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Autonomic-epithelial interactions in
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of ponies and horses

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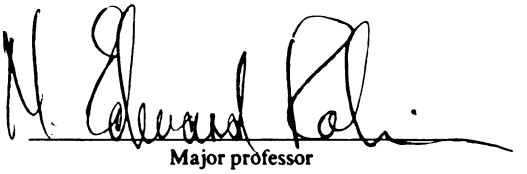

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AUTONOMIC-EPITHELIAL INTERACTIONS IN RECURRENT OBSTRUCTIVE PULMONARY DISEASE (HEAVES) OF PONIES AND HORSES

By

Richard V. Broadstone

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ABSTRACT

AUTONOMIC-EPITHELIAL INTERACTIONS IN
RECURRENT OBSTRUCTIVE PULMONARY DISEASE (HEAVES)
OF PONIES AND HORSES

By

Richard V. Broadstone

Animals with heaves develop airway obstruction and airway hyperresponsiveness when exposed to dusts and molds in poorly cured hay. Airway smooth muscle (AWSM) is regulated by a variety of autonomic mechanisms that interact with inflammatory mediators. This dissertation describes the investigation of the autonomic regulation of horse airways and how it is modified in the inflammatory disease "heaves."

Atropine was used to investigate the role of the parasympathetic nervous system in heaves. The airway obstruction was largely eliminated by atropine, suggesting activation of the autonomic nervous system. To investigate the mechanism of the cholinergically mediated obstruction, the excitatory and inhibitory responses of the AWSM were studied in vitro. The AWSM was hyperresponsive to electrical field stimulation (EFS) but not to acetylcholine (ACh) in heavey animals, suggesting a presynaptic augmentation of ACh release. The inhibitory response to the beta agonist isoproterenol was similar in the control and diseased groups. Electrically

induced relaxation of tracheal tissues was identical in both groups. Approximately half of the relaxation was nonadrenergic noncholinergic (NANC) in the trachea. Relaxation of third-generation bronchi (3B) of controls was NANC in origin. Diseased 3B lacked inhibitory innervation, which may explain in part the enhanced response of the diseased 3B to EFS.

The role of the epithelium and prostanoids in modulation of the AWSM of the two groups was investigated. Epithelial-mucosal removal had no effect on the response to EFS, but increased the sensitivity of the tissues to ACh in controls. This suggests that the epithelium in normal equine airways modulates the responsiveness of the AWSM. Administration of a cyclooxygenase blocker mimicked epithelium removal, thus implicating epithelium-derived prostaglandins in this modulatory function to ACh in controls. The differences between the responses of heavey and control groups to EFS and ACh were abolished by epithelium-mucosal removal plus the addition of cyclooxygenase blocker. This effect was primarily due to cyclooxygenase blocker, since epithelial-mucosal removal in the two groups had similar effects and the heavey tissues remained hyporesponsive, suggesting that production of non-epithelially derived prostanoids are primarily responsible for the difference between heavey and control groups.

This work is dedicated to Elaine, Paul, and Russell. Without their perseverance, patience, understanding, inspiration, and love, this research and dissertation would not have been possible.

My efforts were inspired by a tireless working genius, my father, Paul Broadstone.

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"No man is an island, no man stands alone."

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACh	acetylcholine
AT	active tension
AT_{max}	maximum active tension
AWSM	airway smooth muscle
C_{dyn}	dynamic compliance
ΔPpl	change in pleural pressure
ΔPpl_{max}	maximum change in pleural pressure
$ED_{65}C_{dyn}$	dose required to decrease C_{dyn} to 65% of baseline value
ED_{50}	concentration of ACh producing 50% contraction
EFS	electrical field stimulation
EPI	epinephrine
f	respiratory frequency
IC_{50}	concentration of isoproterenol producting 50% inhibition
NANC	nonadrenergic, noncholinergic
NE	norepinephrine
PaO_2	arterial oxygen tension
$PaCO_2$	arterial carbon dioxide tension

Period A	clinical remission
Period B	barn environment
R_L	pulmonary resistance
$R_{L\ 0.1}$	increase in R_L at 0.1 mg/ml drug dose
SP	substance P
$\dot{V}_E$	minute ventilation
VIP	vasoactive intestinal peptide
V_T	tidal volume

INTRODUCTION

Ponies and horses suffer from a spontaneously occurring pulmonary disease, "heaves," characterized by recurrent periods of airway obstruction accompanied by airway hyperresponsiveness to physical and pharmacological stimuli similar to asthma. The hypothesized mechanisms of airway obstruction and bronchial hyperreactivity include decreased baseline airway caliber, alterations in the amount or reactivity of smooth muscle, exaggerated parasympathetic responses to stimulation of pulmonary mechanoreceptors, abnormalities of the sympathetic system, and changes in bronchial epithelial permeability. Several studies have suggested the involvement of the autonomic nervous system in equine airway obstruction and hyperreactivity (Scott et al. 1988a, 1988b, Derksen et al. 1987a, Murphy et al. 1980). However, little information is available on the autonomic regulation of horse airways at different levels of the tracheobronchial tree in normal and diseased horse airways.

Parasympathetic efferent nerve fibers are supplied to the airways by the vagus nerves. Ganglia are located in the walls of the airways, and postganglionic fibers extend to airway smooth muscle. The parasympathetic nervous system is the major bronchoconstrictor network in the lung and cholinergic mechanisms play crucial roles in the maintenance of bronchomotor tone and airway caliber. In obstructive airway diseases such as asthma, the cholinergic pathway is in part responsible for broncho-spasm. My goal was to investigate the involvement of the autonomic nervous system

in the airway obstruction of heaves. Initially, the role of muscarinic receptors in airway obstruction was investigated by the administration of the nonspecific muscarinic antagonist atropine to diseased and control animals at pasture and in the barn. Subsequently, in vitro studies investigated the mechanism of the cholinergic component to airway obstruction.

The purpose of these in vitro investigations was to define excitatory and inhibitory innervation of the trachea and third-generation bronchi in normal and diseased horses.

Recently, the possible role of the airway epithelium and its metabolites has been studied. Tracheal epithelium produces factors that modulate airway smooth muscle activity, and in certain species these substances appear to be arachidonic acid (AA) metabolites. Epithelium disruption occurs in heaves. The final phase of my research was to determine if the influence of the epithelium and prostanoids in modulating the underlying smooth muscle differed in control and heavey horses.

The literature review is presented in three major sections. The first section describes the anatomy and physiology of airway smooth muscle innervation. Reviews of the parasympathetic and sympathetic and (NANC) nervous systems are included in this section. Next, recurrent obstructive pulmonary disease, "heaves," in horses and ponies is reviewed. The third section describes airway hyperresponsiveness, with particular emphasis on the potential mechanisms that cause this increased response of the airways. These three sections of the literature review include much information that has come from human studies and other models of airway disease. Finally,

in the literature review, the specific aims of the research protocols described in this dissertation are briefly outlined.

The first study examines the role of the cholinergic system in the airway obstruction and hyperresponsiveness of heavey ponies. The effects of the muscarinic antagonist atropine on lung function and airway reactivity were measured. The significant decrease in pulmonary resistance following the administration of atropine suggested involvement of the muscarinic receptors in the disease. The second study, using in vitro investigations, was to define excitatory and inhibitory innervation of the trachea and third-generation bronchi in normal and diseased horses and to determine if autonomic regulation is altered in the diseased horses. The final study was designed to determine if the airway epithelium and prostanoids modulate the underlying smooth muscle of the control and heavey horses. The final chapter summarizes the results from all the experiments.

CHAPTER 1

LITERATURE REVIEW

Innervation of airway smooth muscle

Introduction

The autonomic nervous system controls many aspects of airway function (Nadel and Barnes 1984). In addition to regulation of airway smooth muscle tone, autonomic nerves may influence secretion of mucus from submucosal glands, transport of fluid across airway epithelium, permeability and blood flow in the bronchial circulation, and release of mediators from various inflammatory cells. The innervation and autonomic control of the airways is complex and poorly understood (Richardson 1979). The efferent neuropathways are divided into excitatory and inhibitory pathways. Three efferent neural pathways to airway smooth muscle have been described. Two of these pathways, the cholinergic and the adrenergic, have been known for a long time (Larsell and Dow 1933, Gaylor 1934). In addition to classic cholinergic and adrenergic mechanisms, a third component of neural control exists in some species, which is neither cholinergic or adrenergic. The more recently described third pathway, the nonadrenergic, noncholinergic (NANC) pathway is less well known because of lack of knowledge of its neurotransmitter (Richardson 1979, Richardson 1983). Until recently, evidence implied that neuropeptides were the

possible neurotransmitters of this system (Richardson 1981, Barnes 1984b). More recent studies suggest that nitric oxide is the mediator (Li and Rand 1991).

Although the dominant motor system to the airways is usually regarded as that to the tracheobronchial smooth muscle, this is only one component of airway innervation. The innervation of airway secretory tissue, and the pulmonary and vascular bed, may also have indirect effects on airway smooth muscle. Mediator release from motor nerves may act on isolated migratory cells such as leukocytes and mast cells as well as on the amine-containing cells of the epithelium, which also may influence airway smooth muscle.

Several types of afferent nerves have been described in airways, and their purpose is to send information up the vagus nerve so that appropriate changes in the pattern of breathing or in bronchomotor tone may occur. Three types of afferent nerve fibers are apparent: 1) slowly adapting (stretch) receptors; 2) rapidly adapting (irritant) receptors; 3) C-fiber endings. The slowly adapting receptors are myelinated nerve terminals localized mainly to the smooth muscle of conducting airways. The rapidly adapting receptors are also myelinated nerve terminals, but they adapt rapidly. These sensory receptors are predominantly localized to airway epithelium. The C-fiber endings, which are nonmyelinated, contain neuropeptides such as tachykinins and gene-related peptides. The release of these potent neuropeptides from the C-fibers means that these nerves have sensorimotor function, and retrograde activation of the nerves via an axon reflex mechanism. This may lead to inflammatory effects in the airways and to bronchoconstriction. An interesting aspect of afferent

innervation is the interaction between afferent nerves and airway smooth muscle, either by axon reflexes or by local synaptic connections via ganglia.

Our understanding of autonomic regulation of airways is advancing due to improvements in histochemical and ultrastructural techniques for demonstrating nerves, from pharmacologic methods for studying autonomic receptors, and from physiologic methods for measuring functional effects in airway smooth muscle, glands, and the bronchial circulation. These advances underscore the significant differences in autonomic innervation between different species.

Cholinergic control of airway smooth muscle

The parasympathetic nervous system is the dominant neural bronchoconstrictor mechanism in all animals and plays an important role in the regulation of airway tone. Mammalian airways receive a rich cholinergic innervation (Richardson 1979, Barnes 1986a). Cholinergic efferent nerves arise in the vagal nuclei of the brain stem and pass down the vagus nerve to synapse in ganglia situated in the airway wall. From these ganglia relatively short postganglionic fibers pass to smooth muscle cells. Cholinergic nerve fibers are found in smooth muscle of human and dog (Russell 1978) airways from trachea to terminal bronchioles, but the density of innervation markedly decreases in the smaller airways.

Electrical stimulation of the vagus nerve in animals causes bronchoconstriction, which is potentiated by cholinesterase inhibitors and blocked by the muscarinic receptor antagonist atropine (Nadel 1980, Nadel and Barnes 1984). The onset of bronchoconstriction is rapid and readily reversible, suggesting contraction of airway

smooth muscle rather than bronchial wall edema or luminal obstruction with mucus. Rapid freezing of the airways in cats after vagal stimulation has confirmed that muscle contraction is responsible for the airway narrowing (Olsen et al. 1965). The effects of vagal nerve stimulation are not equally distributed between airways. Using tantalum bronchography to outline airways, maximal bronchospasm is seen in intermediate-sized bronchi of dog, with relatively little effect in bronchioles (Nadel et al. 1971), consistent with the distribution of cholinergic innervation.

Efferent cholinergic stimulation causes release of acetylcholine (ACh) from the agranular vesicles in cholinergic nerve terminals, which rapidly diffuses the relatively short distance to cholinergic receptors on the target cell. Acetylcholine is rapidly broken down by the enzyme acetylcholinesterase; this enzyme is inhibited by specific cholinesterase inhibitors, such as edrophonium, which therefore potentiate the bronchoconstrictor effect of nerve stimulation.

Postganglionic cholinergic innervation has been studied directly in isolated airways of many species in a muscle bath. In these studies, field stimulation produces a contractile response that can be blocked by atropine and by the nerve blocker tetrodotoxin, thereby confirming the presence of intrinsic cholinergic nerves (Davis et al. 1982, Taylor et al. 1984). The distribution of cholinergic nerve effects may depend on the size of the airways. In vitro cholinergic nerve effects are less pronounced in bronchioles than in bronchi (Palmer et al. 1986). Similarly, in vivo studies with helium-oxygen flow-volume curves show that the bronchodilator effect of anticholinergic drugs is greater in large than in small airways, whereas beta-agonists relax all airways in normal and asthmatic subjects (Hensley et al. 1978).

Cholinergic receptors

Acetylcholine released from preganglionic vagal fibers in airway ganglia activates nicotinic cholinergic receptors on ganglionic neurones. These receptors are stimulated by nicotinic agonists, such as dimethylpiperazinium, which therefore cause contraction of airway smooth muscle if the ganglia are intact, and this contraction is blocked by the nicotinic antagonist hexamethonium (Skoogh 1983).

Acetylcholine released from postganglionic nerves activates muscarinic cholinergic receptors on smooth muscle cells, which are blocked by atropine and related drugs such as ipratropium bromide (Barnes 1987a). 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, and there is a close relationship between muscarinic receptor occupation and stimulation of phosphoinositide turnover (Grandordy et al. 1986). Activation of muscarinic receptors also inhibits adenylate cyclase, leading to a reduction in the concentration of cyclic AMP (Madison et al. 1985). Direct receptor binding studies have demonstrated a high density of muscarinic receptors in smooth muscle of large airways (Cheng and Townley 1982). This is confirmed by autoradiographic mapping, which also shows that the density of muscarinic receptors decreases in smaller airways, so that terminal bronchioles are almost devoid of receptors (Barnes et al. 1983b). Muscarinic receptors have also been identified in human lung homogenates (Raaijmakers et al. 1984), but their distribution has not been studied. Because human lung strips, in comparison to bronchi, are only weakly contracted by

cholinergic agonists, this suggests that there may similarly be fewer cholinergic receptors on smooth muscle of bronchioles than of bronchi (Goldie et al. 1982).

Muscarinic subtypes have recently been differentiated by the development of selective muscarinic antagonists (Hammer and Giachetti 1982). Three of these subtypes have been identified in lungs of several species, including humans (Barnes et al. 1988, Barnes 1989). M_1 receptors, which are pirenzepine sensitive, are localized to parasympathetic ganglia and may facilitate ganglion neurotransmission (Bloom et al. 1988). These receptors facilitate cholinergic reflex bronchoconstriction in humans (Lammers et al. 1989). M_2 receptors are localized to postganglionic cholinergic nerves and function as autoreceptors, inhibiting the release of ACh (Barnes et al. 1988). These receptors are sensitive to gallamine, which distinguishes them from the muscarinic receptors on airway smooth muscle, which are termed M_3 receptors and are sensitive to 4-DAMP (Roffel et al. 1990). Prejuctional M_2 receptors have been demonstrated to have a potent inhibitory effect on cholinergic neurotransmission (Minette and Barnes 1988). These receptors appear to inhibit cholinergic reflex bronchoconstriction in normal human subjects in vivo, although there is some evidence that they may be dysfunctional in asthma, thus enhancing reflex bronchoconstriction (Minette et al. 1989, Ayala and Ahmed 1989).

Cotransmission

Vasoactive intestinal peptide (VIP) is probably a cotransmitter of ACh in airway cholinergic nerves (Laitinen et al. 1985b), and there may be some functional relationship between this neuropeptide and cholinergic control. Vasoactive intestinal

peptide is a potent vasodilator in the bronchial circulation (Laitinen et al. 1985b), so if released with ACh it may increase the blood flow to contracting airway smooth muscle (Barnes 1987a). Vasoactive intestinal peptide is also a potent relaxant of human bronchi in vitro and may counteract the bronchoconstriction effect of ACh (Barnes 1986b), and thereby act as a braking mechanism for cholinergic nerves.

