of carotid sinus region). The characteristics of the receptors in the carotid sinuses and aortic arch and their reflex responses have been described in several reviews (Heymans & Neil, 1958; Kezdi, 1967; Kirchheim, 1976; Sleight, 1980). Baroreceptors in the carotid sinuses and aortic arch are innervated by medullated (A-type) or nonmedullated (C-type) fibers (Kirchheim, 1976). The receptors respond to stretch of the vessel wall in which they lie and therefore to changes in mean pressure, pulse pressure, and pulse frequency. Changes in the viscoelastic properties of the vessel walls, whether produced by chemical agents, sympathetic activity, or arterial disease and hypertension, can alter the relationship between pressure and the frequency of firing of baroreceptors (Aars, 1968, 1971; Angell-James, 1971, 1973, 1974a,b). Stimulation of baroreceptors in the carotid sinus and aortic arch alters peripheral resistance and heart rate. When compared with the aortic baroreflex, the carotid baroreflex is more sensitive to dynamic than to static stimulation (Angell-James & DeBurgh-Daly, 1970), also, it alters vascular resistance more than heart rate (Glick & Covell, 1968), and seems more effective in hypotensive than in hypertensive states (Donald & Edis, 1971). It has been shown that selective stimulation of baroreceptors induces reflex bronchoconstriction in cats and dogs

(Daly & Schweitzer, 1951) and guinea pigs (Biggs, 1984; Biggs & Perterson, 1981).

Peripheral arterial chemoreceptors are located in the carotid and aortic bodies. Carotid bodies are present in all mammalian species studied and contain two types of cells: type I and type II. Cells are found in the principal mass of the carotid body and in isolated groups in the connective tissue around vessels in the carotid bifurcation, separate from the principal mass - the periadventitial cells (Clarke & Daly, 1981a,b, 1982, 1983). Periadventitial cells are present in cats, dogs, and rabbits but not in guinea pigs, rats, and mice. Chemoreceptors in the carotid bodies are innervated by the carotid sinus nerves. They respond to changes in blood pO_2, pCO_2, and pH. Unlike baroreceptors, chemoreceptor activity is asynchronous with heart rate and activity has no relationship to systole (Heymans & Neil, 1958). Under circumstances in which secondary effects resulting from changes in arterial blood pressure and blood gases are controlled, stimulation of the carotid bodies causes bradycardia, a negative left ventricular inotropic response, a reduction in cardiac output, and an increase in systemic vascular resistance (Daly, 1986). The aortic bodies are located in the aortic arch region (Howe, 1956). The relative contributions of the carotid and aortic bodies to respiratory and circulatory control varies, depending on the species under study, as aortic bodies are absent from rabbits, rats and mice and the carotid bodies are present in all mammals studied. Functional differences are noted between chemoreceptors in the carotid and aortic bodies. Stimulation of the carotid bodies evokes bradycardia and a decrease in left

ventricular (dP/dt)max (where P = pressure and t = time), aortic body stimulation causes tachycardia and an increase in (dP/dt)max (Karim et al., 1980). Also, the carotid bodies exert a greater influence on respiration, whereas the aortic bodies produce a greater hypertensive response (Comroe & Mortimer, 1964).

Pulmonary vascular receptors occur at the adventitial-medial junction in the main pulmonary artery and its two branches or penetrate the outer layers of the media. They are supplied by myelinated fibers running in the vagi, although some fibers run in sympathetic nerves (Daly, 1986). Receptors have also been described in the pulmonary veins. Generally distension of the whole pulmonary vascular bed causes tachycardia, sometimes preceded by brief apnea, bradycardia, and systemic hypotension dependent on the integrity of the vagi (Daly & Hebb, 1966). Endings connected to large myelinated fibers are distributed almost exclusively in the left and right main pulmonary arteries and are active at normal arterial pressures.

Atrial receptors have been reviewed (Linden & Kappagoda, 1982; Linden, 1987; Mary, 1987). Receptors are located in the walls of the atria and atrial appendages at the junctions of the right atrium with superior and inferior venae cavae and of the left atrium with pulmonary veins in animals and humans. Receptors served by myelinated A-fibers are found at all these sites. C-fiber endings are located mainly in the atrial walls, interatrial septum, and atrial appendices. Myelinated afferents run in the vagi and unmyelinated afferents run in the vagi and sympathetic nerves. Of these three groups, only the group of receptors that discharge into myelinated vagal nerve fibers has been shown upon stimulation to

result in the following reflex responses: an increase in the activity in efferent cardiac sympathetic nerves (Linden et al., 1982) and heart rate (Kappagoda et al., 1979); a decrease in the activity in efferent renal sympathetic nerves (Linden et al., 1980) and an increase in urine flow (Sivananthan et al., 1981); a decrease in the plasma level of vasopressin (Bennett et al., 1984) and plasma renin activity (Hicks et al., 1985).

Ventricular receptors are innervated mainly by nonmyelinated vagal C-fibers and their density differs among species (Baker et al., 1979; Coleridge & Coleridge, 1980). They are divided into: those excited predominantly by chemical stimuli, and those responding mainly to mechanical stimuli. Stimulation of ventricular receptors results in decreases in heart rate and in vascular resistance in various parts of the body and dilatation of capacitance vessels. Responses to stimulation of these receptors appear to be influenced by the intensity of the stimuli to other reflexogenic areas and changes in resistance are inhibited at high carotid sinus pressures (Hainsworth & McGregor, 1987).

6.2. Baroreceptors in Carotid Sinus

6.2.1. Arteries

The wall of arteries consists of three layers: **tunica intima, tunica media,** and **tunica adventitia** (Rhodin, 1980). The **intimal layer** is the innermost layer and is composed of the following structures: 1) a single layer of endothelial cells lining the vascular wall; 2) a thin, about 80-nm thick basal lamina; and 3) a subendothelial layer, composed of collagenous bundles, elastic

fibrils, smooth muscle cells, and perhaps some fibroblasts. The subendothelial layer, however, is usually present only in the large elastic arteries such as human aorta. The endothelial monolayer serves as a nonthrombogenic surface for prevention of thrombi and function as a permeability barrier or transport system, or both, for components of the blood to the underlying smooth muscle cells of the media of the vessel wall (Ross & Kariya, 1980). The arterial endothelium is provided with a system of tight, occluding junctions and communicating junctions, which are implicated in transendothelial transport. It has been hypothesized that "injury" to the endothelium is a critical factor in lesion formation in atherosclerosis. The **tunica media** is the middle layer of the arterial wall and made up of smooth muscle cells, a varied number of elastic sheets (laminae), bundles of collagenous fibrils, and a network of elastic fibrils. There are no cells present in the media other than the smooth muscle cells. The **tunica adventitia** is the outermost layer. In all arteries, the adventitia consists of dense fibroelastic tissue without smooth muscle cells. The adventitia also harbours the nutrient vessels of the arterial wall: arterioles, venules, blood capillaries, and lymphatic vessels, collectively referred to as the vasa vasorum. The adventitia renders the arterial wall stable, and serves to connect the blood vessel to its surrounding tissues. Also, it conveys nutrients to the smooth muscle cells of the media.

Generally, arteries are subdivided into two categories, **elastic** and **muscular**. Those with large diameters, in which the media contains both smooth muscle cells and many elastic laminae,

are called elastic arteries. Arteries decrease in diameter as they approach the periphery and become purely muscular arteries in which the media is less elastic and the smooth muscle cells prevail. Branches of some elastic arteries remain elastic throughout their course, e.g. vertebral artery; some demonstrate a gradual transition from elastic to muscular, e.g. internal carotid, axillary arteries; others are elastic only at their origin and become muscular in character, e.g. external carotid artery. Although an elastic artery can give rise to elastic or muscular branches, no elastic artery has been found to originate from a muscular vessel. Elastic arteries include the aorta, brachiocephalic trunk, arteria subclavia, arteria carotis, arteria iliaca, and arteria pulmonalis. Elastic laminae in the tunica media are fenestrated. It has been suggested that the fenestrations are for the passage of nutrition from the lumen to the tunica media, or insertion points for the tendons of smooth muscle cells (Hassler, 1962). The number of fenestrated elastic laminae in the tunica media of elastic arteries varies among species: 40-60 in large elastic arteries such as human aorta and only 20 in the much smaller aorta of the rabbit (Wolinsky & Glagov, 1967). Their number decreases gradually toward the periphery of the arterial system. The elastic laminae are concentrically arranged and spaced equidistantly. A network of delicate elastic fibrils interconnects the elastic laminae. Through this arrangement, the media has a highly structured and elaborate elastic framework which gives it great resilance and strength. The smooth muscle cells are placed within this framework and their arrangement and orientation varies among species. In the aorta of the squirrel monkey (Rhodin, 1974),

the smooth muscle cells are attached to the elastic laminae. Cliff (1976) maintains that alternating elastic and muscular laminae give major mechanical strength to the media, and adapt rapidly to alterations in pressure and flow because of their compliance and plasticity. Wolinsky and Glagov (1964) suggested that the entire wall of an elastic artery is constructed to distribute uniformly the tensile forces to which it is exposed. The elastic components and the smooth muscle cells are held together by a network of collagenous fibrils, all of which are embedded in a viscous and gelatinous mucopolypeptide ground substance (Rhodin, 1980). It has been suggested that the orientation and close relationship among elastic fibers, elastic laminae, collagenous bundles, and smooth muscle cells serve as a continuous fibrous helix, which can withstand longitudinal and lateral forces of expansion. Wolinsky and Glagov (1964) stressed that the lamellar unit of aortic medial structure, made up of elastin, collagen, and smooth muscle, contributes to the viscoelastic properties and could account for many of the static and dynamic mechanical features of elastic arteries.

6.2.2. Carotid Sinus

6.2.2.1. Structural Features

Carotid sinus is located where common carotid artery bifurcates. There are species differences in the anatomical structures involved in the forming of the sinus. An occipital sinus near a carotid sinus has been observed in cat and dog and an occipital-internal carotid sinus has been reported in calf and pig (Muratori, 1967). In guinea

pigs, the carotid sinus is located where common carotid artery bifurcates into the the external and occipital arteries. Despite these anatomical differences, the areas supplied most densely with baroreceptors in all species are elastic in nature. Thus, the media of the sinus region is thinner, contains significantly less smooth muscle and has a higher elastin content than the internal, external, or common carotid arteries (Kirchheim, 1976). Compared with the common carotid artery, the medial-intimal thickness of the carotid sinus was less, the thickness of the adventitia greater, even though the whole-wall thickness was the same (Rees & Jepson, 1970; Bagshaw & Fischer, 1971). In the media of the carotid sinus of rabbits, the elastic sheets alternate with single layers of smooth muscle cells (Rees, 1968). The carotid arterial musculature cranial and caudal to the sinus is helically disposed (the angle between the helical turn and the long axis of the artery varying from 20^{o} to 80^{o}). However, the smooth muscle sheets in the sinus media are arranged circumferentially with respect to the long axis of the vessel (angle of spiraling 90^{o}). Moreover, the neighboring smooth muscle cells were closer to each other, the intercellular distance being 150-200 A [intercellular distance between individual smooth muscle cells in vessels of elastic type was reported to be 10,000 A (Prosser et al., 1960)]. These structural features enable the carotid sinus to decrease its radius more efficiently than other vessels of the elastic type by activation of its smooth muscle cells.

6.2.2.2. Baroreceptors

Generally, the baroreceptors of the carotid sinus are found in

the adventitia, and mainly at the medial-adventitial border (see review, Kirchheim, 1976). Two types of sensory nerve endings have been described in the carotid sinus of man and mammals (Abraham, 1967; Willis & Tange, 1959): type 1 receptors consisting of a few relatively thin myelinated fibers that run together until they form a diffuse arborization in a large loose plexus, and type 2 receptors consisting of a single thick myelinated fiber that runs for quite a distance until an extremely rich arborization begins. In the carotid sinus, the receptors appear to lie between the collagen fibers of the adventitia parallel to the longitudinal axis of the vessel. The majority of the baroreceptors are connected to medullated (A-type) nerve fibers. The A-fibers in the sinus nerve of cat, dog, rabbit, and man range from 2 to 12 um in diameter (Kirchheim, 1976). Of the A-fiber population in the sinus of cat, about two thirds belong to chemoreceptors and one third to baroreceptor fibers (Fidone & Sato, 1969). Also, nonmedullated (C-type) fibers were reported in the sinus nerve of cats (Fidone & Sato, 1969).