Prejunctional modulation

Cholinergic nerve function may be modulated in a variety of ways by other neurotransmitters or by inflammatory mediators. Norepinephrine (NE) reduced the neurally mediated cholinergic contraction of canine tracheal smooth muscle at concentrations that have little effect on ACh responses, suggesting an inhibitory effect on ACh release from postganglionic nerve terminals (Vermiere and Vanhoutte 1979). Similar inhibition of human airway cholinergic nerves has been found with beta agonists (Rhoden et al. 1988). Prostaglandin E_2 and the alpha$_2$-agonist, clonidine, have a similar inhibitory effect on cholinergic transmission (Walters et al. 1984, Anderson et al. 1986). Serotonin, a thromboxane mimetic, and substance P have the reverse effect and appear to potentiate ACh release from cholinergic nerves in animal airways (Sheller et al. 1982, Chung et al. 1985, Tanaka and Grunstein 1984). To date, it has proved difficult to measure ACh release from airway nerves, which has made further investigation of prejunctional modulation difficult.

Cholinergic reflexes

Animal studies have clearly demonstrated that several sensory stimuli may influence airway tone. Stimulation of nasal mucosa, larynx, central and peripheral chemoreceptors, baroreceptors, and esophagus may cause bronchoconstriction. Because the stimulus is separated from the response, this must mean that a reflex mechanism is involved, and the bronchoconstrictor response can be abolished by section of the vagus nerve or by cholinergic antagonists (Nadel 1980). Stimulation of afferent vagal fibers in the airways lead to bronchoconstriction by reflex pathways. Reflex bronchoconstriction has been shown in human airways by documenting a protective effect of anticholinergic drugs on various bronchial challenges. Anticholinergic drugs are generally effective against bronchoconstriction induced by sulfur dioxide, carbon dust, ozone, and acid challenges to the esophagus; however, they are less protective against allergen challenge, perhaps because reflex effects by stimulation of afferents by mediators are less important than the direct effects of the mediators on airway smooth muscle. In some studies, the bronchoconstrictor effect of histamine is reduced by anticholinergic drugs or by ganglion blockade with hexamethonium (Holtzman et al. 1980), suggesting a reflex component, presumably because of stimulation of airway afferent receptors. However, most studies have shown little or no protective effect of anticholinergic drugs against histamine challenge, indicating that bronchoconstriction induced by histamine is largely a direct effect of histamine on airway smooth muscle.

Adrenergic control of airway function

Adrenergic control of airways concerns sympathetic nerves, which release NE, and the adrenal medulla, which releases predominately epinephrine (EPI). These catecholamines activate alpha- and beta-adrenoceptors on target cells in the airway (Barnes 1984a).

Sympathetic innervation

The sympathetic nerve supply to the lung originates from the upper thoracic segments of the spinal cord, and postganglionic fibers arise from the cervical and upper thoracic ganglia. Postganglionic fibers run from these ganglia to the lung, and enter at the hilum together with the vagus nerves (Richardson 1979, Murray 1986). In contrast to the dense parasympathetic nerve supply to airways in all species, sympathetic innervation is generally sparse, but there is considerable variation between species (Richardson 1979, Doidge and Satchell 1982). Feline airways receive a rich sympathetic innervation (Doidge and Satchell 1982, Silva and Ross 1974), whereas in primates, adrenergic innervation of airways is very sparse (El-Bermani 1978). Adrenergic nerve fibers have been found in close association with submucosal glands (Partanen et al. 1982, Pack and Richardson 1984) and bronchial arteries (Partanen et al. 1982, Doidge and Satchell 1982), but few, if any, adrenergic fibers have been demonstrated in smooth muscle of intrapulmonary airways (Partanen et al. 1982, Richardson and Beland 1976, Doidge and Satchell 1982). Sympathetic stimulation results from activation of alpha or beta receptors either by sympathetic nerves or circulating catecholamines. The sympathetic postganglionic neurotransmitter NE,

following activation of the receptor on the postsynaptic muscle membrane, is primarily removed by re-uptake into the nerves or degraded by monoamine oxidase (Burnstock 1988). Norepinephrine in plasma is derived almost entirely from overspill of sympathetic nerve activity (Brown et al. 1981). Epinephrine is secreted by the adrenal medulla and functions as a circulating hormone and not as a neurotransmitter (Cryer 1980). These catecholamines activate alpha and beta adrenergic receptors on target cells in the airway (Barnes 1984a).

Functional studies by electrical field stimulation (EFS) have demonstrated neurally mediated relaxation, which is blocked by beta antagonists in isolated trachea but not bronchus of guinea pig (Doidge and Satchell 1982, Grundstrom et al. 1981a). This is consistent with the demonstration of adrenergic nerve fibers in tracheal but not in the bronchial smooth muscle of this species (O'Donnell et al. 1978). Fluorescence studies in the cat have demonstrated extensive adrenergic innervation to the pulmonary and bronchial vessels, as well as the airway smooth muscle from the level of the trachea to the respiratory bronchioles (Silva and Ross 1974, Richardson 1979).

Even in those species that appear to not have a functional sympathetic innervation of the airway smooth muscle, it is possible that adrenergic nerves may influence bronchomotor tone indirectly. In dogs, electrical stimulation of thoracic sympathetic nerves causes bronchodilation, which is abolished by beta antagonists and unaffected by adrenalectomy, but the degree of bronchodilation is dependent on the degree of preexisting vagal tone (Cabezas et al. 1971). This suggests that sympathetic nerves may have a modulatory effect on cholinergic neurotransmission. In guinea

pigs, stimulation of sympathetic outflow to the lungs produces bronchodilation (Ainsworth et al. 1981), and yet again there is no evidence for a direct sympathetic nerve supply of intrapulmonary airways (O'Donnell et al. 1978). This suggests either interaction with the cholinergic tone or overflow of NE from the sympathetic nerves that supply other structures, such as pulmonary vessels. Thus, sympathetic nerves might influence bronchomotor tone indirectly.

Beta-adrenergic receptors

Radioligand binding studies have demonstrated a high density of beta receptors in the lung of many species, including humans (Rugg et al. 1978, Barnes et al. 1980a, Engel 1981). More recent autoradiographic techniques have revealed that beta receptors are localized to many different cell types within the lung (Barnes et al. 1982a, Carstairs et al. 1984). Beta receptors are found in smooth muscle of all airways from trachea to terminal bronchioles. This is not surprising, since beta agonists relax the airways at all levels in vitro (Davis et al. 1982, Goldie et al. 1982, Zaagsma et al. 1983, Finney et al. 1985, Guillot et al. 1984, Russell 1984, Olson et al. 1989b). The density of beta receptors increases with decreasing size of airways in both animals and humans (Barnes et al. 1983b, Carstairs et al. 1985).

Beta-agonists also stimulate secretion of mucus in several species (Nadel et al. 1985). In animals, administration of beta-agonists results in a more viscous secretion (Leikhauf et al. 1984), probably because of selective stimulation of mucous rather than serous cells of submucosal glands (Basbaum et al. 1981). Autoradiographic studies confirm a high density of beta-receptors in airway glands, with greater labeling

of mucous cells (Barnes and Basbaum 1983a). In addition in humans, beta-agonists stimulate active ion transport, and therefore water secretion, across human epithelium in vitro (Knowles et al. 1984), and a very high density of beta receptors is found on epithelial cells of all airways (Carstairs et al. 1984). In 1967, Lands and coworkers defined two subtypes of beta adrenergic receptors, termed $beta_1$ and $beta_2$ (Lands et al. 1967). The major physiological difference between $beta_1$ and $beta_2$ receptors is their differential sensitivity to NE. $Beta_1$ adrenergic receptors display approximately equal affinity for EPI and NE, whereas at $beta_2$ receptors EPI is considerably more potent than NE. Therefore, the $beta_2$ receptor has been considered a hormonal receptor, whereas the $beta_1$ receptor is a neuronal receptor (Ariens and Simonis 1983). However, $beta_1$ receptors respond to both neuronal and hormonal stimulation and $beta_2$ receptors can be activated by high concentrations of NE (Ariens and Simonis 1983). Both subtypes of beta adrenergic receptors appear to be coupled in a stimulatory fashion to the enzyme adenylate cyclase, which generates cyclic AMP from ATP (Sutherland et al. 1971). Stimulation of this enzyme system, with consequent enhancement of the activity of cyclic AMP-dependent protein kinases represents the biochemical mechanism of action of beta-adrenergic agonists (Glass and Krebs 1980).

Studies using radioligand binding have demonstrated a high density of beta receptors in the lungs of all species examined (Barnes et al. 1980b, Rugg et al. 1978). Beta receptors are found in smooth muscle of all airways from trachea to terminal bronchioles. The density of beta receptors increases with decreasing size of airways in all species studied (Carstairs et al. 1985, Barnes et al. 1983b). When Lands and

coworkers subdivided beta adrenergic receptors into $beta_1$ and $beta_2$ they classified airway smooth muscle receptors as $beta_2$ (Lands et al. 1967). Since then both $beta_1$ and $beta_2$ receptors have been demonstrated in airways (Furchgott et al. 1975). Relaxation of tracheal smooth muscle in response to beta adrenergic agonists in some species is intermediate between a $beta_1$ and $beta_2$ mediated response (Furchgott et al. 1975). Relaxation of feline tracheal smooth muscle, which has a dense sympathetic innervation, was found to be predominantly mediated by $beta_1$ receptors (O'Donnell and Wanstall 1983, Silva and Ross 1974). In canine tracheal smooth muscle, relaxation in response to exogenous beta agonists is mediated by $beta_2$ receptors, whereas relaxation in response to sympathetic nerve stimulation is mediated by $beta_1$ receptors (Barnes et al. 1983c). These findings are consistent with the theory that $beta_1$ receptors are activated by sympathetic nerves and $beta_2$ receptors are activated by circulating catecholamines (Ariens and Simonis 1983). Human airway smooth muscle appears completely devoid of sympathetic nerves with the inhibitory nerves being entirely nonadrenergic (Richardson 1981, Richardson and Ferguson 1979, Barnes 1986b). Furthermore, relaxation of human airway smooth muscle by catecholamines, including NE, is solely mediated by $beta_2$ receptors (Zaagsma et al. 1983). Using receptor binding techniques the ratio of $beta_1$:$beta_2$ receptors in the human lung was found to be approximately 3:1 (Rugg et al. 1978, Engel 1981). Airway beta receptors account for less than 5% of total lung beta receptors with > 90% being localized to alveolar walls (Barnes et al. 1982a, Carstairs et al. 1984).

Human submucosal glands, which receive a sparse adrenergic innervation, have beta receptors of which approximately 10% are $beta_1$ receptors (Carstairs et al. 1985, Pack and Richardson 1984, Meyrick and Reid 1970). Activation of $beta_1$ and $beta_2$ receptors stimulates mucus secretion (Phipps et al. 1982). Epithelial and mast cells, which are not innervated, have only $beta_2$ receptors (Carstairs et al. 1985, Hughes et al. 1983). The high density of beta receptors in the epithelium from large bronchi to terminal bronchioles of human lung exceeds the density of receptors in the corresponding smooth muscle layers (Carstairs et al. 1985). In bovine trachea, the density of beta receptors is two fold higher and the affinity is six fold greater in the epithelial membranes than in the smooth muscle (Agrawal et al. 1987). Similarly, the epithelium of rat bronchioles has a higher density of beta receptors than the smooth muscle (Xue et al. 1983). These data explain the effects of beta adrenergic agonists on mucociliary transport by enhancing fluid transport across the epithelium and by increasing glandular mucus secretion (Mossberg 1979).

Alpha-adrenoceptors

Alpha-receptors, which mediate contraction of airway smooth muscle, have been demonstrated in many species, including humans (Simonsson et al. 1972, Kneussl and Richardson 1978), although alpha-adrenergic responses can be demonstrated only under certain conditions. No alpha-adrenergic contraction can be demonstrated in canine airways in vitro, unless there is a high degree of beta-adrenergic blockade (Leff et al. 1986); but if airways are pretreated with histamine or serotonin a marked contractile response to alpha-agonists or sympathetic nerve

stimulation is found (Kneussl and Richardson 1978, Barnes et al. 1983d), suggesting that these mediators have enhanced alpha-adrenergic responsiveness or "unmask" alpha-receptors. This increased alpha-adrenergic response is not due to any change in either alpha-receptor density or affinity and is therefore likely to be due to a postreceptor mechanism, possibly involving voltage-dependent calcium channels (Barnes et al. 1983d). Similar activation of canine tracheal alpha-adrenergic responses has also been demonstrated in vivo (Barnes et al. 1983d, Brown et al. 1983). No alpha-adrenergic contraction is found in normal human airways. In the ferret, autoradiographic mapping of pulmonary alpha-receptors has demonstrated few alpha-receptors in large airways, but a high density in small bronchioles (Barnes et al. 1983b).

Alpha-agonists also stimulate secretion from submucosal glands of several species, including humans (Nadel et al. 1985). In animals, direct micropipette sampling of secretions from these glands shows that alpha-agonists produce a watery secretion (Ueki et al. 1980, Leikhauf et al. 1984), with selective stimulation of serous cells (Basbaum et al. 1981). Autoradiographic studies have shown that alpha-receptors are localized to serous rather than mucous cells of submucosal glands (Barnes and Basbaum 1983). It is likely that these alpha receptors are activated by the adrenergic nerves that supply submucosal glands. Alpha receptors may also be present in the airway ganglia, since NE inhibits firing of neurons in airway ganglia via alpha-receptors (Baker et al. 1983), and presynaptic alpha-2 receptors may inhibit ACh release from cholinergic nerve endings (Grundstrom et al. 1981b).

Alpha-receptor subtypes

Alpha-receptor may be classified into subtypes, depending on the selectivity of specific antagonists (Hoffman and Lefkowitz 1980). The alpha-1 receptors are the classic alpha-receptors that are postsynaptic, whereas the alpha-2 receptors are presynaptic and inhibit the release of NE from synaptic nerve terminals. More recently, postsynaptic alpha-2 receptors have also been recognized. It was suggested that the alpha-1 receptors might be regulated by sympathetic nerves and alpha-2 receptors by circulating catecholamines. However, in the canine trachea, the contractile response to both exogenous NE and sympathetic nerve stimulation is mediated entirely by alpha-2 receptors, and direct binding studies have demonstrated the presence of alpha-2 receptors, with very few alpha-1 receptors (Barnes et al. 1983e).

Nonadrenergic, noncholinergic nervous system

In addition to classic cholinergic and adrenergic pathways, neural mechanisms that are neither cholinergic nor adrenergic have been described (Richardson 1979, Barnes 1984b). The existence of an NANC nervous system in the gastrointestinal tract, which controls gut motility, sphincters, and secretions, has been established for many years and has been demonstrated in vertebrates from fish to humans (Burnstock 1972). Because the airways develop embryologically from the foregut, the existence of NANC nerves is not surprising. Burnstock originally proposed that the neurotransmitters of NANC nerves might be purine nucleotides, such as adenosine triphosphate or adenosine, since in the gut these purines are released on nerve

stimulation, and both exogenous adenosine triphosphate and adenosine mimic some of the effects of NANC nerve stimulation (Burnstock 1972). These nerves were therefore termed "purinergic." Other studies have suggested and provided convincing evidence that regulatory peptides are more likely to be the neurotransmitters, so that the NANC nerves are probably "peptidergic" (Costa and Furness 1982). More recently it has been suggested that the neurotransmitter of the inhibitory-NANC may be partly due to nitric oxide since L-N^G-nitroarginine, an inhibitor of endogenous nitric oxide synthesis from L-arginine, has an inhibitory effect on the inhibitory-NANC nerve responses in guinea-pig trachea (Li and Rand 1991).

Nonadrenergic inhibitory nerves

Nonadrenergic inhibitory nerves, which relax airway smooth muscle, have been demonstrated in vitro in several species, including humans (Richardson and Beland 1976, Davis et al. 1982, Taylor et al. 1984, Palmer et al. 1986, Doidge and Satchell 1982). Since there is no functional sympathetic innervation in humans, the non-adrenergic inhibitory nerve system is the only direct neural bronchodilator. Nonadrenergic, noncholinergic inhibitory nerves have also been demonstrated in animals in vivo by electrical stimulation of the vagus nerve after cholinergic and adrenergic blockade (Diamond and O'Donnell 1980, Irvin et al. 1980). In cats, tantalum bronchography in cats has shown that NANC bronchodilation is predominantly seen in large airways (Matsumoto et al. 1985). Stimulation of this pathway produces pronounced and long-lasting bronchodilation, and this response can be inhibited by ganglion blockers, suggesting that these NANC nerves are preganglionic

as well as postganglionic, as demonstrated by EFS. Nonadrenergic, noncholinergic nerves regulate secretion of airway mucus in animals. Stimulation of the vagus nerves promotes mucus secretion in cat trachea, which is reduced but not abolished by cholinergic and adrenergic blockers (Peatfield and Richardson 1983), and EFS of ferret tracheal segments in vitro has shown NANC stimulation of mucus secretion (Borson et al. 1984).