One model for baroreceptor function (Brown & Wilson, 1980) suggests two linked events: mechanical and electrical. The mechanical sequence links intrasinus pressure to receptor deformation while the electrical sequence links receptor deformation to axonal discharge. According to this model, pressure produces circumferential wall strain which deforms the baroreceptors and causes them to discharge. In the steady state, the relationship between pressure and diameter or strain (defined as the ratio of the change in radius to the radius at zero pressure) is non-linear as is the relationship between pressure and discharge. However the

relationship between discharge and strain is linear (Andresen et al., 1978). These findings indicate that steady-state non-linearities result from the properties of the tissues in which the receptors are embedded. These findings further imply that for small perturbations, receptor strain, receptor potential, and axonal discharge are probably linear functions of displacement (Brown & Wilson, 1980).

In this section, the mechanical link will be discussed in more detail considering its relationship to the structural features of the carotid sinus. The nonneural wall components, i.e., the fibroelastic tissues, transmit the intensity of stimuli by causing mechanical deformation of the receptor-generator region (Paintal, 1972). Baroreceptors respond to distortion of their receptor regions by generating action potentials and, thus, provide information to the central nervous system for cardiovascular control. It is believed that distortion activates a relatively nonselective cation-conducting channel through the membrane (Andresen & Kunze, 1987). In a model system for mechanotransducer ion channels, it has been shown that membrane tension is coupled to the channel by cytoskeletal strands that concentrate the strain energy from a large area of membrane and thereby provide high sensitivity (Sachs, 1987). In these channels, transduction is accompanied by a strain-dependent increase in the probability of being open. Thus, the baroreceptors are stimulated by the mechanical deformation of certain areas within the arterial wall rather than by the intra-arterial pressure changes per se (Heymans & Neil, 1958; Peterson, 1962). There is evidence that the deformation that determines the electrical activity of

carotid sinus baroreceptors is stretch, and that transmural pressure is the factor determining the stretch or "tone" of the receptors (Kirchheim, 1976). The extent to which the receptors, or the fibroelastic tissues associated with receptors, will become stretched for a given transmural pressure will depend on two factors: 1) the geometry of the vessel wall, i.e., its internal radius and wall thickness; and 2) the elastic and viscous properties of the vessel wall - determined to a large extent by the tissue composition of the arterial wall (Burton, 1954). It has been shown that for a given transmural pressure the (stretching) stress is highest at the innermost part of the vessel wall and decreases in a nonlinear fashion toward the outer part of the wall (Timoshenko, 1962). In the carotid sinus, the main baroreceptor-bearing area (i.e., the medial-adventitial border) is placed close to the sinus lumen, as the media-intima layer of the sinus is thin (see above). Thus, the geometry of the carotid sinus (Bagshaw & Fischer, 1971; Rees, 1967a,b; Rees & Jepson, 1970) seems to provide a special advantage for the transformation and transduction of transmural pressure to radial; i.e., circumferential stretch at those sites within the vessel wall that bear the receptor endings.

6.2.2.3. Sympathetic Innervation

In addition to its afferent innervation, the carotid sinus region receives sympathetic efferent innervation. Sympathetic efferent fibers have been repeatedly described in the carotid sinus of mammals with traditional microscopic methods (Eyzaguirre & Lewin, 1961). Rees (1967b) demonstrated the presence of NE-containing

sympathetic nerve endings in the carotid tree of rabbits using parallel electron and fluorescent microscopic techniques. In the walls of the internal and external carotid arteries, sympathetic nerves closely associated with smooth muscle were observed along the medial-adventitial border. In the wall of carotid sinus, sympathetic nerves were not observed along the medial-adventitial border. However, naked or partly naked sympathetic fibers were observed in the adventitia in close association with smooth muscles; these muscle cells, which are not identified elsewhere in the carotid tree, are circumferentially and longitudinally arranged and are set back from the medial-adventitial border by distances of up to 20 um. Confirming Rees' findings (1967b), Reis & Fuxe (1968) reported fluorescence studies that revealed a rich noradrenaline-containing network in the deeper layers of the adventitia in the rabbit and cat carotid tree. The fluorescent beads, indicative of sympathetic nerve terminals, were distinctly set back from the media and were more dispersed in the sinus wall compared with the neighboring carotid wall, in which the nerve terminals were more close to the media. Both groups (Rees, 1967b; Reis & Fuxe, 1968) demonstrated that the fluorescence was reduced or disappeared after chronic stimulation of the cervical sympathetic ganglion or after administration of reserpine.

It is generally acknowledged that the transducer properties of arterial baroreceptors are influenced by the mechanical behavior of the baroreceptor regions. Also, there is evidence that direct application of vasoconstrictor agents to the carotid sinus could modify its mechanical properties, and thereby influence baroreceptor

nerve output (Heymans & Neil, 1958). However, It is controversial whether sympathetic efferents influence the sensitivity or set point of the sinus baroreflexes. The effects of stimulating sympathetic efferents have been negative (Moncada & Scher, 1963) or variable and modest (Koizumi & Sato, 1969; Sampson & Mills, 1970; Wurster & Trobiani, 1973). Bergel et al. (1980) has shown that local application of norepinephrine to the sinus of anesthetized dogs constricted the sinus; the radius was reduced at all intrasinus pressures except the highest studied. Norepinephrine increased both wall tension and nerve activity at a given radius. Similar findings were obtained with sympathetic stimulation in the sinus of this species (Peveler et al., 1980). Thus, these findings in dogs with norepinephrine and sympathetic stimulation suggest that sympathetic efferents can increase the sensitivity of baroreceptors in the carotid sinus, probably by an increase in the active tension of smooth muscle cells coupled in series with receptor endings. Also, sympathetic nerves may have a direct effect on carotid sinus baroreceptors. Munch & Brown (1987) showed that orthodromic electrical stimulation of the efferent sympathetic nerves to the aortic arch caused baroreceptor unloading via vasoconstriction and, possibly, direct excitation of some units. In vivo, the sensitivity of baroreceptors may be controlled by a feedback burst of sympathetic activity which coincides with diastole; this would accord the pulse a major role in reflex control of the cardiovascular system.

6.3. Preamble

The carotid sinus is the "elastic segment" of the bifurcation region of carotid arteries; it receives a rich sensory innervation from branches of the glossopharyngeal nerve. In various species, ultrastructural studies of this region showed mixed findings on the location of baroreceptors in sinus wall and on their structural characteristics (see Shin et al., 1987). Also, it has been suggested that baroreceptor function is modulated by adrenergic nervous activity. The density of the adrenergic innervation and presumably the degree of adrenergic modulation varies among species. Therefore, we examined the fine structure of the baroreceptors and the adrenergic innervation of the guinea-pig carotid sinus by transmission electron microscopy (TEM).

The sinus region has unique pressure sensing functions, and this has been related to the structural differences among the carotid sinus and its adjacent regions. TEM studies of the carotid sinus demonstrated a close association between baroreceptor terminals and surrounding elastic tissues, emphasizing the importance of elastic tissues. Despite this, three dimensional studies of elastic tissues in the bifurcation region of carotid arteries have not been conducted in any species to date. Therefore, we compared the organization and fine structure of the elastic laminae of the carotid sinus and its adjacent arteries by scanning electron microscopy (SEM).

6.4. Hypothesis

The structural features of baroreceptors in carotid sinus show

interspecies homogeneity, and the elastic laminae of the carotid sinus, coupled with its dense innervation by baroreceptors, relate to its pressure-transducing functions.

6.5. Objectives

Overall: Characterize the morphology of carotid sinus in guinea pigs.

1. Investigate the distribution and ultrastructure of baroreceptors in the carotid sinus from normal, control guinea pigs.
2. Study the adrenergic innervation in the carotid sinus from 6-hydroxydopamine-treated guinea pigs.
3. Compare the elastic networks of the carotid sinus and its adjacent arteries to study structural basis for the physiological role of the carotid bifurcation.

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7. OBSERVATIONS ON THE FINE STRUCTURE OF THE BARORECEPTORS AND ADRENERGIC INNERVATION OF THE GUINEA-PIG CAROTID SINUS*

7.1. Introduction

In guinea pigs, unlike most other species studied, the carotid sinuses are located where the common carotid arteries bifurcate to form the occipital and external carotid arteries (Rees, 1967). The sinus regions receive rich sensory innervation from branches of the glossopharyngeal nerves, and the medial layer of the sinuses contains large amounts of elastic tissue. However, ultrastructural studies of the sinus regions in various species have yielded mixed findings: baroreceptive endings have been demonstrated in the adventitia or extending along the medio-adventitial border in rabbits, cats, and dogs (Knoche & Schmitt, 1964; Knoche & Addicks, 1976, Knoche et al., 1977, 1980), in the outer medial layers in rabbits and dogs (Dropmann, 1967; Chiba, 1972), and in the media even in the innermost layers adjacent to the intima — in guinea pigs (Böck & Gorgas, 1976; Gorgas et al., 1983). Furthermore, not all structural characteristics of baroreceptor terminals are present in every terminal, and they vary among species. A classification of the terminals, based on their ultrastructural characteristics, has been proposed (Böck & Gorgas, 1976) but is not widely used (Knoche

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et al., 1980). These characteristics include densely packed and heterogeneous mitochondria, osmiophilic homogeneous and lamellated bodies, neurotubuli, neurofilaments, glycogen granules, clear and granular vesicles, translucent axoplasmal matrix, lamellar systems (laminar, Knoche et al., 1980), lipid droplet-like material, and vacuoles (Rees, 1967; Chiba, 1972; Böck & Gorgas, 1976; Knoche et al., 1977, 1980).

Studies of the adrenergic innervation of the carotid sinus with fluorescence histochemistry have shown species-related variations in the distribution and number of nerve fibers (Reis & Fuxe, 1968; Böck & Gorgas, 1976; Stanton & Hinrichsen, 1980). In all species studied, however, 6-hydroxydopamine (6HD) induces rapid, selective degeneration of adrenergic nerve terminals, leaving cholinergic neurons, Schwann cells, and glial cells intact (Thoenen & Tranzer, 1968; Lever et al., 1971), and the degenerated fibers become electron-dense or swollen (Tranzer & Thoenen, 1968, Tranzer & Richards, 1971; Cobb & Bennett, 1971).

We examined the distribution and ultrastructure of baroreceptors in guinea pigs, and investigated the adrenergic innervation of the walls of the carotid sinus after treatment with 6HD.

7.2. Methods

Guinea pigs of either sex, weighing 400-750 g, were anesthetized with urethane, 1.5 g/kg, injected ip. The trachea was cannulated (PE 240), both common carotid bifurcations were isolated, and the common carotid arteries were cannulated centrally (PE 50). Both bifurcations were perfused at a rate of 1 ml/min with 10 ml of

heparinized saline containing sodium nitroprusside (20 µg/ml) followed by 10 ml of ice-cold 4% glutaraldehyde containing 0.1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4. Both bifurcations were resected, further fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, washed in 0.2 M cacodylate buffer (pH 7.35), and refixed for 1.5-2.0 h in 1:1% osmium tetroxide with potassium ferrocyanide in 0.1 M sodium cacodylate. The tissues were then rinsed in distilled water, refixed in saturated uranyl acetate for 2 h, dehydrated in a series of graded ethanols and embedded in LX112 resin. Ultrathin serial sections were cut with a diamond knife fitted to a Sorvall Porter-Blum MT-2 ultramicrotome, mounted on naked copper grids, stained with uranyl acetate and lead citrate, and examined with a Phillips 410 transmission electron microscope (TEM). Degeneration of adrenergic fibers was induced by injecting 6HD ip, 50 mg/kg, 12 h before the animals were killed.

7.3. Results

Innervation. Large numbers of nerve bundles were found in the outer layers of the tunica adventitia. Each bundle was surrounded by a perineurium enclosing one or two myelinated fibers and several unmyelinated fibers. Groups of unmyelinated fibers, surrounded by Schwann cells and a basal lamina, were embedded in collagen fibers (Fig. 1). Several large perineuria were seen; they enclosed many unmyelinated fibers (0.36-0.70 µM diameter) and some contained myelinated fibers also (Fig. 2), most of which were much thicker (0.36-1.5 uM diameter). The ratio of unmyelinated to myelinated fibers was 13-18:1.

Penetration. Many large myelinated fibers lost their myelin sheath as they penetrated the adventitia. Others, however, lost their sheath only when they had reached varying depths of the media (Fig. 3), and thus resembled the "premyelinated axons" described by Rees (1967). The fibers, now unmyelinated, accompanied by their Schwann cells, branched and penetrated deeper into the media through gaps in the medial elastic laminae (Fig. 4) and formed rounded endings (Fig. 5). These receptor endings contained cytoplasmic inclusions, most commonly densely packed mitochondria, various types of vesicles, osmiophilic bodies and glycogen granules. In some sections, a thick myelinated fiber was seen between two medial elastic laminae. Serial sections revealed accumulations of mitochondria and osmiophilic bodies along the cytoplasmic membrane, and neurofilaments in the central part of the receptor adjacent to where the myelin sheath disappeared (Fig. 3).

Distribution of baroreceptor nerve terminals. Baroreceptors (sensory-nerve terminals) were distributed throughout the adventitia and media. Most of them were extremely rich in organelles and each was enveloped by a Schwann cell (the "terminal cell" of Knoche & Schmitt, 1964). The nerve complex was enclosed by basal laminae, and embedded in densely packed collagen fibers (Fig. 6). In some sections the receptor surface was partly or completely [illegible] of the cytoplasm of the terminal cell and covered only [illegible] lamina. In a few sections a complicated basal laminar [illegible] observed between the receptor and its adjacent Schwann [illegible]

elastic membrane (Fig. 7). In the adventitia, the receptors were embedded in the matrix of elastic and collagen fibers (Fig. 8).

Ultrastructural characteristics of receptors. Nerve terminals in the adventitia or media showed the ultrastructural features of baroreceptors, varying from a few types of structure to the entire range within individual terminals. Some receptors contained numerous mitochondria, densely packed, of varied size and shape (Figs. 3,5,9), whereas others contained only a few. Other features observed were strongly osmiophilic lamellated or homogeneous bodies that resembled mitochondria in size and shape (Figs. 6,10), aggregates of osmiophilic granules (Fig. 11), vacuoles and vesicles (Fig. 12), abundant glycogen granules (Fig. 7) and a lamellar system. Examination of serial sections revealed that the numbers and sizes of vacuoles, and the numbers of mitochondria, varied according to the plane of sectioning.

Several types of vesicles were observed. Those in one receptor surrounded by adventitial collagen fibers were of varied electron density and granular texture, ranging from 100 to 1000 A in diameter (Fig. 8). Receptors in the media were packed with granular vesicles of almost identical electron density and size; they and other types of receptors appeared to be within the cytoplasm of the same terminal cell (boxed area of Fig. 7). The glycogen granules were interspersed among the mitochondria or clumped in the cytoplasm. The lamellar system consisted of layers of osmiophilic membranes; in one receptor, in the media, numerous vacuoles and lamellated bodies were present inside and outside the lamellar system and there

was an adjacent area of translucent amorphous cytoplasm (Fig. 13).

Effect of 6-hydroxydopamine on adrenergic nerves. The carotid sinuses of guinea pigs given 6HD (50 mg/kg ip) contained a few adrenergic nerve fibers or terminals in various stages of degeneration: some were strongly osmiophilic (Fig. 14), others greatly swollen. These degenerated fibers were in the adventitial matrix of collagen fibers, mostly around the vasa vasorum. No fibers of this type were found in the media.

7.4. Discussion

Innervation. Our findings indicate that the elastic segment (the carotid sinus) of the carotid bifurcation has the densest innervation. TEM of the various areas (common carotid artery, external carotid artery, occipital artery) of this bifurcation revealed that the carotid-sinus region contained the highest density of elastic tissue and least smooth-muscle cells. These guinea-pig carotid sinuses were similar to those of humans, dogs, rabbits and cats; they were unlike those of rats and mice, which contain no clearly defined elastic segment (Rees, 1967; Böck & Gorgas, 1976).

The carotid sinus was densely innervated by both unmyelinated and myelinated nerve fibers. Perineuria enclosing bundles of the fibers were common; however, although some contained only unmyelinated fibers, none contained only myelinated ones. The medial layer contained occasional nerve fibers that appeared to be losing their myelin sheaths and taking on the features characteristic of baroreceptors. Thus, as in rats (see Brown,

1980), a proportion of baroreceptors in guinea pigs possess myelinated afferents. Saum et al. (1976), using electrophysiological techniques, established that baroreceptors possess both myelinated and unmyelinated afferents; however, the anatomical features of baroreceptors subserved by both types of afferents have not been clearly determined. Our findings suggest that the myelinated afferents innervate baroreceptors with characteristics similar to those presumably innervated by unmyelinated afferents; but, in the absence of precisely oriented serial sections, one cannot be sure of this.

In both normal and 6HD-treated animals, some unmyelinated fibers were sympathetic efferents. Stanton and Hinrichsen (1980) showed with fluorescence microscopy that adrenergic nerves are sparse in the guinea-pig carotid sinus, a finding we confirmed with TEM after treatment with 6HD.

C. Ultrastructure of receptors. We identified baroreceptors by their content of densely packed mitochondria, together with osmiophilic, lamellated, and homogeneous bodies, vacuoles, aggregates of osmiophilic granules, conspicuous accumulations of glycogen granules or neurofilaments and vesicles of various types, and lamellar systems. These characteristics have been described in many other species (Chiba, 1972; Knoche & Addicks, 1976; Knoche et al., 1980), as well as guinea pigs (Rees, 1967; Böck & Gorgas, 1976). We also found some features not previously described for guinea-pig carotid sinus. Accumulations of glycogen granules in baroreceptors have been reported for rabbits, cats, mice and dogs

(Böck & Gorgas, 1976; Knoche & Addicks, 1976; Knoche et al., 1977, 1980), but not for guinea pigs (Böck & Gorgas, 1976). We also found two types of receptors containing large numbers of granular vesicles. Some were almost filled with these structures, 400 1000 A in diameter, of widely varied electron density and granular texture; in others, electron density and size were invariant. It is unlikely that these endings containing densely packed vesicles represent efferent adrenergic endings, because the granular textures of the vesicles did not resemble those of catecholamine-containing endings (Knoche & Addicks, 1976); moreover some of the endings were in the tunica media, where degenerated adrenergic fibers were not found in the carotid sinuses from 6HD-treated animals. Similar baroreceptor endings have been reported only in mice (Böck & Gorgas, 1976). Some sections contained large lamellar systems in association with accumulations of glycogen granules or numerous vacuoles, similar to those described in carotid-sinus baroreceptors of rabbits, dogs, and cats (Chiba, 1972; Knoche & Addicks, 1976; Knoche et al., 1977, 1980). They were not noted, however, in the study of guinea pigs by Böck & Gorgas (1976).

The vacuoles in endings that contained lamellated bodies, homogeneous osmiophilic granule-containing and densely osmiophilic bodies (see Fig. 11) are similar to those reported in rabbit carotid sinus by Knoche et al., (1980). These workers suggested that the vacuoles and densely osmiophilic bodies represent end-products of mitochondrial degeneration. Our observations, however, could be interpreted to suggest that the process may be continuous rather than divergent: mitochondria ——> osmiophilic bodies ——>

organelles that contain osmiophilic granules —> vacuoles. It is noteworthy that endings containing large numbers of osmiophilic bodies and vacuoles contained few mitochondria, even though the mitochondria were readily apparent in the cytoplasm of the terminal cells. The greatly varying ratios between mitochondria and osmiophilic bodies support the suggestion that the latter may represent a stage in mitochondrial degeneration. Finally, we noted areas of translucent amorphous axoplasm that have been described in cats, rabbits, and dogs (Knoche & Addicks, 1976; Knoche et al., 1977, 1980).

Having examined many receptors, with various features, we believe the baroreceptors we identified are representative of those in guinea-pig carotid sinuses. Thus, guinea-pig baroreceptors resemble those of other species, and previous reports of differences from cats, mice, and rabbits could arise from failure to examine large enough numbers.

We were unable to distinguish receptors innervated by myelinated or unmyelinated afferents, but we did observe myelinated fibers penetrating the media through gaps in the elastic laminae and developing characteristics typical of baroreceptors.

Location of baroreceptors. We found baroreceptors in the adventitia and at almost all levels in the media, even the inner layers. In following the route of a few myelinated fibers in serial sections, we found that both myelinated and unmyelinated fibers penetrate the media through gaps in the elastic laminae (e.g., Fig. 4). We traced one myelinated axon, filled with numerous

mitochondria, osmiophilic bodies, and neurofilaments, that ran between two elastic laminae: in one section it appeared to lose its myelin sheath and become a typical sensory ending with a terminal cell and basal lamina. This finding is particularly interesting, others having claimed that myelinated fibers lose their sheaths in the inner layers of the adventitia or at the adventitial—medial junction (Knoche & Schmitt, 1964; Rees, 1967; Knoche & Addicks, 1976; Knoche et al., 1977, 1980; Kimani & Mungai, 1983), before becoming the "stem fibers" or "stem axons" described by Böck & Gorgas (1976).

Aumonier (1972), using light microscopy, confirmed earlier reports that baroreceptor endings were confined to the adventitia in dog, cat, and rabbit carotid sinuses. However, electron microscopy has revealed baroreceptors in the outer part of the tunica media (Chiba, 1972; Knoche et al., 1980), and Böck & Gorgas (1976) observed them in even the innermost layers of the media, adjacent to the intima, in guinea pigs. Thus, our findings concur with others'.

Associations between connective tissue and receptors. The frequent association of receptors, terminal cells, and basal laminae indicate that these three form morphological and functional units (Knoche et al., 1980). These units were surrounded by collagen and elastic fibers and fibroblasts in the tunica adventitia and media. In some instances the unit's terminal-cell covering was deficient or absent, so that only the basal lamina was visible between the receptor and surrounding tissues.

The role of basal laminae in baroreceptor function is unclear,

but the closeness of the receptors to their surrounding tissues via the basal laminae implies contact between the receptor unit and the deformable tissue of the sinus wall (Fig. 7). Krauhs (1979) also emphasized the importance of the basal laminae. He observed extensive, prominent basal laminae around rat aortic baroreceptors, closely associated with muscle cells, elastic fibers and collagen bundles, and postulated that the laminae protect the sensory terminals from distending forces. Recently, in the carotid sinus of giraffes, Kimani & Mungai (1983) observed basal laminae juxtaposed between elastic fibers and nerve terminals and, in some places, encapsulating bundles containing these fibers and collagen.

Location of adrenergic nerves in the carotid sinus. The density and distribution of adrenergic nerve fibers in the carotid sinus wall varies among species (Reis & Fuxe, 1968; Böck & Gorgas, 1976; Stanton & Hinrichsen, 1980). Stanton & Hinrichsen (1980) noted sparse innervation of the carotid sinus in guinea pigs, and Böck & Gorgas (1976) reported that the adrenergic innervation was only to the adventitia in this species. Our failure to locate degenerated adrenergic fibers in the tunica media of the carotid sinus in the 6HD-treated animals confirms these findings.

Our findings suggest closer similarity of structural features of baroreceptors in guinea pigs and other species than reported to date; i.e., many of the structures we have now identified for the first time in guinea pigs have been described for rats, mice, cats, dogs and giraffes. These include the accumulations of glycogen granules, large lamellar structures, and distinctive areas of

translucent cytoplasm in the baroreceptors. Thus it seems that these and other features are simply additional observations that confirm interspecies homogeneity - but they militate against a ready explanation for the guinea pig's lower mean resting systemic arterial blood pressure.