The neurotransmitter

Even with the demonstration of the existence of NANC nerves in the lung both in vitro and in vivo, the fact that the neurotransmitter is not certain and no specific blocker is yet available makes it difficult to investigate the physiologic role of this nervous system. Even though the purines have been suggested as neurotransmitters in the airways, there is evidence that argues against a purine in the airways. For example, even though exogenous ATP relaxes airway smooth muscle (Ito and Takeda 1982), an antagonist quinidine does not block NANC relaxation either in vitro or in vivo. Similarly, adenosine fails to mimic nonadrenergic relaxation, and its antagonist theophylline does not block nonadrenergic relaxation (Karlsson and Persson 1984).

Several different regulatory peptides have now been localized to nerves in the airway in several species, including humans (Polak and Bloom 1982, Hakanson et al. 1983). Electron microscope studies have also demonstrated peptide type granules in addition to cholinergic and adrenergic vesicles in the nerves of human airways (Laitinen et al. 1985). Of the several peptides isolated from airways, only VIP and

a related peptide, peptide histidine isoleucine, relax airway smooth muscle. In addition to being an established neurotransmitter in the gastrointestinal tract (Costa and Furness 1982, Said 1984), there is compelling evidence that VIP is at least one of the neurotransmitters of nonadrenergic inhibitory nerves in airways (Said 1982). Vasoactive intestinal peptide is a 28 amino acid peptide that was discovered as a vasoactive substance in lung extracts and that relaxes airway smooth muscle in vitro. Vasoactive intestinal peptide has been localized in both animal and human lungs to neurones and nerve terminals in airway smooth muscle, around submucosal glands, and in bronchial and pulmonary vessels (Uddman and Sundler 1979, Dey et al. 1981, Laitinen et al. 1985). In addition, VIP produces prolonged relaxation of animal airway smooth muscle in vitro, which is unaffected by adrenergic or cholinergic blockers (Ito and Takeda 1982, Cameron et al. 1983, Altiere and Diamond 1984). This relaxation is unaffected by propranolol and indomethacin, and is therefore likely to be due to a direct action of the peptide on airway smooth muscle. Electrical field stimulation of tracheal preparations releases VIP into the bathing medium, and this is blocked by tetrodotoxin, indicating that VIP is derived from nerve stimulation (Cameron et al. 1983, Matsuzaki et al. 1980). To date unfortunately, no specific blocker of VIP is yet available, but in cats, prolonged incubation of airway smooth muscle with VIP reduces subsequent responses to VIP, suggesting tachyphylaxis. Prolonged incubation with VIP also reduces the magnitude of NANC relaxation response, whereas relaxation to sympathetic nerve stimulation and isoproterenol is unaffected (Ito and Takeda 1982).

Histochemical studies have demonstrated the density VIP-immunoreactive nerves diminishes in the small airways, and is virtually absent from bronchioles (Dey et al. 1981, Laitinen et al. 1985). Similarly, the magnitude of nonadrenergic relaxation is very small in bronchioles, and these airways fail to relax in response to exogenous VIP. All this evidence points to VIP as a candidate for the role of neurotransmitter of nonadrenergic inhibitory nerves. The proof awaits the development of a specific antagonist.

Noncholinergic excitatory nerves

Electrical stimulation of guinea pig bronchi, and occasionally trachea, in vitro produces bronchoconstriction that is not inhibited by atropine (Lundberg et al. 1983a, Andersson and Grundstrom 1983). This response is mimicked by substance P (SP) and inhibited by peptide analogues that are SP antagonists, providing evidence that SP might be a neurotransmitter of noncholinergic excitatory nerves. Substance P, an 11 amino acid peptide, is localized to nerves in the airways of several species (Lundberg et al. 1984, Polak and Bloom 1982, Wharton et al. 1979). The SP-immunoreactive nerves in the airway are found beneath and within the airway epithelium, around blood vessels, and to a lesser extent within airway smooth muscle. Substance P appears to be localized to afferent nerves in the airways and is synthesized in the nodose ganglion of the vagus nerve and transported down the vagus to peripheral branches in the lung. Treatment of animals with capsaicin releases SP from sensory nerves acutely (Lundberg et al. 1983). In vitro, SP contracts airway smooth muscle of several species, including humans (Lundberg et al. 1983,

Palmer et al. 1986). In addition, capsaicin is capable of inducing a similar contraction, indicating the release of SP from intrinsic nerves within airway smooth muscle (Lundberg et al. 1983). The contractile effect of SP on airway smooth muscle in vitro may be inhibited by SP antagonists, suggesting a direct effect on smooth muscle cells. However, the specificity of these antagonists have been questioned (Karlsson and Persson 1984a, Karlsson et al. 1984b).

Recurrent obstructive pulmonary disease (heaves)

Introduction

Heaves is one of the most commonly diagnosed conditions affecting the equine tracheobronchial tree. Since domestication, this disease has plagued horses in the temperate parts of the world and is associated with housing or confinement with exposure to improperly cured hay and straw (Williams 1874, Cook 1976). Normal horses and ponies are not affected by barn exposure and hay feeding (Derksen et al. 1985a). The disease is rare in warmer areas where animals are able to be kept outside on grass year round. The disease incidence increases with age but there appears to be no predisposition to the condition due to breed, gender, or genetics.

Heaves is a term applied to a complex of clinical signs rather than to a specific disease (Breeze 1979). Clinical signs of the disease include the following: dyspnea characterized by an accentuated abdominal effort at end expiration, intermittent or chronic purulent nasal discharge, cough, and exercise intolerance (Willoughby and McDonnell 1979, Gillespie and Tyler 1969, McPherson et al. 1978). Synonyms for this condition include chronic obstructive pulmonary disease, recurrent obstructive

pulmonary disease, chronic bronchitis, chronic bronchiolitis, broken wind, and equine pulmonary emphysema.

Etiology

The causes of recurrent obstructive pulmonary disease are unknown, but proposed factors include exposure to allergens, prior respiratory infection, and pneumotoxin ingesion. Heaves is unlikely to be caused by one etiologic agent. The strongest evidence supports an allergic basis in many cases. This evidence comes from several immunologic, physiologic, and epidemiologic studies (Halliwell et al. 1979, McPherson et al. 1979a, Armstrong et al. 1986). Evidence for other contributing factors to this disease is largely circumstantial.

Lowell showed a relationship between the occurrence of acute disease exacerbations and the feeding of hay, and he suggested that the disease was precipitated by the inhalation of "heaves producing factors" in hay (Lowell 1964). Horses with recurrent obstructive pulmonary disease have a greater prevalence of serum antibody titers against antigens commonly found in the horse's environment than do unaffected animals (Halliwell et al. 1979, McPherson 1979a, 1979b). These antigens include Actinomycetes such as *Micropolyspora faeni* and *Thermoactinomyces vulgaris* and molds such as *Aspergillus fumigatus, Alternaria, Penicillium, and Rhizopus sp. Micropolysporum faeni and Aspergillus fumigatus* are the two most commonly implicated antigens (Halliwell 1979). The greatest number of reactions are associated with *M. faeni* challenge. *Micropolysporum faeni* has also been associated with farmers lung in humans, a form of hypersensitivity pneumonitis (Fink 1976). Many affected

horses also have a positive skin test against these antigens (McPherson et al. 1979a). However, many horses without clinical signs of heaves also have serum antibody titers and positive skin tests using the same antigens. Also, some affected horses do not have serum antibody titers or positive skin tests. Therefore, as a group, horses with heaves have serum antibody levels and positive skin tests against several environmental antigens more commonly than control horses (Lawson 1979, Asmundson 1983). Experimental sensitization of normal ponies with *M. faeni* followed by aerosol challenge results in airway inflammation but does not reproduce the characteristic changes in pulmonary function observed in ponies with heaves (Derksen et al. 1987b). Because of the considerable overlap existing between normal and affected horses, serum antibody titers and skin testing cannot be used to diagnose recurrent airway obstruction in individual horses.

Factors that increase environmental contamination by these antigens, such as inadequate ventilation or feeding poor quality hay, are associated with an increased incidence of heaves. Airway obstruction, exhibited as increased R_L and decreased dynamic compliance (C_{dyn}), airway hyperresponsiveness to aerosol histamine, and hypoxemia can be repeatedly induced by placing heavey animals in the barn and feeding them poor quality hay. Remission of signs occurs when horses were returned to pasture and not exposed to hay or dust (Armstrong et al. 1986, Scott et al. 1988a, 1988b, Derksen et al. 1985a)

An increased incidence of heaves has been found in horses following an outbreak of equine influenza in Switzerland (Gerber 1970). Others have reported increased levels of hemagglutination inhibiting activity against influenza A equine 1

in serum and tracheal mucus samples from horses with heaves (Thorsen et al. 1983). Viruses that produce respiratory disease and abnormal airway function have been reported in humans (Busse 1985, Lemanske et al. 1989), cats (Gaskell and Povey 1979, Killingsworth et al. 1990), rats (Chan-Yeung et al. 1980), and dogs (Lemen et al. 1990). Viral infection may induce airway smooth muscle hyperreactivity (Buckner et al. 1985, Lemen et al. 1990), epithelial hyperplasia with subsequent airway narrowing (Moreno 1986), impaired epithelial function (Jacoby et al. 1988), or altered production of inflammatory mediators (Kimman et al. 1989).

Ingestion of 3-methylindole, a metabolite of dietary L-tryptophan, has been suggested as a possible cause of heaves (Breeze et al. 1978, Derksen et al. 1982a). Oral administration of 3-methylindole (0.1 to 0.2 gm/kg bodyweight) to ponies induces lung disease characterized by necrotizing bronchiolitis and alveolar emphysema (Breeze et al. 1978, Derksen et al. 1982). Treated animals became dyspneic, with increases in respiratory rate, minute ventilation ($\dot{V}_E$), functional residual capacity, and total lung resistance. There was also a significant decrease in C_{dyn} and $PaCO_2$ but no change in PaO_2. The changes in C_{dyn} and $PaCO_2$ are associated with pulmonary dysfunction, but they are not characteristic of those seen with recurrent obstructive pulmonary disease. In horses and ponies with heaves there is a significant decrease in PaO_2 but no change in $PaCO_2$, and the changes in C_{dyn} and R_L are more marked (Armstrong et al. 1986, Derksen et al. 1985a, Scott et al. 1988a, 1988b). Tryptophan is found on pasture where horses with heaves remain in remission making it unlikely that only the affected animals are exposed to the pneumotoxin. This makes 3-methylindole an unlikely cause of heaves in a natural setting.

Pulmonary function

Prior to the development of pulmonary function tests, horses with heaves were diagnosed on the basis of clinical signs and history only. Amoroso and Sporri were the first to adapt pulmonary function equipment for use in horses (Amoroso et al. 1962, Sporri and Leeman 1964). The ability to perform pulmonary function tests aids in the diagnosis of recurrent obstructive pulmonary disease, follows the progress of the disease, and helps evaluate the functional changes that occur before and after therapeutic drugs are administered. The first lung function measurements developed evaluated the work of breathing by measuring the maximum change in pleural pressure (ΔP_{pl}) using an esophageal balloon or a pleural cannula during tidal breathing, and pulmonary gas exchange using the PaO_2 and $PaCO_2$ (Obel and Schmiterlow 1948, Alexander 1959, Gillespie et al. 1966, Gillespie et al. 1964, Derksen and Robinson 1980). Heavey horses have a greater ΔPpl than normal horses and they are hypoxemic (Gillespie et al. 1964, Gillespie et al. 1966, Muylle and Oyaert 1973, Sasse 1971). Various techniques have been developed and used on horses to measure air flow, tidal volume (V_T), respiratory frequency (f), C_{dyn}, and R_L (Muylle and Oyaert 1973, Sasse 1971, Beadle 1986, Derksen et al. 1982b, 1985a, Gillespie and Tyler 1969, Gillespie et al. 1966, Lekeax 1986).

Integrated pneumotachography is one common method to measure air flow and V_T in unanesthetized horses (Derksen et al. 1982b, 1985a, Muylle and Oyaert 1973, Sasse 1971, Sporri and Leeman 1964, Gillespie et al. 1966). The pneumotachograph consists of a resistance, either a fine mesh screen or a bundle of fine tubes, and a differential pressure transducer to detect the pressure drop across the resistance

during air flow. The pressure difference across the resistance is proportional to the flow rate, and conversion of flow to volume is accomplished by electronic integration of the flow signal. The V_T and air flow signals can then be displayed and recorded. The pneumotachograph must be attached either to a mask sealed around the face or to an endotracheal tube with an inflated cuff so that it measures all air entering and leaving the lungs. Recorded values for the ΔPpl, V_T, and flow can be used to calculate C_{dyn} and R_L. Dynamic compliance is calculated by dividing the V_T in liters by the changes in ΔP_{pl} (cm H_2O) between points of zero flow. The two points of zero air flow are at end expiration and end inspiration. Pulmonary resistance is the ratio of change in ΔP_{pl} to the change in flow (liters/sec) at points of equal lung volume (Amdur and Mead 1958). Dynamic compliance is lower and R_L higher in horses with recurrent obstructive pulmonary disease compared to normal horses (Derksen et al. 1985a, Armstrong et al. 1986, Scott et al. 1988a, 1988b, Willoughby and McDonnell 1979). Tidal volume, *f*, and $PaCO_2$ values are the same as for normal horses (Derksen et al. 1985a, Armstrong et al. 1986, Scott et al. 1988a, 1988b).

Airway hyperresponsiveness

Airways of ponies and horses with heaves, like those of asthmatics, are hyperresponsive to various stimuli (Armstrong et al. 1986, Derksen et al. 1985a, 1985c, Obel and Schmiterlow 1948). Airway responsiveness is used to define the ease with which airways narrow in response to a variety of stimuli. These stimuli can be classified as nonspecific or specific. Nonspecific stimuli include drugs such as

methacholine or histamine, air pollutants such as ozone, physical stimuli such as cold air or water, and exercise. Specific stimuli include allergens and occupational agents such as toluene diiocyanate and western red cedar dust. This hyperresponsiveness causes the equine airway to constrict in response to stimuli that do not affect normal animals to the same degree. Heavey animals are hyperresponsive to aerosol and intravenous histamine, aerosol methacholine, aerosol citric acid, and aerosol water (Armstrong et al. 1986, Derksen et al. 1985a, 1985c, Obel and Schmiterlow 1948). These heavey horses and ponies are hyperresponsive only during acute disease exacerbations and not during clinical remission when the airway responsiveness is similar to normal ponies (Derksen et al. 1985a).

Airway responsiveness in ponies and horses is measured in the following manner. Increasing concentrations of agonist drug are administered either by aerosol or intravenously in some cases. Pulmonary function measurements such as R_L and C_{dyn} are measured following each dose of the drug. A dose-response curve is constructed plotting C_{dyn} and R_L versus the dose of the drug. The dose required to decrease C_{dyn} to 65% of the baseline value ($ED_{65}C_{dyn}$) is calculated by interpolation between points on the dose-response curve. Therefore, heavey animals during acute exacerbations of the disease show a greater decrease in $ED_{65}C_{dyn}$ or increase in R_L at 0.1 mg/ml drug dose ($R_{L\,0.1}$) when compared to normal subjects or heavey animals in remission.

Mechanisms suggested to account for hyperresponsiveness include 1) changes in the autonomic regulation of airways with an exaggeration of constrictor effects and a loss of dilator effects (Gold et al. 1972, Szentivanyi 1968, Barnes et al. 1980a);

2) altered intrinsic airway smooth muscle responsiveness; 3) epithelial damage that alters the permeability and allows mediators to reach smooth muscle and sensory nerves in greater concentration than normal (Jones et al. 1980, Boucher et al. 1977); 4) epithelial damage that alters the local production of relaxing factors and impairs the degradation of pro-inflammatory neuropeptides (Hogg and Eggleston 1984, Laitinen et al. 1985, Empey et al. 1976); 5) cellular dysfunction and inflammation that alters the profile of eicosanoids in the airways (Metzger et al. 1986, O'Bryne et al. 1984); and 5) geometric factors leading to a narrowed airway.