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Fig. 7-1. A typical fiber bundle surrounded by a perineurium (p) in the outer layer of the guinea-pig sinus wall. Myelinated fibers (my) and groups of unmyelinated fibers (u) are sheathed in Schwann cells (sch) and embedded in collagen (c). (magnification, x9,100)

Fig. 7-2. Large perineuria (p) in the outer layer of the guinea-pig sinus wall, containing many unmyelinated (u) or mixed myelinated (my) and unmyelinated fibers. (magnification, x2,800)

Fig. 7-3. Thick myelinated fiber (my) adjacent to medial elastic laminae (el); numerous mitochondria (m), neurofilaments (f), and osmiophilic bodies (o) are visible at the site where the fiber loses its myelin sheath. A Schwann-cell cytoplasm (sch) containing mitochondria (m) also can be seen. (bl, Basal lamina; c, collagen.) (magnification, x9,100)

Fig. 7-4. An unmyelinated axon (u) has penetrated the tunica media through gaps in several layers of medial elastic tissue (el). It is accompanied by a Schwann cell (sch). (c, Collagen.) (magnification, x2,800)

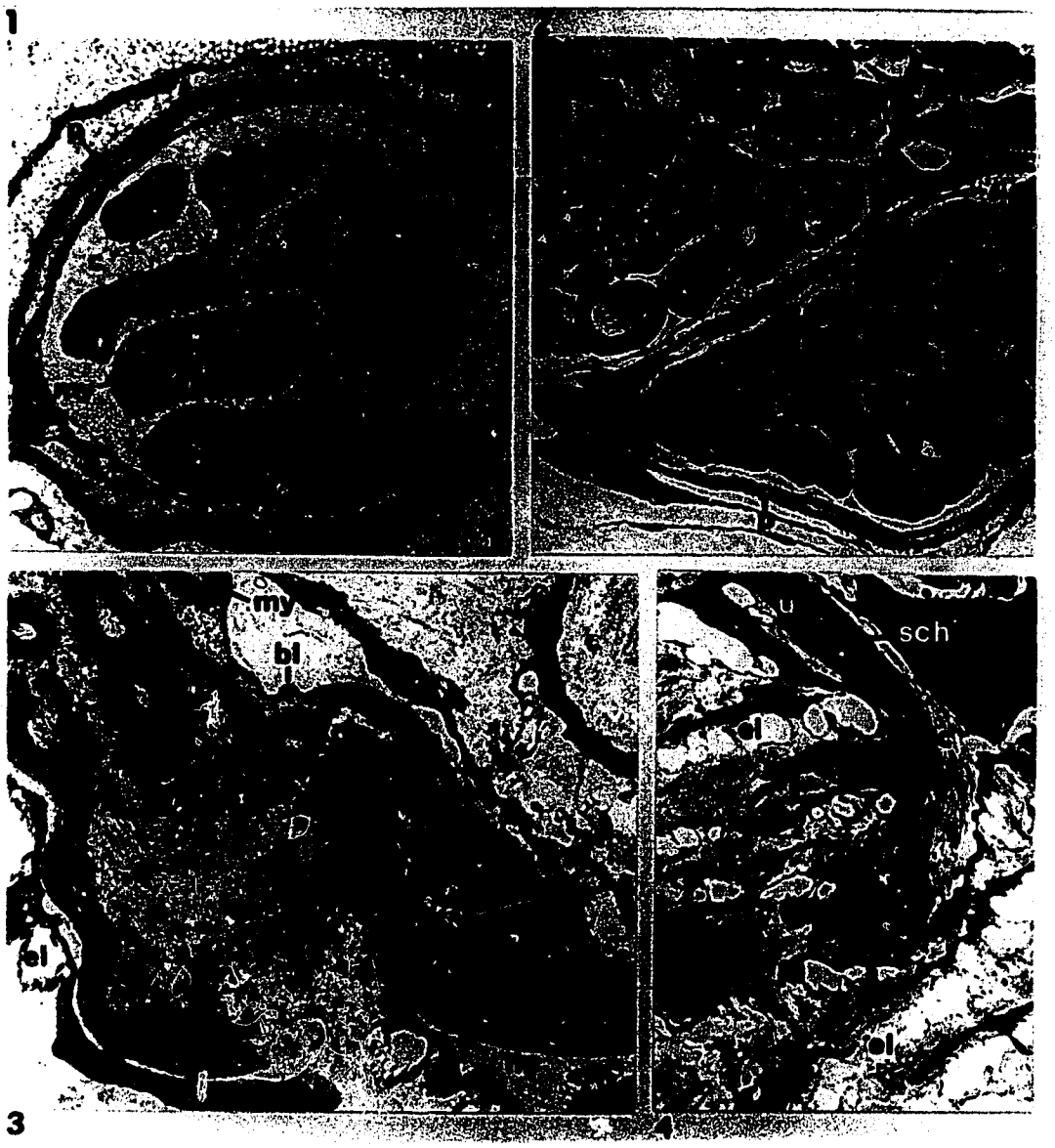
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Fig. 7-5 A typical baroreceptor nerve terminal (r) in the media of the guinea pig sinus wall, showing enlargement of an axon (a) to form a terminal. The receptor contains numerous mitochondria (m) and is enveloped in cytoplasmic Schwann-cell (sch) processes. (c, Collagen.) (magnification, x9,100)

Fig. 7-6. A baroreceptor nerve terminal in the carotid sinus wall is filled with osmiophilic bodies (o). Schwann-cell cytoplasm enwrapping the receptor axon contains mitochondria (m) and endoplasmic reticulum (er). (bl, Basal laminae; c, dense collagen fibrils; n, nucleus of Schwann cell.) (magnification, x12,000)

Fig. 7-7. Baroreceptor nerve terminals located between layers of medial elastic laminae (el). One receptor contains a large collection of glycogen granules (g) and a lamellar system (ls). The receptor was denuded of Schwann-cell cytoplasm in some areas (arrows) and covered only by basal lamina. A basal lamina complex (bl) connects a Schwann cell to another terminal (t). (c, Collagen; er, endoplasmic reticulum of Schwann cell; n, Schwann-cell nucleus.) (magnification, x 14,000)

Inset: Higher magnification of the boxed area reveals a receptor embedded in the Schwann cell. It contains neurotubuli (nt), and numerous granular vesicles (v) of similar size and electron density. (magnification, x33,600)

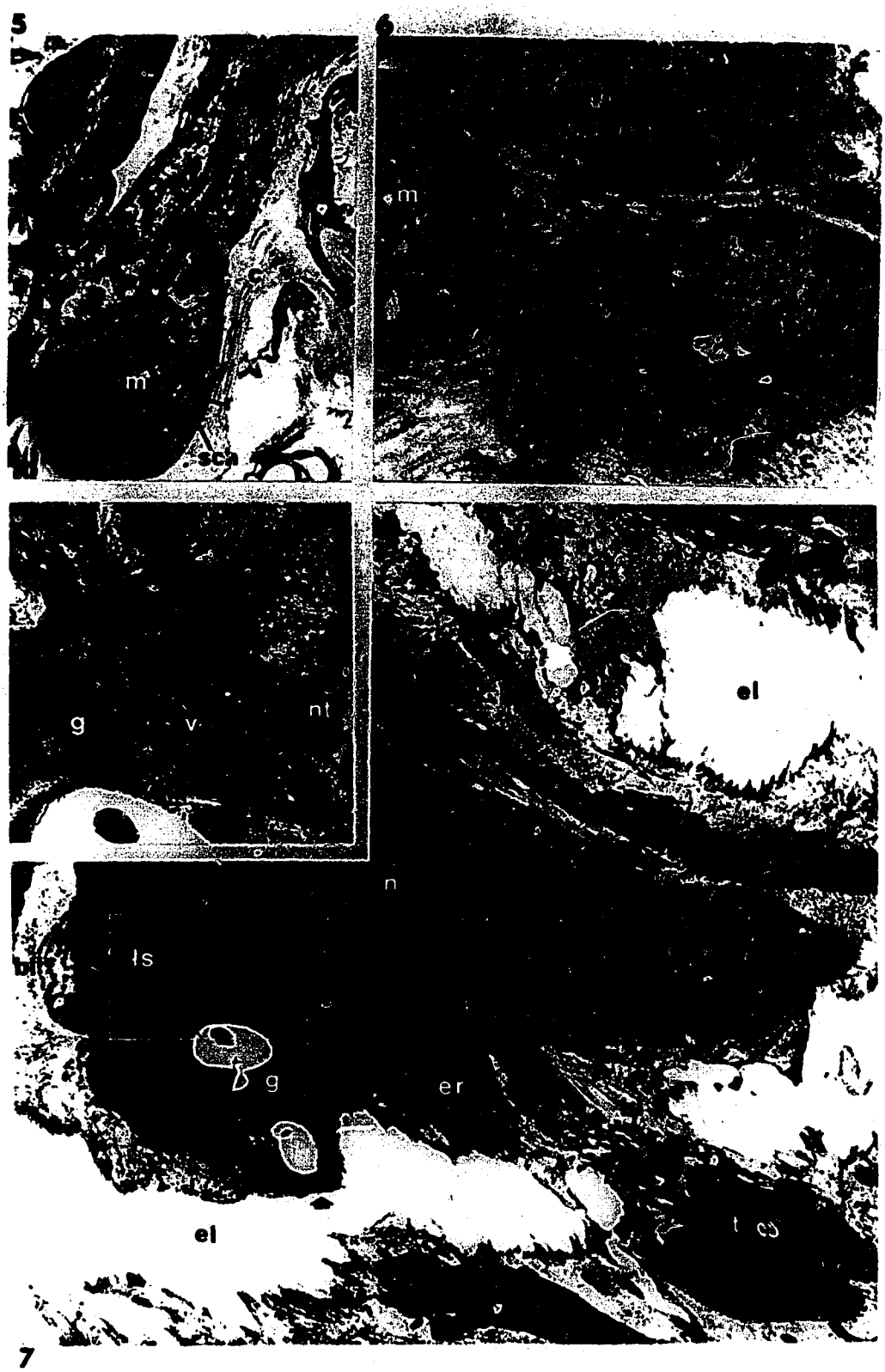
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Fig. 7-8. A baroreceptor nerve terminal in the tunica adventitia of the carotid sinus wall contains predominantly granular vesicles of varied electron density and granular texture. (c, Collagen.) (magnification, x64,800)

Fig. 7-9. A baroreceptor nerve terminal adjacent to the nucleus (n) of a terminal cell. Note the densely packed mitochondria (m) in the receptor and an aggregation of organelles in the cytoplasm of the terminal cell. (c, Collagen; er, endoplasmic reticulum of the terminal cell.) (magnification, x17,000)

Fig. 7-10. A baroreceptor nerve terminal in the carotid sinus wall is enveloped in Schwann-cell cytoplasmic processes (sch). It contains many osmiophilic homogeneous bodies (hb) and some osmiophilic lamellated bodies (lb). (bl, Basal laminae.) (magnification, x29,000)

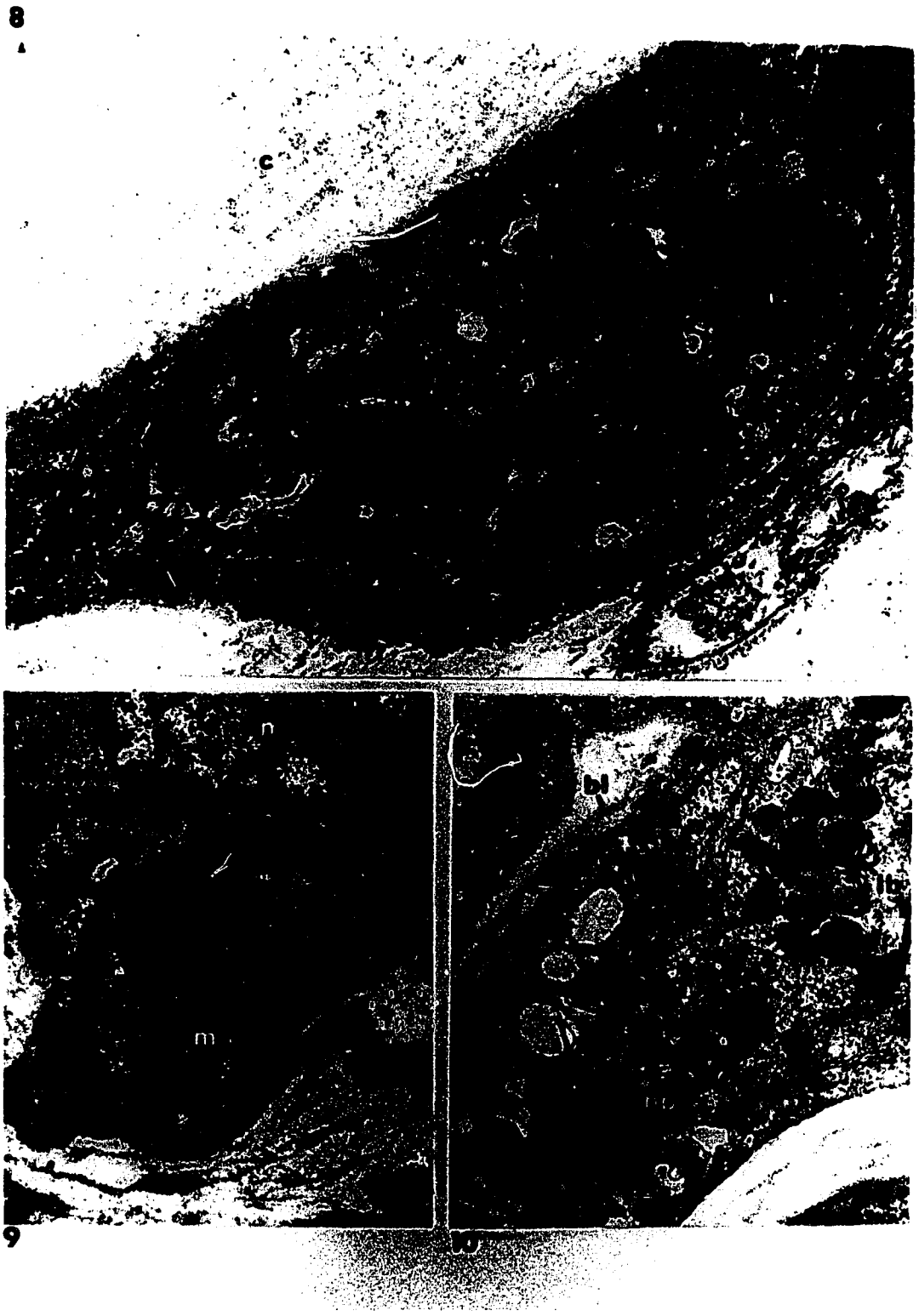
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Fig. 7-11. A baroreceptor nerve terminal in the carotid sinus wall is enveloped in Schwann-cell cytoplasmic processes (sch). It contains many homogeneous osmiophilic bodies (ob), aggregates of osmiophilic particles (op), and vacuolar organelles (vac). (c, Dense collagen fibrils.) (magnification, x40,000)

Fig. 7-12. Baroreceptor nerve terminals located close to the medial elastic laminae (el). One receptor contains a large number of vacuoles (vac), and vesicles (v) of various sizes. (c, Collagen; n, Schwann- cell nucleus.) (magnification, x29,000)

Fig. 7-13. A baroreceptor nerve terminal located close to the nucleus (n) of a terminal cell rich in organelles. Note the large intraneural lamellar system (ls), numerous vacuoles (vac) and lamellated bodies (lb) inside and outside this system, and a translucent amorphous axoplasm (*). (ga, Golgi apparatus.) (magnification, x31,200)

Fig. 7-14. Electron micrograph of degenerated adrenergic fibers (a) from guinea- pig carotid sinus 12 h after the injection of 6-hydroxydopamine. Well-preserved nonadrenergic fibers (na) are visible. (c, Dense collagen fibers in the adventitia.) (magnification, x14,500)

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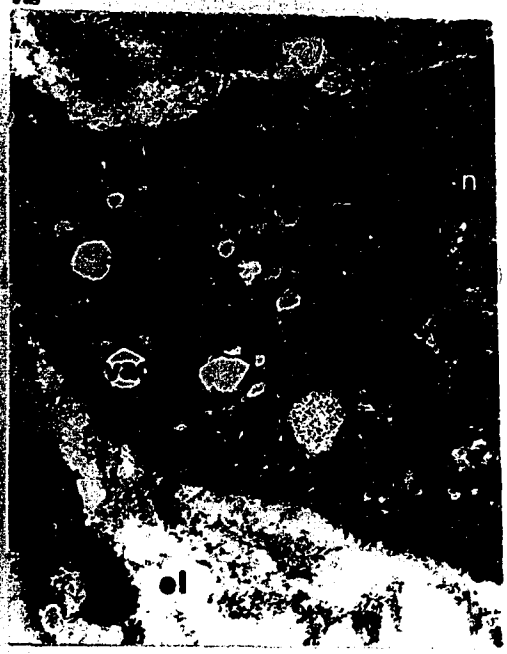

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8. SCANNING ELECTRON MICROSCOPY OF ELASTIC NETWORKS FROM THE BIFURCATION REGION OF GUINEA PIG CAROTID ARTERIES*

8.1. Introduction

In all species studied, including humans, the walls of the arteries contain elastic laminae, termed the fenestrated membranes of Henle by Dees (1923). The internal surface of these laminae is a felt-like sheet composed of fine elastic fibers that merges into a coarse plexus of much larger elastic fibers (Dees, 1923). The internal elastic laminae (IEL) contain numerous small holes that vary in size and shape among arteries in the same species (Hassler, 1962; Cook et al., 1975).

The carotid sinus (CS) has unique pressure-sensing functions, and in guinea pigs, unlike most of other species studied, it is located where the common carotid arteries (CCA) bifurcate to form the occipital arteries (OA) and external carotid arteries (ECA) (Rees, 1967) (Fig. 1). Light and transmission electron microscopic studies (Rees, 1967; Rees & Jepson, 1970; Knoche et al., 1980; Shin et al., 1987) indicate that the sinus region receives rich sensory innervation from branches of the glossopharyngeal nerves and contains a thinner, more elastic and less muscular tunica media

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H.S. Shin, W.C. Hulbert, and D.F. Biggs, Blood Vessels 25:63-74 (1988); Proc. West. Pharmacol. Soc. 29:97-100 (1986)

compared with its contiguous arteries - presumably a reflection of differences in function. Scanning electron microscopy (SEM) was used in this study to compare the organization and fine structure of the elastic laminae of the CS and its adjacent arteries, in an attempt to relate any structural differences to the physiological role of the carotid bifurcation.

8.2. Methods

Female guinea pigs of the Hartley strain were purchased from Charles River Laboratories, St-Constant, P.Q., Canada. They were 8-10 months old and weighed 450-550 g. They were anesthetized with urethane, 1.5 g/kg body weight, injected intraperitoneally, and the trachea was cannulated with a polyethylene tube (PE240) at the midcervical level.

Both CCA were isolated in the midcervical region; they were cannulated (PE50) with the tip pointing cephalad, and their bifurcations were exposed. Both arteries were perfused at 1 ml/min (transmural pressure not measured) with solutions at 20-22^{o}C, first with 10 ml of heparinized saline containing sodium nitroprusside (20 µg/ml) and then with 10 ml of fixative (2.5% glutaraldehyde in 0.1 M Sörensen's phosphate buffer, pH 7.4). Cardiac and respiratory arrest occurred after perfusions of about 2 ml of the latter. Each arterial region containing the bifurcation was then removed en bloc. For this study, seven bifurcations were obtained from 4 animals and 2 of them were processed for scanning IEL, 3 for adventitial elastic layer (AEL), and the other 2 for both IEL and AEL.

To aid examination: IEL, the bifurcations were opened along several planes (Fig. 1); AEL, PE50 tubes were inserted into the CCA and ECA to help prevent their collapse during the remainder of procedures. No special care was taken to maintain the shape of the other arteries (ascending pharyngeal, occipital). Specimens were immersed in 0.1 M NaOH at 95°C for 1.25 or 2.0 h, then transferred to 0.1 M NaOH at 20-22°C (5 min), distilled water (5 min), 0.1 M HCl (2 min), and 0.9% saline (5 min), fixed in 2.5% glutaraldehyde fixative for 2 days, and rinsed 3 timed in 0.15 M phosphate buffer (Song & Roach, 1983). Finally, they were freeze-dried (Grut et al., 1977), mounted on aluminum SEM stubs with conductive silver paint, dried overnight, and gold-coated (10 nm thick) in a cool sputter coater (1.2 kV, 30 mA, 30 s). Adluminal and adventitial surfaces of the specimens were examined at 20 kV with a Cambridge 250 scanning electron microscope.

8.3. Results

Longitudinal sections of the region of bifurcation showed that the walls of the CCA and ECA consisted of a continuous sheet of IEL, about 2.0 μm thick, that was attached to an outer mesh of elastic tissues arranged in a web-like honeycomb pattern (Fig. 2). The web was fine beneath the adluminal surface but became coarser as it approached the adventitial surface (Fig. 2a, b). By contrast, cross sections of the CS revealed attachment of the outer surface of the IEL to a much coarser mesh of elastic tissues (Fig. 2c). Cross-sectional thickness of the elastic networks was 87.5 μm (±6.9 SD, n = 9; measurements from the same bifurcations) in the CCA and

ECA and 46.8 μm (±2.4 SD, n = 4 measurements) in the CS.

Internal Elastic laminae. The IEL fenestrated. Throughout each specimen, the adluminal surface appeared as a membranous sheet (Fig. 3a) of varied texture of elastic fibers. In the OA, high magnification of the adluminal surface revealed two distinct patterns (Fig. 3b): much of the surface was coarse and felt-like, but in some regions, continuous with this, was a loose arrangement of unidirectional fibers. Higher magnification of the transitional areas showed that some adjacent fibers had fused to form thicker fibers, and both types had a coarse surface comprising fine fibrillar components (Fig. 3c). In other regions, the surface was composed of a very fine, open-meshed plexus of fibrillar components continuous with an underlying membranous sheet (Fig. 3d).

In specimens from ECA, a coarse felt-like surface, ridged in placed, was also visible on the adluminal surface of the IEL (Fig. 4a). High magnification of a ridged area revealed tightly fused bundles of unidirectional fibers (Fig. 4b), some of which were composed of fine fibrillar material. The mainly fibrous surface was seen over a wide area (Fig. 4c), particularly in the ECA adjacent to the apex of the bifurcation. In other regions, the adluminal surface consisted of either a complex network of elastic fibers contiguous with the underlying coarse surface (Fig. 4d) or a membranous sheet of multidirectional fine fibrillar components that were fused together in a lacy pattern (Fig. 4e). In some areas, this sheet underlay wide "ribbons" of elastic tissue (Fig. 4f).

The adluminal surface of the CCA comprised a felt-like mass similar to that in the OA, ECA, and CS.

In the CS, a similar felt-like mass on the adluminal surface appeared to be composed of tightly fused fine fibrillar components (Fig. 5a, b). In part of the cranial sinus, a coarse open-meshed plexus of elastic fibers was superimposed on and continuous with the underlying membranous sheet (Fig. 5c). In some regions, unidirectional loose fibers were seen on the adluminal surface of the IEL; these were not as prominent as in the OA (Fig. 5d). The cranial sinus exhibited several unique structures. Firstly, the adluminal surface of the IEL of the interior of the cranial sinus bore many blister-like structures (Fig. 5e), rounded or apparently collapsed and of various shapes and sizes, maximally accumulated near the circumferential center line of the cranial sinus. Secondly, there were large numbers of fenestrations, of various sizes and shapes, separated by fine elastic fibers; these were most numerous in the region between the entrance to ascending pharyngeal artery (APA) (not shown in Fig. 5e) and the maximal accumulation of blister-like outgrowths (Fig. 5e). Finally, a unique honeycomb-like mesh arose at the entrance to the APA and extended over about one-fourth of the sinus's circumference; higher magnification revealed that the orientation of adjacent IEL changed from horizontal to vertical at this site to form the honeycomb rugae (Fig. 5f).