Autonomic abnormalities in airway disease

The idea that autonomic control might be abnormal in obstructive airway diseases was first suggested by Alexander and Paddock, who observed that wheezing was precipitated in asthmatics, but not in normal people, by injection of the cholinergic agonist pilocarpine, and that it was relieved by an injection of EPI (Alexander and Paddock 1921). Such an observation could involve an increase in excitatory mechanisms, or a lack of inhibitory influences, so that the balance is tipped in favor of excitation, resulting in hyperreactive airways. This led to the speculation that the basic defect in asthma was an imbalance between cholinergic and sympathetic nervous systems. Several different autonomic abnormalities have been proposed, including enhanced cholinergic (Simonsson et al. 1967, Reed 1974), alpha adrenergic (Reed 1974, Szentivanyi 1968), or noncholinergic excitatory mechanisms (Lundberg et al. 1983a), or reduced beta adrenergic (Reed 1974, Szentivanyi 1968), or nonadrenergic inhibitory mechanisms (Richardson 1981, Barnes 1984b). The idea that neural

control may be abnormal in asthmatics has been supported by the lack of correlation between the contraction of airway smooth muscle in vitro and the responsiveness to the same agonist in vivo (Armour et al. 1984, Vincenc et al. 1983, Roberts et al. 1984). Therefore, these studies suggest that the hyperresponsiveness lies in the control of the airway smooth muscle rather than in the smooth muscle itself.

Cholinergic mechanisms in airway disease

Because parasympathetic nerves are the dominant bronchoconstrictor neural pathway in most species studied, it is logical to suggest that overactivity of cholinergic mechanisms might contribute to airway obstruction and airway hyperresponsiveness in airway disease. The hyperresponsiveness seen in asthma and obstructive pulmonary disease could be due to many potential mechanisms. There could be an increase in the afferent receptor (irritant or C-fiber) discharge, either as a result of inflammatory mediators and/or because afferent nerve endings below the epithelium have been exposed due to damage or loss of epithelium. Inflammatory mediators could also potentially facilitate neurotransmission through cholinergic ganglia and efferent nerves (Hahn et al. 1978, Sheller et al. 1982). Finally, the smooth muscle may be hyperresponsive to ACh, either by an increase in muscarinic receptor number or affinity, or by a postreceptor mechanism.

Since the data suggest that there may not be an abnormality of the airway smooth muscle per se, Holtzman investigated where methacholine acts to cause bronchoconstriction in asthmatics (Holtzman et al. 1980). The effects on the bronchomotor responses to methacholine with and without pretreatment with

hexamethonium and atropine were investigated. The findings of this study suggest that methacholine acts directly at the smooth muscle muscarinic receptor to cause bronchoconstriction and raises the question of whether muscarinic receptors are dysfunctional in airway disease. Mita and co-workers showed that the muscarinic receptor numbers are increased in guinea pigs sensitized with ovalbumin and then challenged with ovalbumin aerosol, but the receptor affinity is unchanged (Mita et al. 1983). Supporting the theory of dysfunctional muscarinic receptors, McKay and Brooks demonstrated an increased sensitivity of guinea pig airways to carbachol following exposure to toluene diisocyanate and attributed this to an increase in the number or affinity of muscarinic receptors (McKay and Brooks 1983). What this suggests is that muscarinic receptors might be abnormal in some models of airway disease; however, since the in vitro studies do not support an increased cholinergic sensitivity in the diseased airway smooth muscle, this makes implication of the cholinergic receptor system in the pathogenesis of airway disease difficult. In fact, the increased responsiveness is not confined to cholinergic agonists, but is also seen with other spasmogens, such as histamine, prostaglandins, bradykinin, and leukotrienes (Boushey et al. 1980).

The contribution of the reflex cholinergic mechanisms to airway obstruction can be tested by examining the protection provided by anticholinergic drugs. These drugs are effective against the bronchoconstrictor stimuli that are known to activate afferent receptors, but are less effective against bronchoconstriction induced by antigen, histamine, and exercise (Gross and Skorodin 1984, Mann and George 1985). Anticholinergic drugs give no protection against the direct effect of locally released

mediators in the airways, unlike beta agonists and theophylline, which relax airway smooth muscle regardless of the contractile agent used. In acute severe asthma, anticholinergics have a significant bronchodilator effect, suggesting that the reflex component of bronchoconstriction is considerable (Ward et al. 1981).

Adrenergic mechanisms in airway disease

Possible abnormalities of sympathetic function that could result in bronchoconstriction include 1) decreased circulating catecholamines; 2) a defect in Beta receptor function; and 3) enhanced alpha adrenergic activity.

The fact that humans have an absence of a direct functional adrenergic innervation, but yet airway smooth muscle of humans and other species is potently relaxed by beta agonists in vitro (Davis et al. 1982, Goldie et al. 1982, Zaagsma et al. 1983) and in vivo suggests that circulating catecholamines might be important in regulating human airway tone. Plasma catecholamines are no higher in stable asthmatics at rest than in age-matched normal subjects, nor is there any relationship between plasma catecholamine concentration and severity of bronchoconstriction (Barnes et al. 1982b). Even in subjects who bronchoconstrict in response to infused propranolol, plasma concentrations of catecholamines are within the normal range (Ind et al. 1984). Even in acute severe asthma, no elevation of plasma EPI has been found (Ind et al. 1985). The low concentrations of EPI found in plasma (< 0.5 nmol/L) is difficult to understand, since these concentrations of EPI could exert little effect on airway smooth muscle itself. One possibility is that EPI may be exerting an inhibitory effect on the airway ganglia. Thus, beta blockers could enhance cholinergic

tone, which would have a greater effect in asthmatic subjects because of the enhanced sensitivity to ACh. This idea is supported by the fact that anticholinergic drugs prevent and reverse beta-blocker induced bronchoconstriction (Grieco and Pierson 1971). Scott showed similar results in ponies with recurrent airway obstruction to those found in human asthmatics (Scott et al. 1988b). In this study, treatment with propranolol did not alter R_L, C_{dyn}, or airway responsiveness to histamine in control ponies or affected ponies in remission, but significantly increased pulmonary resistance in affected ponies during acute exacerbations of the disease (Scott et al. 1988b). However, this propranolol-induced bronchoconstriction was prevented by atropine, suggesting the bronchoconstriction resulted from unopposed cholinergic activity (Scott et al. 1988b).

An enhanced alpha-adrenergic response in asthma has been proposed as a cause of hyperresponsiveness (Reed 1974, Szentivanyi 1968). In a guinea pig model asthma, there is a marked increase in pulmonary alpha-1 receptor density in the affected animals (Barnes et al. 1980a). Similarly in humans, the density of alpha receptors is much higher in lung taken from patients with airway obstruction than from normal lung (Barnes et al. 1980a). Asthmatic subjects bronchoconstrict in response to inhaled alpha agonists (Simonsson et al. 1972, Snashall et al. 1978), even in the absence of beta-blockade (Black et al. 1982), whereas normal subjects are unaffected. The bronchoconstrictor effect of the alpha-agonist methoxamine is inhibited by the selective alpha-1 antagonist prazosin, suggesting that alpha-1 receptors are involved (Black et al. 1984). The bronchoconstrictor response to methoxamine is also inhibited by anticholinergic drugs, therefore suggesting the

involvement of cholinergic pathways (Black et al. 1986). Others have failed to demonstrate a bronchoconstrictor effect of alpha agonists in asthmatic patients (Thomson et al. 1982). To prove that an alpha-adrenergic mechanism contributes to the pathogenesis of asthma requires the demonstration that alpha-adrenoceptor antagonists have a beneficial effect in asthma.

Since most alpha-antagonists have other pharmacologic actions, such as antihistamine activity, direct effects on airway smooth muscle, and release of circulating catecholamines, the effect of alpha-antagonists in asthma remains controversial (Barnes 1984a, 1985). Prazosin, a specific alpha-antagonist selective for alpha-1 receptors, has been reported to cause bronchodilation after oral administration (Marlin et al. 1982). This effect could be explained by a baroreceptor response to the drop in blood pressure. Inhaled prazosin has no bronchodilator effect in asthmatic patients (Barnes et al. 1981a, 1981b) and no effect on histamine-induced bronchoconstriction (Barnes et al. 1986). The role of alpha-receptors in the pathogenesis of asthma thus appears limited, and alpha-antagonists have no useful role to play in the therapy of asthma.

Scott and coworkers (1988a) studied the response of ponies with recurrent obstructive disease to aerosol challenge with alpha-adrenergic agonists. These ponies were pretreated with atropine and propranolol, then challenged with the alpha-agonist phenylephrine. Phenylephrine had no effect on control ponies, but decreased C_{dyn} and increased R_L in the affected ponies during both remission and periods of airway obstruction. This suggested that ponies with recurrent obstructive pulmonary disease had increased density and/or activity of alpha receptors compared to the

control ponies. However, the involvement of the alpha receptors in airway obstruction was thought to be only minimal, because treatment with the alpha-1 antagonist, prazosin, produced no improvement in pulmonary function in the heavey ponies (Scott et al. 1988a). This lack of response to an alpha-antagonist is similar to that reported for asthmatics (Barnes et al. 1981a, Utting 1979). The results of these studies suggest that increased alpha receptor activity is not the primary problem in asthma or heavey ponies.

Possible abnormalities of the nonadrenergic, noncholinergic nervous system

Although it has been possible to demonstrate the existence of the NANC nerves in several species both in vitro and in vivo, the physiological role of this system in health and disease is difficult to investigate, because the neurotransmitter is not certain and no specific blocker is presently available. If the neurotransmitter is a peptide, then a functional defect in this system may develop in airway disease as a result of inflammation. Increased degradation of peptide transmitters by inflammatory cell peptidases could disrupt the modulating action of the non-adrenergic inhibitory system on cholinergic nerves and lead to exaggerated bronchoconstrictor responses (Barnes 1986b). Airway inflammation is reported in ponies and horses with heaves during acute exacerbation of airway obstruction. Ollerenshaw and co-workers reported an absence of VIP-immunoreactive nerves in lung tissue from asthmatics (Ollerenshaw et al. 1989). The lack of VIP is not known to be either a cause or an effect of the disease. However, the lack of VIP could be important, since the nonadrenergic inhibitory system is the sole inhibitory innervation of human airways.

The increased levels of proteases in asthmatic airways were thought not to be responsible for the lack of VIP, because SP-containing nerves were present.

Decreased baseline airway caliber

The caliber of the airways in the baseline state can influence the subsequent response to agents that induce bronchoconstriction. Most in vivo tests of airway narrowing depend directly or indirectly on changes in airflow resistance. Resistance is inversely proportional to the fourth power of the radius when the flow in the airways is laminar; therefore, any decrease in the radius of a narrow airway causes a greater change in airway resistance than does the same decrease in the radius of a dilated airway. Differences in airway caliber during bronchoconstriction and smooth muscle hypertrophy may be further exaggerated by folding of the mucosa or mucosal edema (Bouhuys 1963, Freedman 1972). Based on this, airway hyperreactivity is due primarily to the fact that the airways are narrower in the control state before bronchial provocation (Benson 1975).

Although changes in baseline airway caliber may be important under some circumstances, the differences in bronchial hyperreactivity in a variety of circumstances are not explained by a decrease in airway caliber, because the baseline airway caliber was similar. For example, asthmatic subjects in clinical remission (Townley et al. 1971, 1975, Cockcroft et al. 1977), subjects with hay fever (Townley et al. 1965, Fish et al. 1976), and normal subjects following a brief exposure to ozone (Golden et al. 1978, Holtzman et al. 1979), who have no evidence of airflow obstruction in the baseline state, may have increased bronchial reactivity.

Alteration in smooth muscle

Hypertrophy and hyperplasia of airway smooth muscle occur in asthmatic patients (Takizawa and Thurlbeck 1971) and in some patients with chronic bronchitis (Hossain 1970). These alterations in the airway smooth muscle could play a role in the hyperreactivity associated with these diseases, because an increased amount of muscle is capable of developing greater tension and may narrow the airways more than the normal amount. These changes in airway smooth muscle and airway wall thickness probably contribute to the increased responsiveness of severely asthmatic patients, but they cannot be the cause of the hyperreactivity that occurs transiently during viral infections (Empey et al. 1976) or after exposure to ozone (Golden et al. 1978, Holtzman et al. 1979, Lee et al. 1977), because the mass of smooth muscle is not likely to change in such a short time.

Modification of smooth muscle behavior itself may account for bronchial hyperreactivity. For example, the exposure to ozone has been shown to cause a decrease in the acetylcholinesterase concentration in circulating red blood cells (Goldstein et al. 1968), and may have a similar effect in airway smooth muscle (Boushey et al. 1980). Ozone could also damage the epithelium or drug-receptor binding sites, which could lead to airway hyperreactivity (Lee et al. 1977).

Changes in airway smooth muscle that could alter airway response include an alteration in myosin content. Mapp et al. concluded that in isolated canine airways the active force generated was dependent on the content of myosin in the muscle (Mapp et al. 1989). Other changes that could alter the airway responses would be post-receptor mechanisms, such as changes in smooth muscle calcium release. A

decrease in cCAMP or cCAMP-dependent kinase activity would result in increased smooth muscle tension development. A relationship between airway reactivity and cAMP levels has not been investigated.

It is not known whether airway hyperreactivity is characterized by changes in the intrinsic properties of the airway smooth muscle itself. Dose-response curves to carbachol in subjects with asthma show wide variations in the dose needed to decrease specific airway conductance and in the slopes of the curves (Orehek et al. 1977). This could be due to changes in the smooth muscle mass, changes in the contractile mechanisms or their regulation within the airway smooth muscle (Boushey et al. 1980).

Airway epithelium

Many researchers have reported that airway epithelium produces substances that modulate the responsiveness of underlying airway smooth muscle to contractile agonists and have coined the term "epithelial-derived relaxant factor" to describe these substances (Flavahan et al. 1985, Morrison et al. 1990, Tschirhart et al. 1987). Decreased production or release of such a factor could increase the responsiveness of airway smooth muscle to contractile stimuli and thus contribute to the hyper-responsiveness seen in diseases such as recurrent airway obstruction in horses and asthma. In some species this epithelial-derived relaxant factor appears to be a cyclooxygenase metabolite, because the effects of epithelial removal can be reproduced by treating tissues with indomethacin, a cyclooxygenase inhibitor (Barnett et al. 1988, Butler et al. 1987, Folkerts et al. 1989, Tschirhart et al. 1987). Prostaglandin

E_2 is produced by intact airway epithelium in response to a variety of stimuli, including eosinophil-derived major basic protein (Jacoby et al. 1987), granulocytes and reduced oxygen molecules (Kazura et al. 1987), bradykinin, platelet activating factor, and calcium ionophore (A23187) (Widdicombe et al. 1989). In vivo, PGE_2 is a bronchodilator in both normal and asthmatic humans (Cuthbert 1971, Kawakami et al. 1973) and reverses bronchoconstriction caused by administration of $PGF_{2\alpha}$ (Smith et al. 1975). In vitro, it relaxes isolated human (Sweatman and Collier 1968) and equine (Gill and Kroeger 1990) tracheal smooth muscle, possibly by inhibiting ACh release at a prejunctional site (Barnett et al. 1988). Decreased production of PGE_2 by tracheal epithelium is also associated with endotoxin-induced hyperreactivity in guinea-pig isolated trachea (Folkerts et al.1989).

As mentioned, one of the features of both bronchial asthma in humans and horses with "heaves" is loss of, or damage to, airway epithelial cells (Kaup et al. 1990a, 1990b). Histological examination of airway epithelial biopsies from asthmatics during remission (Laitinen et al. 1985a) and following death in status asthmaticus (Dunhill 1960) shows widespread epithelial damage, affecting principally ciliated epithelial cells. A relationship between epithelial damage and bronchial hyperreactivity is supported by reports that exposure to agents such as ozone that are known to damage the airway mucosa, results in increased bronchial reactivity. In guinea pigs, this hyperreactivity is related to signs of airway mucosal injury and precedes neutrophil infiltration (Murlas and Roum 1985).

Epithelial damage may result in bronchial hyperresponsiveness through many potential mechanisms. Stimuli could more readily interact with superficially located

sensory nerve endings, which terminate in the epithelial layer, resulting in exaggerated vagal reflexes. Epithelial damage may also cause release of chemotactic factors, resulting in an influx of neutrophils into the airways. Inflammatory mediators released by these cells may then result in tracheal smooth muscle hyperresponsiveness. Alternatively, epithelial cells may synthesize and release an epithelium-derived relaxant factor that normally modulates the response of tracheal smooth muscle to contractile stimuli. Epithelial cell destruction could decrease production of such a factor, with a consequent increase in tracheal smooth muscle responsiveness. Finally, epithelial cells may possess metabolic pathways for inactivating inflammatory mediators released into the airway lumen. For example, epithelial cells contain enzymes such as enkephalinase, which break down inflammatory peptides. Reduced levels of these enzymes during influenza infection have been associated with airway hyperreactivity (Jacoby et al. 1988).

The identity or existence of an epithelium-derived relaxant factor is the subject of much debate. In the rabbit, mechanical removal of the epithelium from bronchial segments increases the sensitivity of bronchial smooth muscle to bethanecol (Butler et al. 1987). In the guinea-pig, relaxation of tracheal smooth muscle exposed to arachidonate is converted to contraction following epithelium removal (Tschirhart et al. 1987), while in ovalbumin-sensitized animals, epithelium removal causes a greater increase in sensitivity to antigen than to either methacholine or histamine (Hay et al. 1986). In humans, contractile responses of bronchial smooth muscle to ACh, histamine, and $PGF_{2\alpha}$ are significantly increased following epithelium removal, while

subsequent addition of chopped epithelium to the muscle bath significantly decreases the contractile response to ACh (Aizawa et al. 1988).

Eicosanoids and airway epithelium

A potent group of chemical mediators that may play an important role in the production of lung inflammation and airway hyperresponsiveness are the 20-carbon oxygenated derivatives of arachidonic acid-termed eicosanoids (5,8,11,14-eicosa-tetraenoic acid). Two major pathways of AA metabolism have been described. The cyclooxygenase pathway leads to the formation of prostaglandins and thromboxanes. The lipoxygenase pathway results in the production of metabolites of 5-,12- and 15-lipoxygenases.

There is increasing evidence that eicosanoids play a role in the pathogenesis of airway hyperresponsiveness. Some arachidonate metabolites are potent broncho-constrictors in humans (Weiss et al. 1982, Dahlen and Hedqvist 1980) and animals (Drazen et al. 1980, Hedqvist et al. 1980) and also alter mucus production (Marom et al. 1982, 1983), cause inflammatory cell accumulation (Palmer et al. 1980, Goetzl et al. 1980), and vascular permeability changes (Kuehl and Egan 1980). These are all characteristic features of human asthma and heaves. After an attack of asthma, plasma levels of 15-keto,13,14-dihydro-prostaglandin $F_{2\alpha}$ (a metabolite of $PGF_{2\alpha}$) are increased and the degree of increase correlates with the severity of the attack (Green et al. 1974). Furthermore, antigen challenge of human asthmatic lung leads to the release of leukotriene C_4, D_4, and E_4 in vitro (Dahlen et al. 1983a). Allergen chal-lenge of asthmatic patients results in increased bronchoalveolar lavage concentrations

of PGD_2 (Wenzel et al. 1989, Murray et al. 1986), thromboxane B_2 (Wenzel et al. 1989), and 15-HETE (Murray et al. 1986). The urinary LTE_4 concentration following antigen challenge is also significantly increased (Taylor et al. 1989). There is no change in the concentration of these eicosanoids in body fluids of control populations. Even asthmatics in clinical remission have elevated levels of proinflammatory prostanoids in bronchoalveolar lavage fluid (Liu et al. 1990). Doucet and co-workers in preliminary studies report increased levels of LTE_4 in the urine of heavey horses in remission compared to normal horses (Doucet et al. 1991).

The epithelium is able to produce both cyclooxygenase (Xu et al. 1986, Churchill et al. 1989) and lipoxygenase products of arachidonate metabolism (Hunter et al. 1985, Holtzman et al. 1988, Eling et al. 1986, Hansbrough et al. 1989, Holtzman et al. 1983). PGE_2 is the major product in cultured sheep and human airway epithelium (Churchill et al. 1989, Holtzman 1987. Gray and co-workers (1992b) have recently shown that PGE_2 is also the major eicosanoid produced by equine tracheal epithelium. In the guinea pig trachea, increased contractility after endotoxin treatment coincided with a decrease in PGE_2 production by the epithelial layer (Folkerts et al. 1989). Also, arachidonate causes relaxation of guinea pig airway smooth muscle in vitro (Farmer et al. 1987).

Within the lung, bioactive arachidonate acid metabolites have been implicated in numerous homeostatic and pathologic processes. However, a contrast in the predominant biologic activities of lipoxygenase products and cyclooxygenase metabolites has become apparent. PGE_2 is a potent bronchodilator in a number of species (Smith and Cuthbert 1976); leukotrienes are powerful bronchoconstricting

agents (Dahlen et al. 1983b, Dahlen 1983a, 1983b, Hamel et al. 1983). PGE_2 is a major arachidonate metabolite of respiratory epithelium, and its production is stimulated by a number of inflammatory mediators, including bradykinin, platelet activating factor, reactive oxygen species, eosinophil major basic protein, interleukin 1, and tumor necrosis factor (Hamel et al. 1983, Bachwich et al. 1986, Godfrey et al. 1988, Elias et al. 1987, Widdicombe et al. 1989, Kazura et al. 1987, Jacoby et al. 1987). PGE_2 in turn can decrease production of some of these same mediators, including interleuklin 1, tumor necrosis factor, and LTB_4 (Kunkel et al. 1986a, 1986b, Ham et al. 1983, Brigham et al. 1988). In contrast, lipoxygenase products such as 12-HETE can stimulate the production of pro-inflammatory substances (Kanaji et al. 1986, Maclouf et al. 1982). Attraction, adherence, and activation of inflammatory cells such as neutrophils are stimulated by lipoxygenase products (LTB_4) (Sirois et al. 1980), but are inhibited by PGE_2. Lipoxygenase eicosanoids augment, whereas PGE_2 suppresses, lymphocyte proliferation and antibody production. In addition, PGE_2 can inhibit ACh release pre- and post-synaptically or abolish excitatory post-junctional electrical potentials (Shore et al. 1987, Daniel et al. 1987).

Rat alveolar epithelium predominantly metabolizes AA to PGE_2 and PGI_2 Chauncey et al. 1988). Similarly, equine airway epithelium produces PGE_2 as the major AA metabolite. In contrast, alveolar macrophages and inflammatory cells such as neutrophils and eosinophils predominantly generate lipoxygenase products (Ford-Hutchinson et al. 1980). The significance of this eicosanoid profile is that it endows the epithelium with the potential to down-regulate inflammatory processes and prevent smooth muscle contraction in the airways, a potential not shared by other

resident cells such as the alveolar macrophage or by inflammatory cells such as neutrophils. In inflammatory airway diseases, epithelial damage may result not only in decreased PGE_2 production and a decreased ability to down-regulate inflammation, but AA made available may also be metabolized by damaged epithelial cells, alveolar macrophages, or inflammatory cells to pro-inflammatory lipoxygenase products. This imbalance in epithelial eicosanoid metabolism may well contribute to airway obstruction and inflammation.

Specific aims

Horses with heaves have clinical signs that include dyspnea characterized by an accenuated abdominal effort at end expiration, cough, intermittent or chronic purulent nasal discharge, and exercise intolerance. During acute exacerbations of the disease, heavey horses have a greater ΔPpl than normal horses and are hypoxemic. Dynamic compliance is lower and R_L is higher in horses with heaves. The airways of horses with heaves are hyperresponsive to various stimuli during acute exacerbations of the disease. All of these signs and findings are consistent with an obstructive pulmonary disease.

The literature has shown an intimate relationship between airway smooth muscle, autonomic innervation, and airway epithelium. My overall objective, therefore, was to examine the role of cholinergic mechanisms in the hyperresponsiveness and airway obstruction of heaves. To accomplish this objective, experimental protocols were designed and performed to:

1. Determine in vivo the role of muscarinic receptors in the bronchoconstriction of heaves by administration of the muscarinic antagonist atropine to both normal and heavey animals.

2. Determine if the autonomic regulation of airway smooth muscle is altered in horses with heaves. The smooth muscle of the trachea and third-generation bronchi was studied. Excitatory and inhibitory innervation of the trachea was defined in normal and diseased horses.

3. Determine if the role of epithelium and prostanoids in modulating the underlying smooth muscle differs in control and heavey tissues.

CHAPTER 2

EFFECTS OF ATROPINE IN PONIES WITH RECURRENT AIRWAY OBSTRUCTION

Introduction

Patients with asthma and chronic obstructive bronchitis show greater airway responsiveness to inhaled agonists than do normal subjects (Nogrady and Bevan 1978, Snapper et al. 1986). Ponies with a history of recurrent obstructive pulmonary disease (heaves) also demonstrate airway hyperresponsiveness to histamine and other aerosol agonists, but the cause of this increased responsiveness is unknown (Chung et al. 1982). In other species, histamine produces bronchoconstriction by acting directly on airway smooth muscle H_1 receptors (Laitinen 1974, Thomson 1983) and possibly via vagal reflexes (Richardson 1979). Airway hyperresponsiveness to histamine may therefore be due to increased sensitivity of the smooth muscle itself, or may be related to augmented vagal reflexes.

If cholinergic pathways are involved in the hyperreactive response to inhaled histamine in ponies with recurrent airway obstruction, atropine should attenuate this response. I therefore evaluated the ability of atropine to modify the histamine-induced bronchoconstriction in ponies with recurrent airway obstruction to determine whether parasympathetic activity is responsible for the airway hyperreactivity in this

model. I also wished to investigate the role of the parasympathetic nervous system in the airway obstruction which occurs in this disease.

To investigate the role of the parasympathetic nervous system in the airway obstruction and hyperreactivity of the principal ponies, I established an atropine dose for muscarinic blockade. Atropine has a narrow therapeutic window, because it is easily absorbed and readily crosses the blood-brain barrier, causing excitement in ponies. The first protocol established an effective atropine dose regime by challenging the ponies with increasing concentrations of aerosol methacholine, while the second protocol examined the effects of intravenous (I.V.) atropine on the dose-response curves to aerosol histamine. I then examined the difference in response to aerosol and I.V. atropine administered prior to aerosol histamine challenge in the principal ponies during acute exacerbations of airway obstruction.

Methods

Pony preparation

Mixed-breed ponies with a history of heaves were matched for age and gender with a control group of ponies with no history of heaves. Chronic tracheostomas were created in the midcervical region and the carotid arteries were relocated to a subcutaneous site. To ensure that both pony groups received the same environmental exposures, pairs were housed together, transported together, fed together, and studied on the same day. Measurements were made when principal ponies were in clinical remission (period A), at which time all animals were kept on pasture with no exposure to hay, straw, or a barn environment. Ponies were also studied during an

acute attack of airway obstruction in the principals precipitated by housing principal and control ponies in a barn (period B).

Pulmonary function measurements

The ponies were studied unsedated standing in stocks. A 20-mm-ID cuffed endotracheal tube (45 cm in length) was introduced into the trachea via the tracheostoma. A pneumotachograph (Fleisch No. 4, Dynasciences, Blue Bell, PA) and associated pressure transducer (Validyne DP 45-22, Northridge, CA) were attached to the endotracheal tube. The pneumotachograph transducer system produced a signal proportional to flow that was electronically integrated to give V_T. Prior to each experiment, this system was calibrated by forcing known volumes of air through the pneumotachograph, using a 2-liter syringe (Super Syringe, Hamilton Syringe Co., Warminster, PA).

An esophageal balloon (10 cm length, 3.5 cm perimeter, 0.06 cm wall thickness) was sealed over the distal end of a polypropylene catheter (3 mm ID, 4.4 mm OD, 140 cm length) that had several spirally arranged holes in the portion covered by the balloon. The distance from the nares of the ponies to the mid-thoracic portion of the esophagus was visually approximated and marked on the esophageal balloon catheter. The esophageal balloon was passed via the nares into the mid-thoracic portion of the esophagus. Balloon volume was adjusted to 0.5 ml of air (Derksen and Robinson 1980). The balloon was attached to a pressure transducer (Model PM 131, Statham Instruments, Hato Rey, Puerto Rico) that was taped to the halter. Transpulmonary pressure (P_L) was defined as the pressure

difference between atmospheric and esophageal pressure. Transpulmonary pressure, V_T, and flow ($\dot{V}$) were recorded on light-sensitive paper (VR_{12}, Electronics for Medicine, White Plains, NY).

Tidal volume, *f*, C_{dyn}, and R_L were calculated by a pulmonary function computer (Buxco Electronics, Inc., Pulmonary Mechanics Analyzer, Model 6, Sharon, CT). These values were checked against calculations from the physiograph recording. Dynamic compliance was calculated by dividing V_T by the difference in P_L between points of zero flow. Pulmonary resistance was calculated using the isovolume method of Amdur and Mead (1958). To prevent phase differences between pressure and flow, frequency responses of catheter systems were matched to 10 Hz (Derksen and Robinson 1980).

Arterial O_2 tension (PaO_2), CO_2 tension ($PaCO_2$) and pH were measured using a blood gas analyzer (Model ABL 3, Radiometer, Copenhagen, Denmark) immediately prior to baseline pulmonary function measurements.

Airway responsiveness to aerosol histamine and methacholine

Dose-response curves were generated for aerosol histamine and methacholine in the following manner. Aerosolized sterile 0.9% NaCl was used as the "control" for the dose-response curves. Solutions of 0.001, 0.01, 0.1, 1.0, 3.0, 10.0, 30.0, and 100 mg of agonist per ml were prepared by dissolving either histamine diphosphate (Sigma Chemical, St. Louis, MO) or methacholine chloride in sterile 0.9% NaCl (Sigma Chemical, St. Louis, MO). Aerosols were generated by an ultrasonic nebulizer (Model 65, DeVilbiss, Somerset, PA). The output of the nebulizer averages

0.11 ml/2-liter breath and delivers a particle size of 0.5-3.0 μm (7). Delivery of aerosol to the ponies was accomplished by attaching a 2-way non-rebreathing valve to the endotracheal tube and attaching the valve to the nebulizer. The ponies were allowed to breathe the aerosol spontaneously for 2 minutes. The nebulizer was then disconnected from the endotracheal tube, and the pneumotachograph was attached for measuring lung function.

Beginning one minute after each aerosol challenge, P_L, V_T, and $\dot{V}$ were recorded for 2 minutes. The C_{dyn} and R_L were calculated and averaged over this time period. Exactly 4 minutes after the end of the first challenge, another aerosol challenge began. The concentrations of aerosol histamine or methacholine were increased until C_{dyn} decreased by 35% or more of the value recorded following saline aerosol. At this time ponies exhibited respiratory distress.

Dose-response curves of C_{dyn} and R_L were plotted as a function of methacholine or histamine aerosol concentration. By interpolation between points on the dose-response curves, I calculated the $ED_{65}C_{dyn}$. Airway reactivity was also measured by determining the change in R_L between the aerosol saline and the 0.1 mg/ml histamine or methacholine aerosol ($\Delta R_{L0.1}$).

Experimental protocols

Protocol 1: Determination of intravenous atropine dose

Three pairs of ponies (19.7 ± 3.24 years, body weight 223.7 ± 15.3 kg) were studied at periods A and B. Pulmonary function measurements and airway reactivity were measured in both principal and control ponies at each measurement period on

consecutive days. On day 1, baseline measurements were performed. Each pony received an I.V. injection of phosphate buffered saline, then fifteen minutes later the aerosol methacholine challenge started. On day 2, baseline measurements were repeated, then each pony received an I.V. injection of 0.02 mg/kg atropine sulfate (Atropine Injectable L.A., Fort Dodge Laboratories, Inc., Fort Dodge, IA). This was followed by a continuous infusion of atropine at 0.0013 mg/kg/minute (Harvard Infusion/Withdrawal Pump, Model 911, Harvard Apparatus, Dover, MA). The atropine infusion continued throughout the aerosol methacholine challenge period. Fifteen minutes elapsed between the atropine I.V. bolus and the first aerosol challenge dose of methacholine.

Protocol 2: Effects of intravenous atropine on aerosol histamine responsiveness

Six pairs of ponies (6 principals, 6 controls 16.2 ± 3.9 years, body weight 227 ± 19 kg, means ± SE) were studied to determine the effects of I.V. atropine on the response to aerosol histamine challenge. This protocol was identical to protocol 1 except histamine was the agonist.