Adventitial Elastic Laminae. Unlike the adluminal surface of the IEL, the adventitial surface consisted of a complex network of elastic fibers. In the CCA, individual elastic fibers fused and divided repeatedly to form interwoven bands of varying width that

made up a complex network of elastic tissue (Fig. 6a). The continuous membranous sheet characterizing the IEL was absent. Higher magnification revealed that the outermost elastic networks were linked to deeper ones; also, fine fibrous or fibrillar components connected adjacent intra- or interlaminar wide elastic ribbons (Fig. 6b). Where deeper layers were exposed, they appeared as a continuous fenestrated sheet; again, the fenestrations were of varying shapes and sizes (Fig. 6c). Marked regional heterogeneity in the density of fibers was also noted (Fig. 6d).

Generally, findings similar to those described for the CCA were observed in the other arteries; however, there were differences. Particularly in the OA, a continuous membranous sheet similar to the IEL was seen over a wide area (Fig. 7); it was fenestrated, with holes resembling those in the IEL. In the CS, in addition to the radiating type of network like that in the CCA and ECA (Fig. 6a), networks whose directionality was roughly maintained were seen at low magnification (Fig. 8a). Some regions of the CS contained bundles of tightly fused, multidirectional fibers (Fig. 8b); this type of fusion was also visible in the junctional region of the CS and OA, much finer networks in the CS enwrapped the OA (Fig. 9a). At the junction of the CS and ECA, the elastic networks of the CS were much finer than those of the ECA (Fig. 9b). Patches of densely packed fibers found in the CCA were observed in the ECA.

Fenestrations. Fenestrations were seen on the IEL throughout the entire bifurcations, and their distribution varied among vessels and among regions of the same vessel. They appeared much more unevenly

distributed in the CS and OA than in the CCA and ECA.

In the OA, fenestrations were in groups (Fig. 3a). In the interior cranial sinus, there were clusters of fenestrations separated by fine elastic fibers near the area of maximal accumulation of blister-like structures (Fig. 5e) but none in contiguous regions in any of the specimens examined, indicating uneven distribution even in the same vessel. The number of fenestrations per square millimeter, counted from randomly chosen micrographs of the same bifurcation representing an area 40 X 40 μm^2, varied among branches: CCA and ECA, 13,625 (±3,075 SD, n = 5 micrographs); CS, 26,250 (±10,508 SD, n = 4), and OA, 36,094 (±10,660 SD, n = 4). The fenestrations varied in shape and size; all bifurcations examined contained either simple pits 0.2-5.0 um in diameter, or holes 2.0-6.0 μm in diameter, which were spanned by delicate but intact elastic fibers (Fig. 3b, d, 5a) that were continuous with the sheet of coarse elastic fibers surrounding the hole (Fig. 5b). There was some overlap in size between these two types of fenestrations. Particularly in some regions of the CS, clusters of simple pits were closely associated with fibrous components of the adluminal surface of the IEL (Fig. 5d).

Fenestrations were also seen on the AEL of all arteries in the bifurcation region, but only in regions where individual fibers fused to form wide bands or continuous sheets of elastic tissue (Fig. 6c, 7).

Fibrous or Fibrillar Components. Fine fibrous or fibrillar components were identifiable on the surface of the IEL and AEL. In

the IEL, these components (0.1-0.2 µm) fused to form thicker fibers (0.3-0.5 µm) or the adluminal surface of the IEL (Fig. 3c, 4b, e). Intact fenestrations were structures bridged by thread-like spans about 0.3 µm thick (Fig. 5b). In the AEL of the CCA, the densely packed elastic fibers were 1.8-2.6 µm thick (Fig. 6d) and the fibrous or fibrillar components that connected bands of elastic tissue (Fig 6b) were 0.1-0.3 µm thick.

8.4. Discussion

All bifurcations examined contained a well-defined continuous fenestrated IEL, as has been shown by others in the aorta and other arteries of many species. In 1923, Dees described the elastic lamina of human and bovine aorta and renal arteries as a thin continuous sheet that on closer examination was a felt-like mass of fine elastic fibers fused to a coarse, open-meshed plexus of thicker elastic fibers whose meshes were elongated in the direction of the longitudinal axis of the blood vessel. Smith (1976) determined the orientation of the elastic fibers in laminar from the pulmonary trunks and aorta from rabbits, and Song & Roach (1983) confirmed many of Dees's original findings in their SEM studies of thoracic aorta from sheep. In a later study, whose main objectives was closer examination of the fenestration, Song & Roach (1984) described three patterns of the adluminal surface of the IEL of canine thoracic and abdominal aorta - a coarse felt-like surface, a smoother surface (figure not shown), and a smooth membranous sheet continuous with a loose arrangement of unidirectional fibers. In the present study, however, I distinguished five distinct patterns in guinea pig

carotid bifurcations: (a) a coarse felt-like surface (Fig. 3b); (b) a coarse felt-like surface continuous with a loose arrangement of unidirectional fibers (left lower corner of Fig. 3b); (c) a coarse surface composed of a very fine, open-meshed plexus of fibrillar components continuous with an underlying membranous sheet (Fig. 3b); (d) a ridged surface composed of tightly fused bundles of unidirectional fibers (Fig. 4b) and (e) a membranous fine fibrillar components that were fused together in a lacy pattern (Fig. 4e).

The predominant pattern of the AEL was of individual elastic fibers fused in a complex lacy network that on closer examination was found to be continuous with underlying network structures. In some regions, however, particularly near junctions between the CS and its adjacent arteries, the AEL appeared to be a membranous sheet similar to that in the IEL although less well defined.

In sheep aortae, Song & Roach (1983) showed that the adluminal surface of the IEL kept its felt-like membranous appearance, and the AEL its fibrous appearance, when aortae were digested in 0.1 N NaOH for 1-48 h. These workers noted that numbers of fenestration in both IEL and AEL correlated positively with digestion time, but that the AEL appeared "more fibrous" after long digestion times. Crissman (1986, 1987) recently reported that networks prepared form canine femoral arteries by two different digestion techniques were generally similar in appearance. However, he claimed that there were minor differences, among networks, attributable to the method of preparation. Although it has not been demonstrated conclusively that the preparative technique for this study has not effect on the elastin fibers in the networks, it is felt that findings in this

study and those of others (see above, Song & Roach, 1983; Crissman, 1986), strongly suggest that the features of the IEL and AEL observed in this study are predominantly morphological and not artifactual.

Examination of the IEL of the CS and its adjacent arteries revealed fenestrations in all laminae. However, the fenestration were unevenly distributed and varied greatly in size and shape, precluding comparison of measurements of density and size. Like Song & Roach (1984), who studied aortae, I could not relate the fenestrations to the functions of the CS or contiguous vessels. Nevertheless, they could be divided into two groups: simple pits or holes (diameter 0.2-5.0 μm), and holes spanned by delicate elastic fibers (diameter 2,0-6.0 μm). The simple pits may have arisen from the latter, as a few intermediate forms with apparently collapsing spans were observed and there was some overlap in size between the two types. However, the existence of simple pits much smaller than those spanned by delicate elastic fibers seems to suggest that they are always thus. Cook et al. (1975) noted larger holes in arteries that had been distended during preparation. In the present study, great care was taken to ensure that all of the bifurcations were perfused at constant rates and with a medium containing sodium nitroprusside to relax the smooth muscle. Thus, the holes observed in the present study should have been comparable in all specimens - although, of course, it cannot be absolutely certain that the method of preparation did not affect their size.

Fenestrations were observed throughout the IEL and in those regions of the AEL where individual fibers were fused into a band or

continuous sheet - and appearance more characteristic of IEL structures - but none in those AEL regions that consisted of fibrous networks. In studies of cerebral arteries, Hassler (1962) suggested that the fenestrations are for the passage of nutritional materials from the lumen to the tunica media, or insertion points for the tendons of smooth muscle cells, whereas Campbell & Roach (1983) suggested that their size and distribution probably related to the elastic properties of the laminae. Thus, the function of fenestrations in the IEL characterized by a well-defiend continuous membranous sheet may be different from that of fenestrations in AEL characterized by fibrous networks.

Very fine fibrous or fibrillar components 0.1-0.3 μm thick identified in both IEL and AEL were of similar diameter to those in bovine ligamentum nuchae (Gotte et al., 1972). In the IEL, they merged into an uneven, ridged surface of large fibers, similar to Crissman's (1984) finding in canine saphenous veins, or fused to form its adluminal surface. By contrast, in the AEL, they connected wider bands of elastic tissue.

Longitudinal sections of the carotid bifurcations show that the walls of the CCA, ECA and CS consist of a continuous IEL, attached to an outer mesh of elastic tissue that is arranged in a web-like, honeycomb pattern, a structural feature that may contribute to maintaining elasticity of the arteries (Wolinsky & Glagov, 1967; Carnes et al., 1977). It is noteworthy that, in the CCA and ECA, the web was fine beneath the adluminal surface and became coarser as it approached the adventitial surface. Surprisingly, the cross-sectional thickness of the entire elastic layers in the CS was about

half that of the CCA and ECA; also, the CS had fewer elastic layers and thus formed a coarser mesh than the CCA and ECA. The relatively coarse mesh seen adjacent to the adventitial surface of the CCA and ECA and throughout the CS did not appear to be due to the method of preparation: in all experiments, the fine honeycomb of rugae beneath the adluminal surface was well preserved.

Some investigators (Wolinsky & Glagov, 1967; Bergel, 1961a, b) have correlated the viscoelastic properties of the arterial walls with the close association among elastic and collagen fibers and smooth muscle cells (Wolinsky & Glagov, 1967; Bergel, 1961a, b) and have stressed the importance of the elastic lamella and the contents of its adjacent interlamellar zone to the proper functioning of the arterial walls (Wolinsky & Glagov, 1967). It may be postulated that the differences of the CS from its adjacent arteries, as shown in this study, are associated with its unique pressure-sensing functions, although one would have predicted more, rather than less, elastin in this structure.

Unlike the other areas of the bifurcation, the cranial sinus exhibited several unique structures - blister-like outgrowths, dense clusters of fenestrations, and an unusual honeycomb-like maze near the entrance to the ascending pharyngeal artery.

The many morphologic differences between elastic laminae of he CS and its adjacent arteries, coupled with the dense innervation of the CS region of the bifurcation by baroreceptors (Rees, 1967; Rees & Jepson, 1970; Knoche et al., 1980; Shin et al., 1987), lead to the conclusion that the unique features of the IEL and the findings in sections of the CS relate to a pressure-transducing function of the

sinus region. The unique structures - blister-like outgrowths, dense clusters of fenestrations, and honeycomb-like mazes - may increase the elasticity of the IEL directly below the endothelial cells (Campbell & Roach, 1983). This increased elasticity, in conjunction with the cross-sectionally thin, uniformly coarse, network of elastic tissue, may help amplify pressure gradients in the vessel wall and enhance the transmission of "stretch" to the sensory receptors that, in guinea pig CS, have been found mainly in the medial layers (Böck & Gorgas, 1976; Shin et al., 1987).

8.5. References

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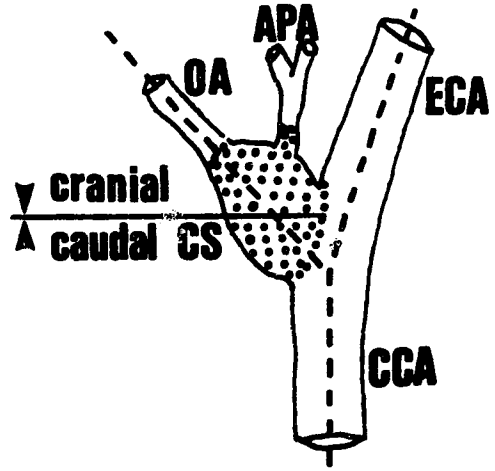
Fig. 8-1. Diagrammatic representation of guinea pig carotid bifurcation (ventral view of right side; after Rees, 1967). CCA = common carotid; ECA = external carotid; OA, occipital; APA = ascending pharyngeal artery; CS = carotid sinus (stippled area). Broken lines indicate the planes along which bifurcations were opened.