Protocol 3: Effects of aerosol atropine on aerosol histamine responsiveness

Six principal ponies (13.8 ± 2.5 years, 211.7 ± 16.7 kg weight) at period B were studied to compare the effects of aerosolized or I.V. administered atropine on the response to aerosol histamine challenge. On day 1, the ponies' dose-response curve to aerosol histamine was generated without pretreatment of atropine. On another day each pony, following a baseline pulmonary function reading, inhaled

aerosol atropine until a dosage of 0.14 mg/kg had been nebulized. This volume was nebulized with spontaneous ventilation in an average time of 9.83 ± 0.65 minutes. Fifteen minutes elapsed between the end of atropine aerosol and the beginning of aerosol histamine challenge. An aerosol atropine supplement was administered for 1 minute every 12 minutes between histamine aerosol challenges. This dosage was determined by challenging with aerosol methacholine as in Protocol 1. On a third day, the I.V. atropine was administered as previously described in protocol 2 prior to histamine aerosol challenge.

Statistical Analysis

The effects of barn exposure, pasture housing, and treatment were analyzed using a split plot factorial analysis of variance. When F values were significant at $P < 0.05$, means from each measurement period were compared using Tukey's omega procedure.

Results

Table 1 shows baseline lung function data of both groups of ponies from protocol 2 (n = 6 pairs) at measurement periods A and B prior to aerosol challenge. At period A, no significant differences existed between principals and controls in R_L, C_{dyn}, $PaCO_2$, PaO_2, V_T, or *f*. Following exposure to the barn environment, principal ponies developed clinical signs of airway obstruction, including recruitment of abdominal muscles on expiration. In the control group, barn exposure caused no change in lung function measurements, but in the principal group barn environment

significantly decreased PaO_2 and C_{dyn}, and increased R_L. Principal ponies had no change in V_T and f at period B. During barn exposure, $PaCO_2$ was greater in the principal ponies than in the controls, yet within the normal range.

Intravenous atropine effects on lung function

Figure 2-1 shows C_{dyn} after I.V. saline or atropine but prior to histamine aerosol challenge in controls and principals at measurement periods A and B. Following I.V. saline, no significant differences were observed between principal and control groups at period A, and barn housing had no effect on C_{dyn} in the control group. In principals at period B, C_{dyn} following I.V. saline was significantly less than in principals at period A and also less than in controls at periods A and B. The intravenous dose of atropine described in protocol 1 shifted the aerosol methacholine dose-response curve at least two logarithmic doses to the right in all ponies but caused no significant change in C_{dyn} in the control ponies at periods A and B or in the principal ponies at period A. Atropine administration caused a non-significant increase in mean C_{dyn} in the principal ponies at period B to a value that was approximately one half of the principals' period A value.

Pulmonary resistance after I.V. saline and atropine at periods A and B is also shown in Figure 2-1. No significant differences in R_L occurred between treatments or measurement periods in the control ponies. In principal ponies, barn exposure significantly elevated R_L measured following I.V. saline to a value significantly greater than in the controls at periods A and B. At period A, atropine had no effect on R_L in principal ponies; however, at period B, atropine significantly decreased R_L.

Intravenous atropine effects on aerosol histamine responsiveness

All twelve ponies responded to histamine with a major decrease in C_{dyn} and a more modest increase in R_L. No significant changes in log ED_{65} C_{dyn} between the I.V. saline and I.V. atropine treatments were present in the control ponies at period A and B (Figure 2-2). At period A, log ED_{65} C_{dyn} was similar in principal and control ponies. At period B, the log ED_{65} C_{dyn} of the principal ponies following saline decreased significantly when compared to the controls at the same time period. At period B after I.V. atropine administration, the log ED_{65} C_{dyn} of principal ponies was significantly less than the log ED_{65} C_{dyn} of the control ponies at period B and also less than the log ED_{65} C_{dyn} of the principals following I.V. atropine at period A. Following atropine, the log ED_{65} C_{dyn} in principals at period B did not differ significantly from the log ED_{65} C_{dyn} following saline. No significant differences were found in $\Delta R_{L0.1}$ of controls between periods A and B or as a result of atropine administration (Figure 2-2). At period A, the $\Delta R_{L0.1}$ of principal ponies did not differ from the $\Delta R_{L0.1}$ of control ponies. Between period A and B, $\Delta R_{L0.1}$ increased significantly in the principal ponies. Following I.V. atropine at period B, the $\Delta R_{L0.1}$ tended to decrease but remained significantly different from the value in control ponies at the same time period and from principal ponies at period A.

Comparison of aerosol and intravenous atropine effects on lung function and aerosol histamine responsiveness

In protocol 3, neither aerosol nor I.V. atropine administration caused a significant increase in C_{dyn} in principals at period B. Dynamic compliance increased

by 0.01 ± 0.01 cm H_2O/L/sec following I.V. saline and by 0.37 ± 1.29 cm H_2O/L/sec and 0.34 ± 0.24 cm H_2O/L/sec following I.V. and aerosol atropine, respectively. Administration of atropine by both I.V. and aerosol routes significantly decreased R_L as shown in Figure 2-3. No significant difference occurred between the R_L values measured following I.V. or aerosol atropine. Although neither I.V. nor aerosol atropine caused significant changes in log ED_{65} C_{dyn} (Figure 2-4), the other measure of reactivity ($\Delta R_{L0.1}$) decreased significantly following both aerosol and I.V. atropine from the value measured following I.V. saline.

Discussion

The changes in lung function observed in principal ponies at period B are consistent with those described previously (Derksen et al. 1985a). Barn housing caused hypoxemia and airway obstruction (increased R_L and decreased C_{dyn}) in the principal group and no changes in lung function in the control ponies.

Following I.V. atropine administration, no significant changes in lung function occurred in the control ponies during either period A or B, suggesting that there is little cholinergic bronchomotor tone in normal ponies. In most other species muscarinic blockade causes modest bronchodilation, and Derksen and coworkers have described a small decrease in R_L measured at functional residual capacity, following vagal cooling in ponies (Derksen et al. 1982b). Above functional residual capacity, Derksen et al. found no effect of vagal cooling on resistance. In the present study, measurement of R_L at 65% of V_T above functional residual capacity may explain the lack of an effect of atropine on R_L in control and principal ponies at period A.

Alternatively, the pony may possess a non-cholinergic excitatory nervous system that is blocked by vagal cooling but not by atropine.

In the principal group of ponies at period B, I.V. atropine significantly decreased R_L but had no consistent effect on C_{dyn}. This suggests that a large portion of the increased R_L in the principal ponies at period B is mediated via muscarinic receptor activity. Our observations do not allow us to distinguish if the increased R_L is due to augmented vagal reflex activity, enhanced reactivity of smooth muscle to a normal level of vagal activity, or loss of inhibitory factors that normally oppose vagally mediated bronchoconstriction. Derksen et al. investigated the role of vagal reflexes in ponies sensitized to ovalbumin and subsequently given an aerosol ovalbumin challenge (Derksen et al. 1987a). Like the findings following atropine administration in the present study, vagal cooling caused a significant decrease in resistance but no increase in compliance. When only one lung was challenged with ovalbumin, however, no increase in resistance occurred in the contralateral lung, suggesting that vagally mediated reflexes were of minor importance as a cause of airway obstruction. They concluded that increased responsiveness to vagal activity was a likely cause of part of the airway obstruction. Similar logic may explain the effects of atropine observed in the present study. Armstrong and coworkers previously described hyper-responsiveness of airways to the muscarinic agonist methacholine in ponies with acute exacerbations of heaves but not when these ponies are in disease remission (Armstrong et al. 1986). A similar change in responsiveness of airways to ACh may explain the effects of atropine observed in the present study. The changes in lung function resulting from atropine administration in diseased ponies are similar to those

observed in humans with asthma and with chronic obstructive pulmonary disease (Gross 1987).

Mechanisms other than parasympathetic activity are contributing to the increase in R_L in principal ponies at period B, because intravenous atropine did not return the R_L to the baseline values at period A and also did not significantly increase C_{dyn}. A part of the obstruction must be due to the lesions of heaves, which are bronchiolitis with excess mucus production and leukocyte accumulation in the peripheral airways (Breeze 1979, Thurlbeck and Lowell 1964, Wilkie 1982).

Consistent with previous observations (Derksen et al. 1985a), ponies with a history of heaves were hyperreactive to aerosol histamine during acute exacerbations of airway obstruction but not during clinical remission. This was demonstrated by both measurements of hyperreactivity used in this study, log ED_{65} C_{dyn} and $\Delta R_{L0.1}$. For aerosol histamine the log ED_{65} C_{dyn} decreased by 360% in principals from period A to period B. $\Delta R_{L0.1}$ increased in principals by greater than 3500% between periods A and B.

The data do not support a role for muscarinic receptors in the response of normal pony airways to aerosol histamine. Intravenous atropine in a dose sufficient to shift the dose-response curve to methacholine by two logarithmic doses did not significantly change the histamine log $ED_{65}C_{dyn}$ or $\Delta R_{L0.1}$. This suggests that in normal ponies as in several other species, the response to histamine is due to a direct action on smooth muscle rather than vagal reflexes (Bleeker et al. 1976, Loring et al. 1977, Snapper et al. 1986, Thomson and Kerr 1980, Yanta et al. 1981). In principal ponies, there also appears to be little role for muscarinic receptors in the response

to aerosol histamine. At period A with principal ponies in clinical remission, atropine had no effect on the response to aerosol histamine and thus principal ponies in remission behave in a similar manner to control ponies (Figure 2-2). At period B, conclusions about reactivity would appear to vary depending on the measure of reactivity used. Neither I.V. nor aerosol atropine had a significant effect on log ED_{65} C_{dyn}; in fact, I.V. atropine tended to increase rather than decrease reactivity (Figure 2-2). In protocol 3, the $\Delta R_{L0.1}$ following both I.V. and aerosol atropine suggests that muscarinic blockade did change the principal ponies' responsiveness to the administration of aerosol histamine. However, it is quite likely that this observation is due to geometric changes in the airways, because the change in resistance and change in reactivity were significantly correlated (Figure 2-5).

In conclusion, this study has demonstrated that a significant portion of the airway obstruction observed in principals during acute exacerbations of recurrent airway obstruction is mediated through the muscarinic receptors. A significant role for muscarinic activity in the mediation of histamine induced bronchoconstriction in ponies with recurrent airway obstruction is not supported by this study.

Table 1. Baseline pulmonary function variables in principal and control ponies at periods A and B.

	Measurement Period	
	A	**B**
Control Ponies		
PaO_2, torr	89.9 ± 2.7	94.7 ± 2.4
$PaCO_2$, torr	37.1 ± 1.1	37.21 ± 1.1
C_{dyn}, L · cm H_2O^{-1}	0.99 ± 0.10	1.09 ± 0.17
R_L, cm $H_2O \cdot L^{-1} \cdot s$	1.05 ± 0.09	0.87 ± 0.08
V_T, liters	1.63 ± 0.16	1.37 ± 0.08
f, min^{-1}	26.0 ± 5.2	30.5 ± 4.3
Principal Ponies		
PaO_2, torr	90.0 ± 2.3	65.3 ± 2.8*†
$PaCO_2$, torr	38.8 ± 1.04	42.6 ± 1.3†
C_{dyn}, L · cm H_2O^{-1}	0.79 ± 0.15	0.15 ± 0.02*†
R_L, cm $H_2O \cdot L^{-1} \cdot s$	1.56 ± 0.31	6.49 ± 0.61*†
V_T, liters	1.90 ± 0.22	1.85 ± 0.10
f, min^{-1}	21.5 ± 3.86	22.7 ± 1.5

Values are baseline means ± SEM.
Measurement Period A = pasture
Measurement Period B = barn environment
PaO_2 = arterial oxygen partial pressure
$PaCO_2$ = carbon dioxide partial pressure
C_{dyn} = dynamic compliance
R_L = pulmonary resistance
V_T = tidal volume
f = respiratory frequency
* Significant difference from period A
† Significant difference between principal and control groups

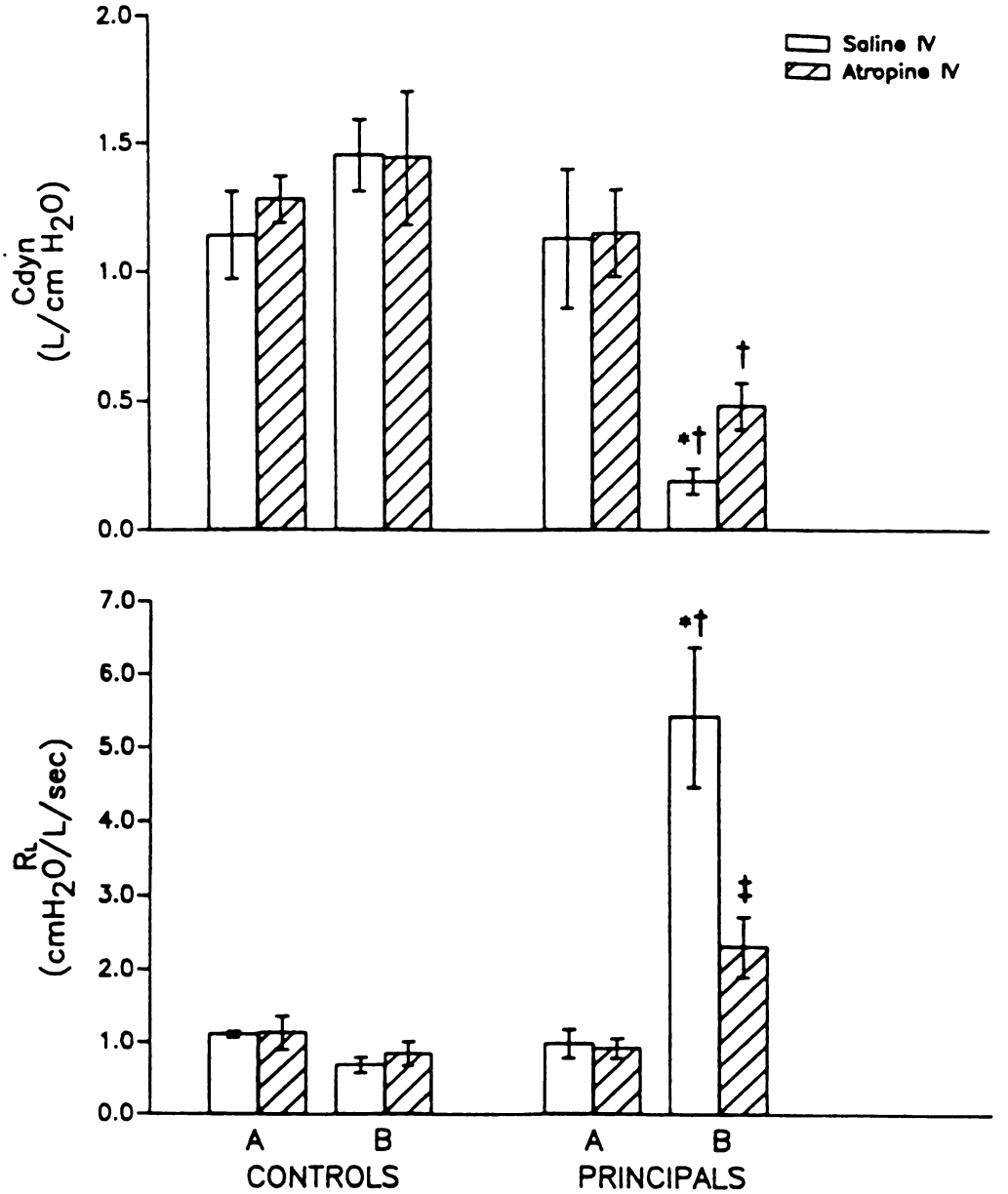

Figure 2-1. Dynamic compliance (C_{dyn}) and pulmonary resistance (R_L) after saline I.V. and after atropine I.V. prior to histamine challenge at periods A and B in control and principal groups. * = Significant difference from Period A. † = Significant difference between principal and control groups. ‡ = Significant difference within group at same period (treatment effect).

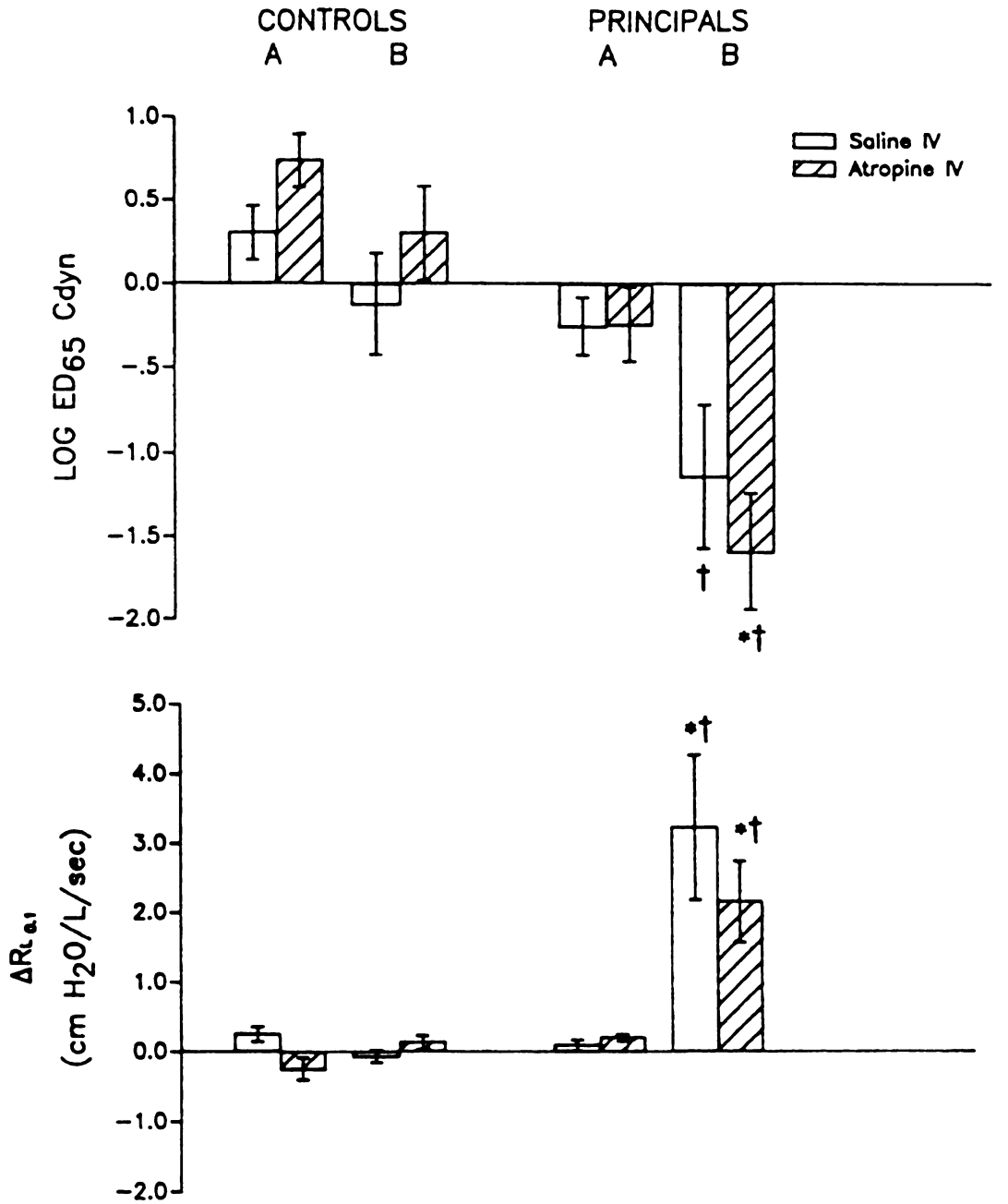

Figure 2-2. Histamine dose required to reduce dynamic compliance to 65% of baseline (ED_{65} C_{dyn}) and the change in pulmonary resistance (R_L) from aerosol saline to 0.1 mg/ml histamine aerosol ($\Delta R_{L0.1}$ mg/ml) following I.V. saline (unshaded) or I.V. atropine (shaded) in principals and control ponies at periods A and B. Histamine is expressed as logarithmic dose. * = Significant difference from Period A. † = Significant difference between principals and controls.

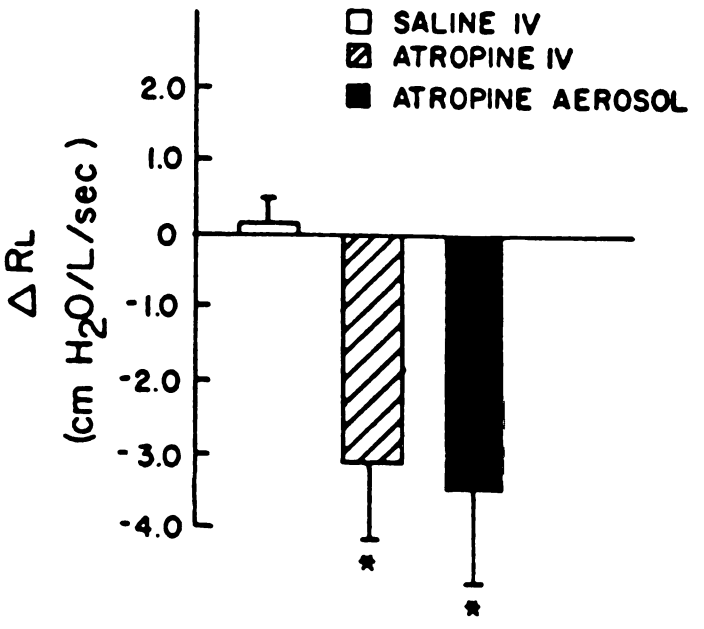

Figure 2-3. The changes in pulmonary resistance (ΔR_L) between baseline and following I.V. saline, I.V. atropine, or aerosol atropine administration in principals at period B. * Significant difference from baseline.

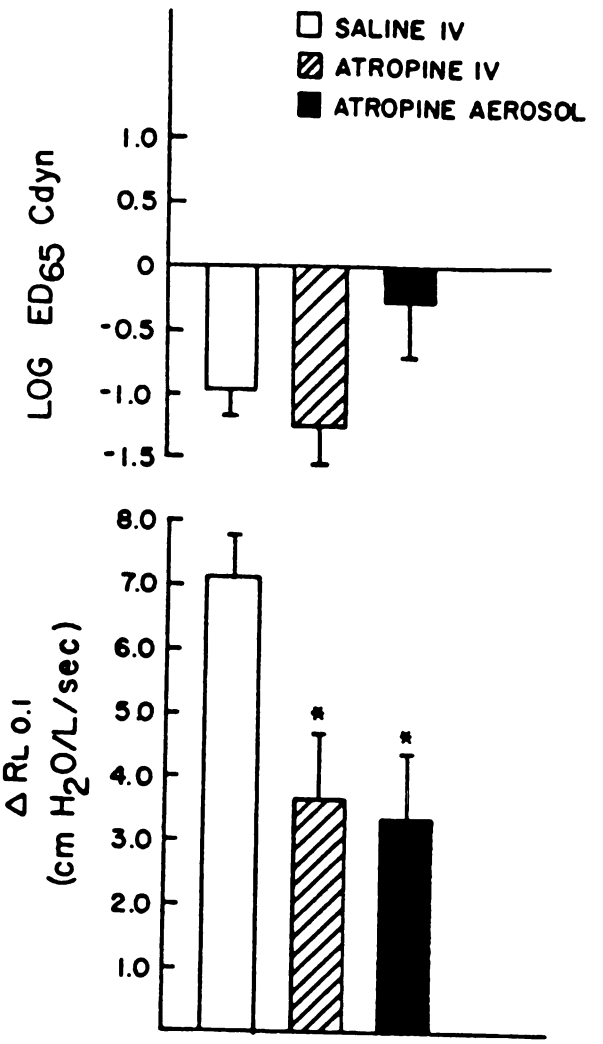

Figure 2-4. The mean values of Log ED_{65} C_{dyn} and the change in pulmonary resistance from saline aerosol to 0.1 mg/ml histamine ($\Delta R_{L0.1}$ mg/ml) with I.V. saline, I.V. atropine, and aerosol atropine in principals at measurement Period B. * = Significant difference from I.V. saline.

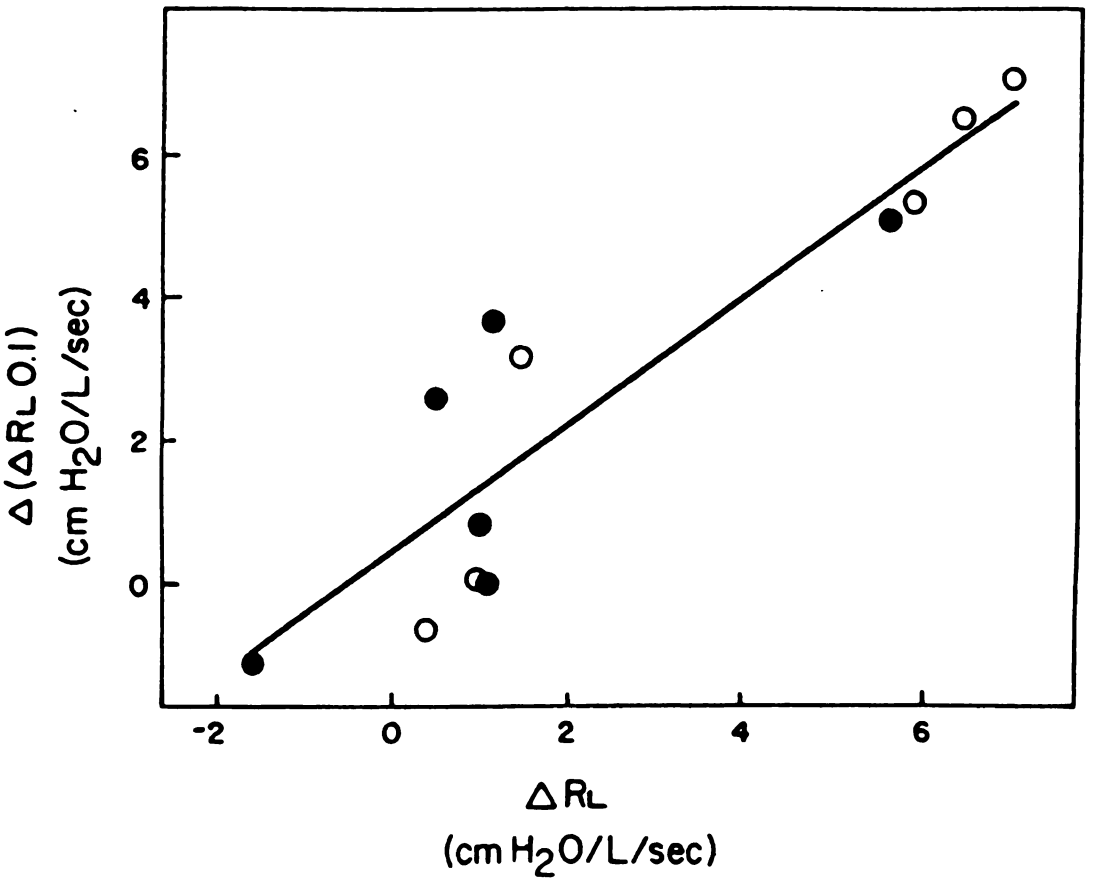

Figure 2-5. The change in reactivity ($\Delta R_{L0.1}$) as a function of the change in pulmonary resistance (ΔR_L) in principal ponies at period B. The ΔR_L equals the change in resistance from I.V. saline to either I.V. (●) or aerosol (○) atropine. The Δ ($\Delta R_{L0.1}$) equals the corresponding change in reactivity ($r = 0.91$, $P = 0.0001$).

CHAPTER 3

IN VITRO RESPONSES OF AIRWAY SMOOTH MUSCLE FROM HORSES WITH RECURRENT AIRWAY OBSTRUCTION

Introduction

Atropine significantly reduced R_L without significantly increasing C_{dyn} in vivo during periods of acute airway obstruction, suggesting that a large portion of the increased R_L in the principal ponies at period B is mediated via muscarinic receptor activity. These observations do not distinguish if the increased R_L is due to augmented vagal reflex activity, enhanced reactivity of smooth muscle to a normal level of vagal activity, or loss of inhibitory factors that normally oppose vagally mediated bronchoconstriction. Although the above observations suggest involvement of the autonomic nervous system in this disease, little information is available on the autonomic regulation of horse airways at different levels of the tracheobronchial tree. I therefore started in vitro studies of the smooth muscle of the trachea and third-generation bronchi. The first purpose of this study was to define excitatory and inhibitory innervation of the trachea and third-generation bronchi in vitro and to determine if it is altered in the diseased horses. A second objective of the study was to determine if the hyperresponsiveness to muscarinic agonists observed in vivo persists in vitro.

Methods

Animal and tissue preparation

Experimental protocols were performed on tracheal strips and third-generation airways taken from two groups of mixed-breed adult horses. The control horses had no history of respiratory disease, showed no clinical signs of respiratory tract disease, and did not develop clinical signs of airway obstruction when housed in a barn, bedded on straw, and fed hay. These control horses were taken from a pasture environment the day before the in vitro studies. Gross pathologic examination of the respiratory tracts of this group revealed no abnormalities. The horses with a history of developing recurrent airway obstruction when exposed to hay and entering remission when pastured and denied access to a hay, straw, or barn environment were studied during an attack of airway obstruction precipitated by housing in a barn environment. Average length of barn exposure was 11.7 ± 1.2 days. These "heavey" horses were studied when they developed clinical signs of airway obstruction, including recruitment of abdominal muscles on expiration, hypoxemia (PaO_2 less than 75 mmHg), and increases in transpulmonary pressures (greater than 15 cm H_2O). These parameters were used to define the acute state of airway obstruction of heavey horses in previous studies (Derksen et al. 1985a, Armstrong et al. 1986, Scott et al. 1988a, 1988b). At this time, neutrophil numbers are significantly increased in bronchoalveolar lavage fluid samples (Derksen et al. 1985b). Immediately following euthanasia with an overdosage of concentrated pentobarbital sodium (85 mg/kg, iv), a portion of trachea 10 cm cranial to the carina and sections of the lung were excised and placed in room temperature Krebs-Ringers solution that had been aerated with

5% CO_2 in oxygen. The tissue was then transported to the laboratory. Rectangular strips of trachealis tissue were cut approximately 2 mm x 20 mm using a template so that the muscle fibers were oriented along the long axis of the tissue. The lung parenchyma and prominent surface vessels were removed from the third-generation bronchi. These airways were cut into rings 2-3 mm in width and placed in the tissue baths. The epithelium and mucosa were left intact in all preparations, but the serosa was trimmed in the trachealis strips. The tracheal strips and bronchi were suspended by 3-0 silk in 30-ml organ baths filled with Krebs-Ringers solution (composition in mM: NaCl 110.5, KCl 3.4, $NaHCO_3$ 25.7, dextrose 5.5, KH_2PO_4 1.2, $MgSO_4$, $7H_2O$ 0.4, and CaCl2 · $2H_2O$ 2.4) bubbled with 95% O_2 - 5% CO_2 and maintained at 37.4°C. The trachealis strips were placed into the tissue baths within 45 minutes after killing the horse and the bronchi within 60 minutes. For both the trachealis muscle strip and the bronchial ring, one end of the tissue was tied to the bottom of a stationary plexiglass tissue holder. The other end was tied to a force transducer (Grass FTO3) mounted on micromanipulators so that the tissue length could be changed when studying the length-tension relationships of the airway smooth muscle. A continuous recording of isometric force generation was obtained by connecting the force transducer to a polygraph (Grass Model 7D). Two rectangular platinum electrodes (dimensions: 8 mm x 40 mm) were placed parallel to the tissue for electrical stimulation of the preparations. Electrical impulses were produced by a Grass S88 stimulator and passed through a stimulus power booster (Stimu-Splitter II, Med Lab Instruments, Fort Collins, Colorado). The electrical impulses consisted of

square waves. The voltage and duration were monitored on an oscilloscope (Model OS-7020 Goldstar Precision Co., Ltd., Cerritos, CA).

Tissues were equilibrated for approximately 90 minutes with an initial passive tension of 2 g applied and maintained. Following equilibration, the length of each tissue was increased progressively in 0.5 mm to 1.0 mm increments until the AT produced by a standard submaximal electrical stimulus (10 v, 15 Hz, 0.5 ms pulse duration, 30-second stimulation period) reached a maximum (AT_{max}). Active tension was determined by subtracting passive tension from total tension. Tissue length was considered optimal when the AT in response to the stimulus no longer increased when the tissue was lengthened. The length at which AT_{max} occurred was held constant throughout each experiment. AT_{max} was checked at hourly intervals as the experimental design allowed and prior to the addition of blockers. If AT_{max} varied by greater than 5%, the tissue was eliminated from the protocol. This methodology was similar to that used by Russell in canine airway smooth muscle (Russell 1978). After completion of a protocol, tissues were removed from the tissue bath and fixed in 10% phosphate-buffered formalin. Sections were then prepared, stained with hematoxylin and eosin, and examined for the presence of epithelium.