Fig. 8-2. SEM of elastic lamina in carotid arteries, showing the IEL and the coarser outer mesh of elastic tissue arranged in a web-like honeycomb pattern. Bar = 20 μm. a CCA. b ECA. c CS, showing the IEL's undulating adluminal surface and a uniformly coarse network of elastic tissue.



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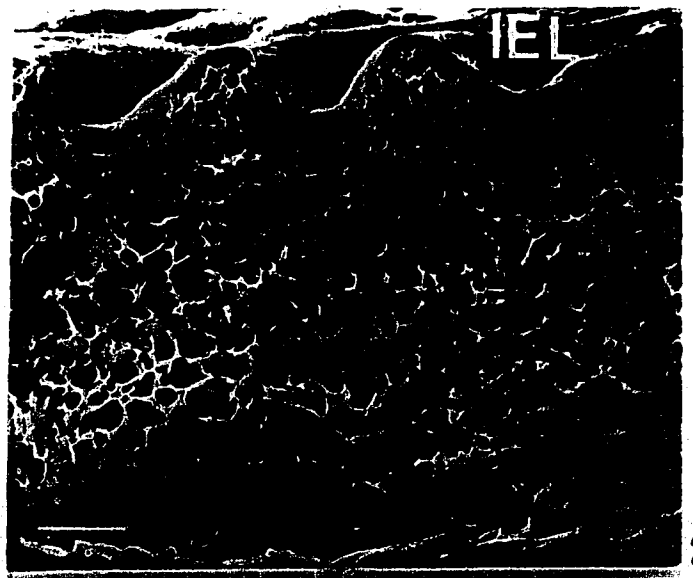

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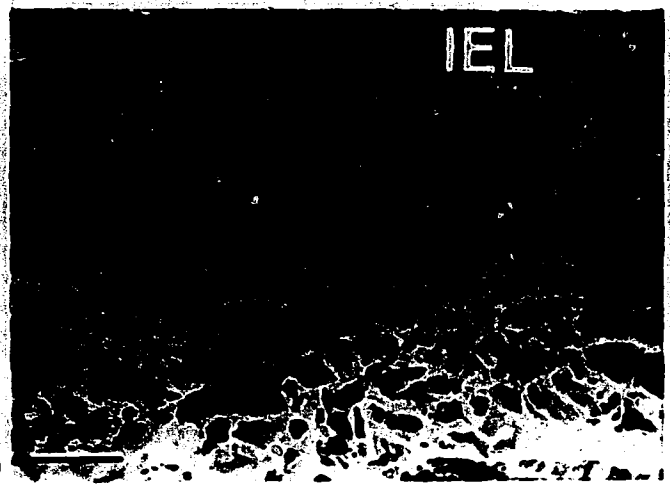

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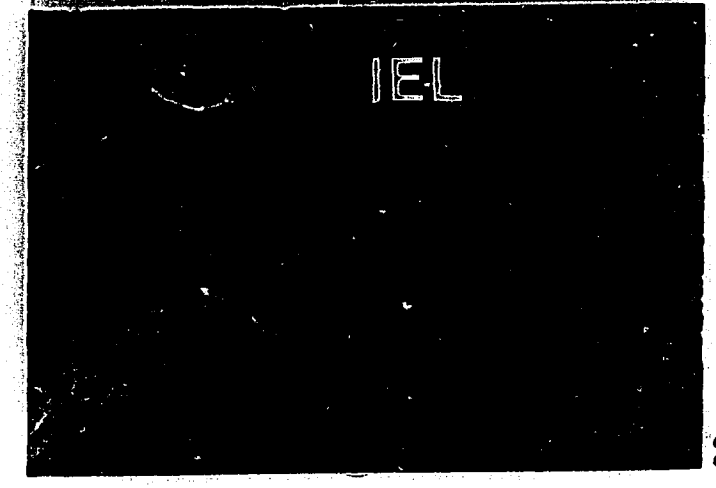

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Fig. 8-3. SEM of the adluminal surface of IEL from an OA. a A membranous sheet is pierced by numerous, unevenly distributed fenestrations. Bar = 20 μm. b High magnification of the boxed area in a shows two patterns - a coarse, felt-like surface, which is distinct from but continuous with a loose arrangement of unidirectional fibers (left lower corner) that on higher magnification (c) are shown to be fine fibers fused into bundles forming thicker fibers. In some areas, the felt-like surface is visible beneath the fibers. Bars = 4 and 1 μm, respectively. d The coarse surface (arrow), composed of a very fine open-meshed plexus of fibrillar components, is continuous with an underlying membranous sheet. Bar = 4 μm.


3a
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Fig. 8-4. SEM of the adluminal surface of an IEL from an ECA. a There are two distinctly different patterns interspersed - coarse and felt-like, and ridged (arrow). Bar = 10 um. b Higher magnification (X9,500) of the ridged surface reveals bundles of tightly fused fibers, mostly unidirectional. Unlike the appearances in OA specimens (see Fig. 3c), no underlying stroma is visible. Bar = 1 μm. c An area close to the apex of the bifurcation has a mainly fibrous surface and bears blister-like outgrowths (arrows). Bar = 10 μm. d A complex network of elastic fibers merges into the underlying coarse surface. Bar =4 μm. e Multidirectional fine fibers and fibrillar components are fused, forming a membranous sheet in which their directionality is maintained. Bar = 4 μm. f Ribbons of elastic tissue cover the surface and form wide, dense bands in some areas (arrows). Bar = 4 μm.

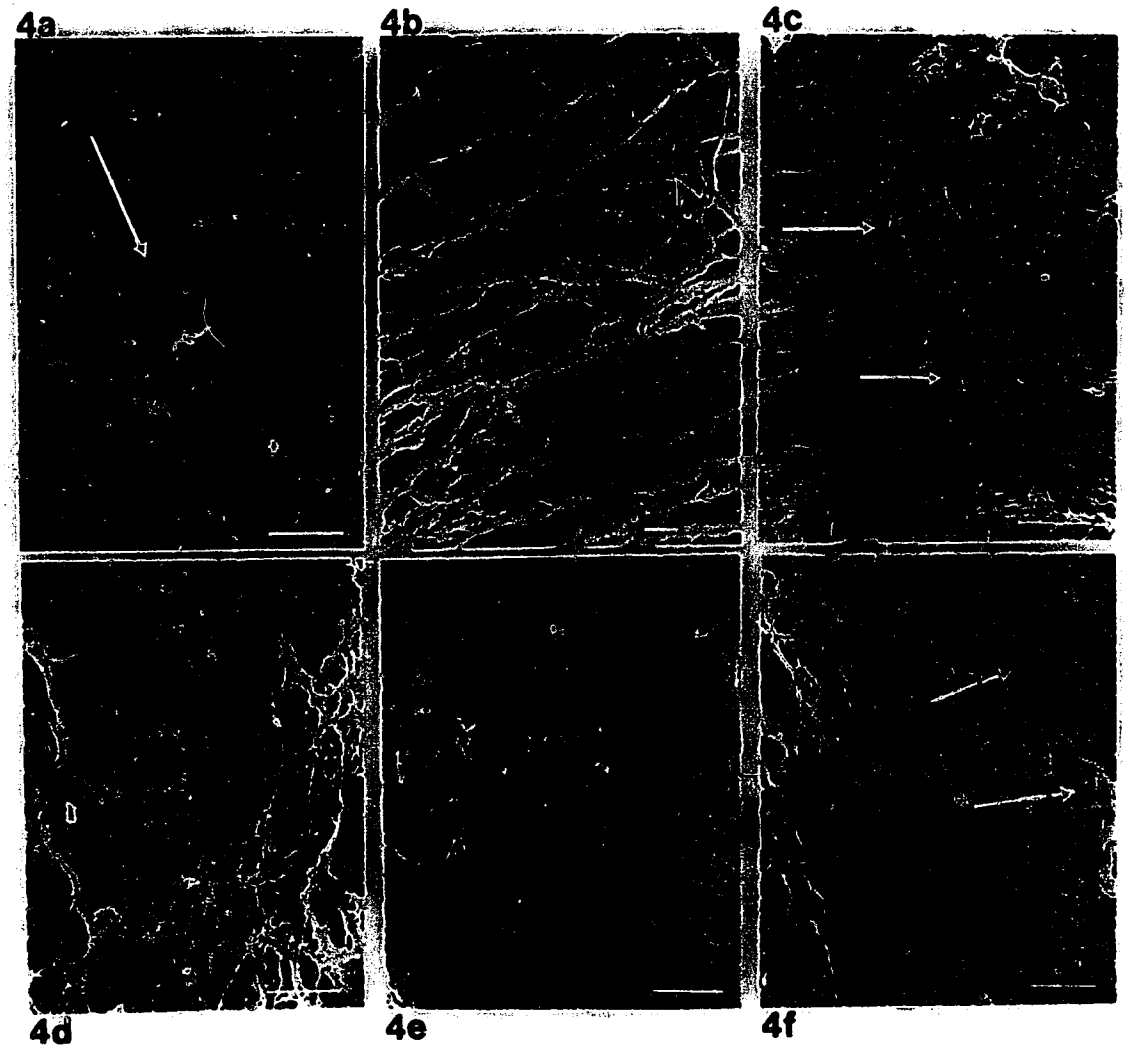
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Fig. 8-5. SEM of the adluminal surface of the IEL from a CS. **a** Felt-like surface showing three distinct types of fenestration: simple holes (arrow), those spanned by intact delicate elastic fibers (solid arrowhead). Bar = 4 μm. **b** High magnification of a fenestration spanned by delicate elastic fibers. Bar =2 μm. **c** Coarse, open-meshed plexus (arrow) of elastic fibers continuous with an underlying felt-like mass at the cranial sinus. Bar = 2 μm. **d** Accumulation of numerous simple fenestrations and fibrous components of the adluminal surface of the IEL. Bar = 4 μm. **e** Accumulation of blister-like outgrowths (solid arrowhead) and fenestrations (open arrowhead) separated by fine elastic fibers on the interior of the cranial sinus. Bar = 20 μm. **f** Honeycomb-like mesh arising from the origin of the APA. Bar = 4 μm.

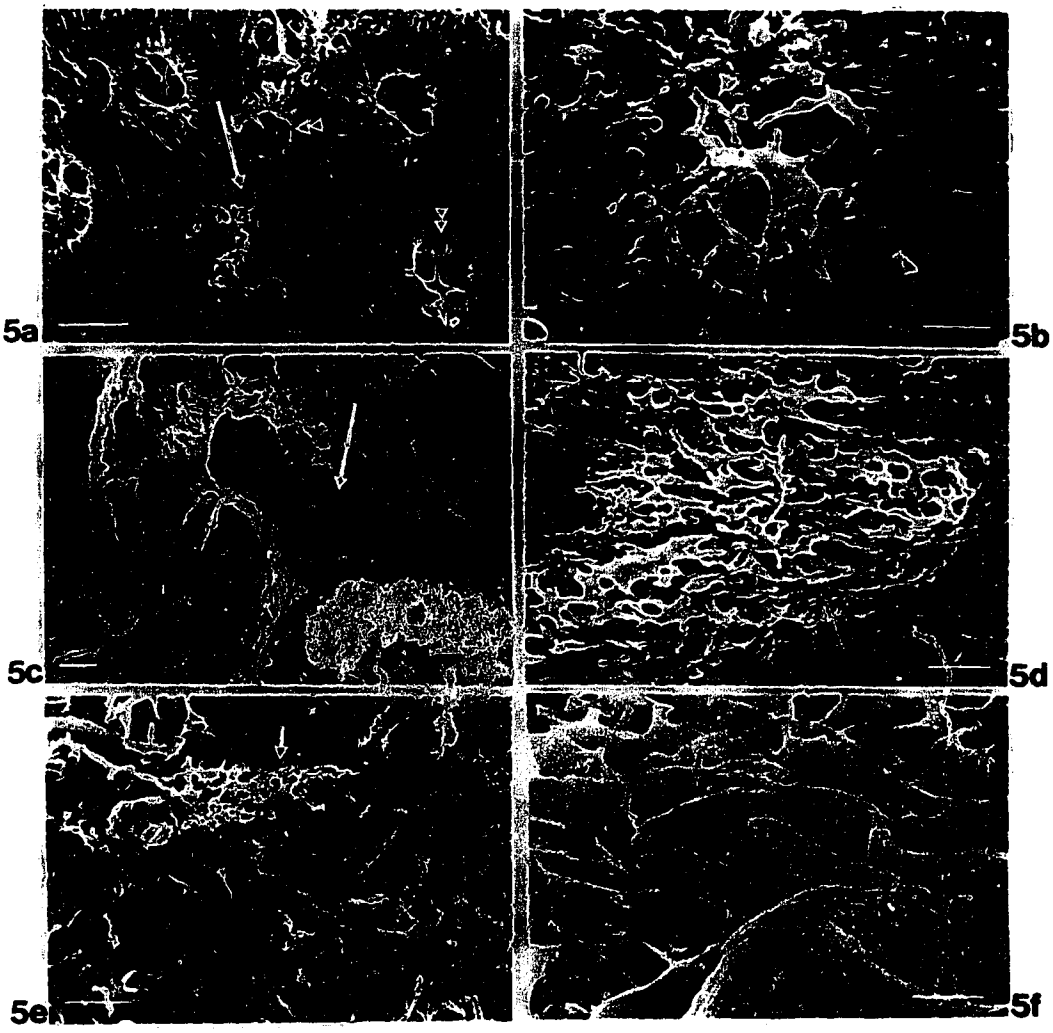
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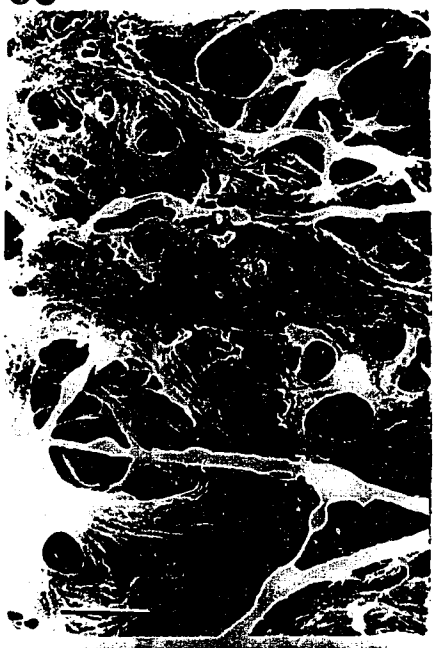
Fig. 8-6. SEM of AEL from a CCA. a Complex multilayered network of elastic tissue. Bar = 20 um. b Higher magnification of the area boxed in a reveals that the continuous membranous sheet characteristic of the IEL is absent. Very fine elastic components (arrows) bridge adjacent intra- and interlaminar wide bands of elastin. Bar = 4 μm. c Exposed deeper layers form a continuous sheet pierced by fenestrations similar to those in the IEL. Bar = 4 μm. d The pattern changes abruptly from an open, patchy network (left) to a densely packed area containing a mass of tangled and some parallel fibers that occludes view of any underlying structure. Bar = 10 μm.

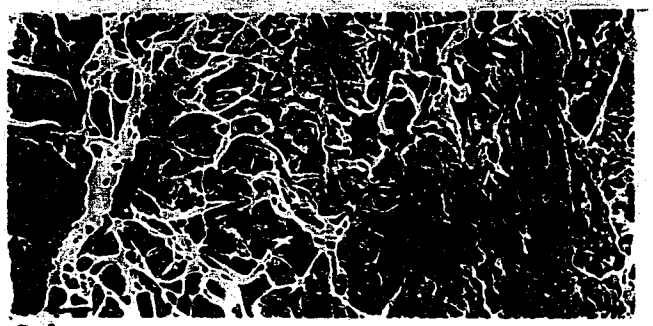
Fig. 8-7. SEM of AEL from an OA, showing a continuous membranous sheet. This sheet is similar to that in the IEL and is more extensive than in any other adventitial region. Bar = 4 μm.

6a

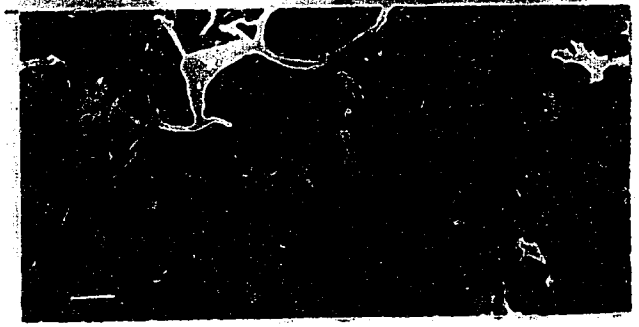
6b

6c





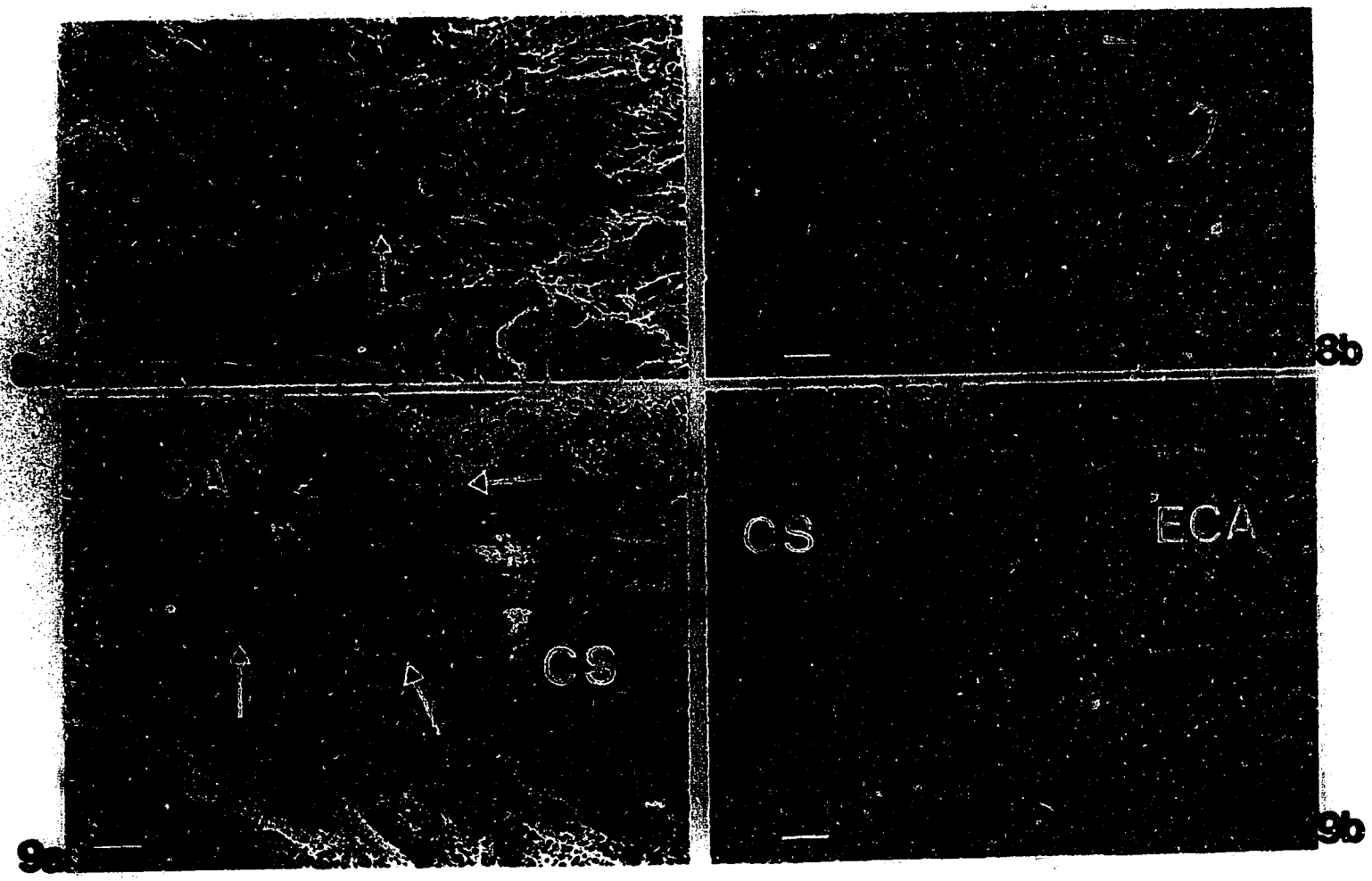
6d



7

Fig. 8-8. SEM of the AEL from the CS. a Tightly packed network of fine fibers whose directionality is roughly maintained, unlike their pattern in Fig. 6a. Bar = 40 μm. The densely packed area (arrow) is similar in some respects to that in Fig. 6d. b Tightly fused multidirectional fibers form a membrane-like sheet. Bar =4 μm.

Fig. 8-9. SEM of the AEL at the junction of the CS.a Junction with the OA: very fine networks (arrowheads) in the CS are enwrapping the OA. Bar = 40 μm. b Junction with the ECA, showing abrupt transition from a very fine, complex elastic network in the CS to a relatively coarse network in the ECA. Bar = 10 μm.


8b
CA
CS
CS
ECA
9b

9. GENERAL DISCUSSION AND CONCLUSIONS

The literature review revealed that:

1. Baroreceptors in the carotid sinus (CS) display morphologic features which vary among species.
2. The location of the baroreceptor endings in the CS of various species is variable - baroreceptors have been demonstrated in the tunica adventitia, along the medio-adventitial border, in the outer media, and even in the innermost layers of the media adjacent to the intima.
3. In the sinus region, baroreceptors are closely associated with elastic wall components, characteristic of the sinus region.

The guinea pig was chosen as an animal model for TEM and SEM studies as the few TEM studies on baroreceptors conducted in this species were particularly at odds.

The present study revealed that morphological features of the baroreceptors in guinea-pig CS are the same as other species. By contrast, previous reports (Böck & Gorgas, 1987; Gorgas et al., 1983) had suggested structural differences of sinus baroreceptors between guinea pig and other species. Thus, many of the structures we found for the first time in guinea pigs had been described in other species. These findings fill the gap in our knowledge of the fine structure of baroreceptors in various species and suggest interspecies homogeneity among their structures. The reported structural differences among species could arise from failure to examine large enough numbers of receptors rather than from species differences. The identification of baroreceptors in the media

confirms the findings of others in guinea pigs. Thus, it is possible that baroreceptors in guinea-pig CS are more suitably placed for pressure-sensing functions than in other species, as it has been shown that, for a given transmural pressure, the (stretching) stress is highest at the innermost part of the vessel wall and decreases towards the outer part of the wall.

TEM studies of baroreceptors indicated the close association between baroreceptors and elastic laminae. In many cases, baroreceptors were located between layers of elastic laminae and were in close contact with adjacent elastic laminae only via basal laminae or a basal laminal complex where terminal-cell covering was absent. These findings of "naked" baroreceptors prompted us to examine the elastic laminae in the CS and its adjacent regions in the second part of the present study.

Our SEM studies on the organization and fine structure of elastic laminae in the CS and its adjacent arteries are the first comparative examination of the elastic laminae from guinea-pig carotid bifurcations. Only one report has dealt with structure of elastic networks from (human) common carotid arteries (Mikhalev et al., 1978). Unlike other areas of the bifurcation, the sinus region exhibited some unique structural features including dense clusters of fenestrations, blister-like outgrowths, and an unusual honeycomb-like maze on the adluminal side of the internal elastic lamina. In addition, the cross-sectional thickness of the entire elastic layers was less in the CS than in adjacent arteries, also the elastic networks in the CS were much finer.

In summary, the results of this study indicate that guinea-pig

carotid sinuses contain baroreceptors with morphologic features similar to those described for other species. Also, the fine structure of elastic laminae of the carotid sinus differs from that of its adjacent arteries - presumably a reflection of differences in function. These findings provide a morphologic basis for functional studies of baroreceptors in this species.