In this study a total of 30 horses was used. Five control horses and five horses with recurrent airway obstruction were used for the EFS and ACh protocol. Five control horses and five horses with recurrent airway obstruction were used for each of the other protocols: electrical stimulation of precontracted tissues and relaxation with isoproterenol. The following drugs were used: ACh chloride, barium chloride, histamine, atropine sulfate, propranolol, indomethacin, tetrodotoxin, ± isoproterenol

(Sigma Chemical Co.), and phentolamine (Ciba Geigy). Solutions were prepared fresh daily with the exception of tetrodotoxin, which was stored in aliquots at -70°C. All drugs were dissolved in deionized water with the exception of indomethacin, which was dissolved in ethanol. The final concentration of ethanol in the tissue bath was 0.2%. Ascorbic acid (0.1%) was added to the isoproterenol solution to retard oxidation. 0.0026 mM CaEDTA was added to the buffer when isoproterenol was used. Solution concentrations were prepared so that all drugs were added in 100-μl aliquots. Drug concentrations are reported as final bath concentrations.

Experimental protocols

Protocol 1: Response to acetylcholine and electrical stimulation

Cumulative concentration response curves to ACh (10^{-9}M to 10^{-3}M) were recorded. Contractions produced by each dose were allowed to reach a plateau before adding the next higher concentration. Acetylcholine was added in 1/2 log increments until the bath concentration was 10^{-3}M. A 60-minute rinse, wash, and recovery period was allowed between the generation of the cumulative ACh response curves with a return of AT to zero prior to generation of the voltage and frequency-response curves. The tissues were stimulated at frequencies of 1, 3, 5, 10, 15, 20, 25, and 32 Hz and random voltages of 6, 9, 12, 15, 18, and 20 volts to determine the optimal electrical stimulus to induce a maximal contraction. These voltages and frequencies were applied at a stimulus duration of 0.5 ms. At least 5 minutes elapsed between each stimulation. The effect of atropine and tetrodotoxin on the response of the tissues to EFS was studied to determine

that the AT achieved was due to the activation of the muscle via cholinergic nerves. Thirty minutes after electrical stimulation, either 10^{-6}M atropine or tetrodotoxin (3 x 10^{-6}M) was added to the baths and, after 30 minutes of incubation, the frequency response was again tested at 18 v and 0.5 ms. Thirty minutes after electrical stimulation, the ACh dose response was then repeated in the presence of atropine.

Protocol 2: Electrical field stimulation of precontracted tissues

Precontraction of tissues was necessary to demonstrate relaxation in response to electrical stimulation. In preliminary studies no relaxation occurred in tissues that were not precontracted. 10^{-3}M ACh was added to the tissues and the AT recorded. The tissues were repeatedly washed with warm buffer until the AT returned to zero. In one series, atropine (10^{-6}M), phentolamine (10^{-6}M), and indomethacin (3 x 10^{-6}M) were added and incubated for 30 minutes. In preliminary protocols, indomethacin (3 x 10^{-6}M) prevented spontaneous phasic contractile activity in response to histamine. Histamine was then added in 1/2 log increments from 10^{-9}M until an AT equal to approximately 60% of the maximum AT (AT_{max}) obtained with 10^{-3}M ACh was reached. While precontracted, the tissues were then stimulated with 18.0 v, 0.5-32 Hz frequency, and 0.5 ms duration. Tetrodotoxin (3 x 10^{-6}M) was added to the baths and stimuli repeated. In a parallel study, the same protocol was conducted with the addition of propranolol (10^{-6}M). A control precontracted strip and bronchial ring were included in all protocols to ensure the stability of AT with time.

Protocol 3: Relaxation with isoproterenol

Trachealis strips and bronchial rings were precontracted with histamine as in protocol 3 to 60% of the AT_{max} achieved with 10^{-3}M ACh. A cumulative isoproterenol (10^{-10}M - 10^{-4}M) dose response was conducted on the precontracted tissues. In a parallel study, propranolol (10^{-6}M) was added and incubated for 30 minutes prior to the cumulative addition of isoproterenol.

Data analysis

Responses to electrical stimulus frequency and concentration of agonist were calculated as a percentage of the maximum contraction produced by 10^{-3}M ACh. Barium chloride (3 x 10^{-2}M) did not produce a contraction larger than that produced by 10^{-3}M ACh. Therefore, the effect on tension development of varying frequency and voltage was determined by normalizing the response of each tissue to its maximal response to 10^{-3}M ACh. The response to isoproterenol and EFS after histamine precontractions was normalized to the precontracted tension.

The EC_{50} values for ACh and isoproterenol were obtained visually from a plot of log concentration versus the percent of maximum response produced by each substance for each animal. The mean EC_{50} results of a group were obtained by averaging the EC_{50} values of individual animals.

Statistical analysis

The results are expressed as means ± S.E. Differences between means of the two groups of horses at a specific concentration of drug agonist or a specific stimulation frequency were compared by the unpaired Student's t test. When $p < 0.05$, means were considered significantly different. Three trachealis strips and three bronchial rings from each animal were averaged to establish a mean value for each horse except when noted otherwise.

Results

Acetylcholine dose response

In the tracheal smooth muscle, the maximal AT at 10^{-3}M ACh was 41.5 ± 3.5 grams in the controls and 39.7 ± 2.7 grams in the group of heavey horses. These values are not significantly different between the two groups. The tracheal strips of heavey animals (Figure 3-1) were less sensitive than the controls to ACh concentrations between and including 3×10^{-8}M and 3×10^{-7}M. The concentration of ACh producing 50% contraction (EC_{50}) at the level of the trachea of the controls ($2.9 \pm 1.7 \times 10^{-6}$M) was not significantly different from that of the heavey group ($6.6 \pm 3.2 \times 10^{-6}$M).

At the level of the third-generation bronchi, the AT_{max} at 10^{-3}M ACh was not significantly different between control and heavey groups (19.4 ± 1.9 grams and 14.7 ± 1.7 grams, respectively). Figure 3-1 also shows the response of the third-generation bronchi to ACh. The bronchi of the heavey group were significantly hyporesponsive at ACh concentrations between and including

10^{-7}M and 3 x 10^{-4}M. The EC_{50} concentration in the bronchi of the controls (1.5 ± 1.1 x 10^{-5}M) was significantly different from the heavey group (8.1 ± 1.7 x 10^{-5}M). Atropine blocked the response to ACh at each concentration used through 10^{-3}M at both levels of the airway.

Electrical field stimulation

At the level of the trachea, the optimal stimulus parameters producing the maximum active tension (AT_{max}) were 18 volts, 25 Hz, 0.5 ms in the controls and 20 volts, 25 Hz, 0.5 ms in the heavey horses. In the bronchi, the optimal parameters were 20 volts, 25 Hz, 0.5 ms in controls and 12 volts, 25 Hz, 0.5 ms in the heavey group. Figure 3-2 shows the data from the trachea normalized to the response to 10^{-3}M ACh. At all voltages, the trachealis of horses with recurrent airway obstruction was more sensitive to EFS than the controls at all frequencies up to and including 10 Hz. At 6 volts, significant differences persisted between the two groups through 25 Hz. The third-generation bronchi of the heavey group were more sensitive than the controls at voltages of 6-18 and at frequencies greater than 10 Hz (Figure 3-3). Atropine and tetrodotoxin totally blocked all responses at the voltages, frequencies, and duration used in this protocol. In preliminary experiments, it was determined that stimulus durations greater than 0.5 ms were not completely tetrodotoxin sensitive at this optimal stimulus parameter.

In the trachea of control horses, the AT_{max} response to EFS was 98.6 ± 1.2% of the contractile response to ACh 10^{-3}M. The ATs developed by EFS and

ACh (10^{-3}M) were not significantly different (Table 2). This optimal response was achieved at 18 volts, 25 Hz, 0.5 ms duration. In the heavey group, however, the maximal response in the trachea to EFS occurred at 20 volts, 20 Hz, and 0.5 ms duration and was 110.6 ± 7.9% of the contractile response to ACh at 10^{-3}M. This response to EFS in the trachea of both groups was not significantly different from the AT developed with ACh (10^{-3}M). The contraction induced by EFS of control third-generation bronchi was 69.3 ± 8.2% of the contraction induced by 10^{-3}M ACh and was significantly less than the 10^{-3}M ACh response. However, EFS of the third-generation bronchi of heavey horses was 105.4 ± 14.7% of the contraction induced by 10^{-3}M ACh and was not significantly different from the ACh response. The EFS to ACh ratio of the diseased third-generation bronchi was significantly different from the control bronchi ratio.

Relaxation with isoproterenol

The AT achieved at the level of the trachea with ACh (10^{-3}M) was 46.0 ± 2.6 grams in the controls and 42.3 ± 3.0 grams in the heavey group. These values were not significantly different. The time-control tissues showed that the histamine precontractions were stable over the 25-30 minutes needed to obtain the isoproterenol concentration response curves. The tissues of control and heavey groups were precontracted to 60.9 ± 3.5% and 60.6 ± 4.1% of the AT achieved with ACh (10^{-3}M). The histamine concentrations used to contract the tissues of the control and diseased tissues were not significantly different (5.1 ± 2.3 x 10^{-6}M and 1.5 ± 0.2 x 10^{-6}M, respectively).

The AT achieved with ACh in the bronchi was not signficantly different between control and heavey groups (18.6 ± 1.4 grams and 15.8 ± 1.2 grams, respectively). The third-generation bronchi were precontracted 61.4 ± 2.8% in the controls and 57.3 ± 2.2% in the heavey horses with histamine concentrations of 1.9 ± 1.2 x 10^{-5}M and 3.7 ± 2.1 x 10^{-5}M, respectively. None of these values were significantly different.

The isoproterenol dose-response curves are shown in Figure 3-4. Relaxation was dose dependent and reached 100 percent in both groups at both levels of the airway. No significant differences existed between the heavey and control group at either level of the airway. The concentration of isoproterenol producing 50% inhibition (IC_{50}) of contraction at the level of the trachea were not significantly different between control and heavey groups (1.8 ± 0.5 x 10^{-9}M and 9.9 ± 0.9 x 10^{-10}M, respectively). There was no significant difference between the control and heavey groups for the IC_{50} of isoproterenol at the level of the third-generation bronchi (1.1 ± .3 x 10^{-7}M and 1.3 ± 0.2 x 10^{-8}M, respectively). Propranolol (10^{-6}M) blocked the effects of isoproterenol up to a concentration of 10^{-5}M and shifted the dose-response curve to the right, as seen in Figure 3-5.

Electrical field stimulation of precontracted tissues

In this protocol, the AT in the trachealis strips developed with ACh (10^{-3}M) was not significantly different between the control and heavey groups (44.3 ± 1.7 grams and 41.6 ± 2.3 grams, respectively). The tissues of heavey and control groups were precontracted with histamine to 61.4 ± 1.6% and 58.7 ± 2.2%

of the AT achieved with ACh response (10^{-3}M), respectively. The histamine precontraction percentages were not significantly different. The concentration of histamine required for this precontraction was not significantly different in the control (1.3 ± 0.8 x 10^{-6}M) versus the heavey group (6.8 ± 1.7 x 10^{-6}M).

At the bronchial level, similar ATs of 21.3 ± 2.1 grams and 17.4 ± 1.8 grams were achieved with 10^{-3}M ACh in the control and heavey group. Using 2.2 ± 1.3 x 10^{-5}M for the controls and 7.0 ± 2.3 x 10^{-5}M of histamine for the heavey group, precontraction reached 60.7 ± 0.9% in the controls and 58.9 ± 1.1% of the maximal response to 10^{-3}M ACh in the heavey group. None of these values were significantly different.

Electrical field stimulation in the absence of propranolol (Figure 3-6) caused frequency-dependent relaxation in the trachealis of 80-90% in both the control horses and heavey horses. At the level of the third generation, the tissues relaxed 21% in the control horses. No relaxation was seen in the third-generation airways of heavey horses. At the level of the trachea in the presence of propranolol, EFS-induced relaxation still occurred but was reduced in amount at all stimulus frequencies (Figure 3-7). In preliminary protocols, higher concentrations of propranolol had no greater effect and in fact caused destabilization of the tissue and actually decreased the AT achieved at a particular voltage, frequency, and duration. Propranolol (10^{-6}M) reduced the inhibition of the trachealis muscle by approximately 50% in the tissues of both control and heavey horses. Maximal relaxation in the presence of propranolol was 46% at the level of the trachea. No differences in response to EFS in the

precontracted tissues were demonstrated between the control and heavey groups after propranolol administration at the level of the trachea. At the level of the third-generation airways, propranolol did not reduce the inhibition caused by electrical stimulation in the control horses.

Discussion

In heavey horses, respiratory distress is due to airway obstruction indicated by increased R_L and decreased C_{dyn} (Derksen et al. 1985a, Armstrong et al. 1986, Scott et al. 1988a, 1988b). Airway hyperresponsiveness to methacholine and histamine is also a feature of the heavey group (Derksen et al. 1985c, Armstrong et al. 1986). The results of the present study show differences in the responses of the two groups to ACh and to EFS.

The response of the smooth muscle of the two levels of airways of both groups of horses to ACh was similar to that observed in other mammals (Russell 1978, Nakanishi et al. 1976, Olson et al. 1988) and is similar to the response found by Mason et al. in equine airway strips (Mason et al. 1989). The smooth muscle tissues contracted in a dose-dependent manner over the range of doses tested. In the present study, the EC_{50} values were similar to other species and also agree with Mason's findings at these two levels of the airway. All contractions were mediated via muscarinic receptors as demonstrated by blockade with atropine.

In the trachealis of the control animals, the maximal contractile response to EFS was almost identical to the maximal response to 10^{-3}M ACh. Similar

observations in the dog have been interpreted to show that there is a high density of parasympathetic innervation or that intercellular junctions allow rapid propagation of the action potential throughout the muscle at this level of the tracheobronchial tree (Russell 1978). A significant decrease was noted in the ratio of the response to optimal EFS to the maximal response produced by exogenous ACh in the third-generation airways in the control animals, a result similar to that observed in the dog (Russell 1978). This decrease may be due to a smaller percentage of innervated ACh receptors in the third-generation bronchi. Alternatively, presynaptic inhibitory effects of mediators released during EFS contractions could be responsible for this decrease at the level of the third generation.

Even though there was no difference in the ACh EC_{50} value of the two groups at the tracheal level, the heavey animals were less sensitive at lower concentrations. At the level of the third generation, the ACh EC_{50} of the tissues in heavey horses was significantly greater than the control EC_{50}. This finding is similar to that in hyperresponsive Basenji-Greyhound mongrels, in which the trachealis in vitro is hyporesponsive to methacholine when compared to non-hyperresponsive Greyhounds (Austin et al. 1987, Downes et al. 1986). The cause of the hyporesponsiveness in vitro in the heavey horses is unknown. These data provide no evidence of increased smooth muscle responsiveness to ACh that would explain the airway hyperresponsiveness to methacholine observed in vivo (Derksen et al. 1985a, Armstrong et al. 1986).

The relaxation in response to isoproterenol in both groups of horses was similar to that described in horse airways by Olson (Olson et al. 1989a, Olson et al. 1989b). Since the horse tissues have no active tone, the tissues were precontracted with histamine to approximately 60% of the tension achieved with 10^{-3}M ACh and relaxed with isoproterenol in a dose-dependent manner down to the baseline tension prior to histamine contraction. Olson reported that, as in other species, total relaxation of horse trachealis in response to isoproterenol requires the presence of the epithelium. In the absence of epithelium, maximal relaxation was approximately 55% (Olson et al. 1989a). In the present study, the magnitude of the relaxation confirms that epithelium was present and apparently normal. The shift in the isoproterenol dose-response curve after propranolol administration confirms that the relaxation is mediated through beta-adrenergic receptors.

An increase in resistance was observed in vivo in the heavey horses but not in the controls after β-adrenergic blockade with propranolol (Scott et al. 1988b). One possible mechanism of this increase in resistance may be an up-regulation of β-receptors in the heavey horses. The observation that the IC50 for isoproterenol did not differ between groups suggests that there is no up-regulation of β-receptors in the heavey horses at either level of airway.

To demonstrate neurally mediated relaxation, the stimulus parameters that had previously been shown to be tetrodotoxin-sensitive and optimal for contraction were used. Electrical field stimulation of indomethacin, atropine, and phentolamine-treated precontracted trachealis tissues caused frequency-

dependent relaxation. After the addition of propranolol to the tissue baths, field stimulation still caused frequency-dependent relaxation, but the magnitude of relaxation was less at each frequency in the trachea. These observations suggest the presence of both sympathetic and non-adrenergic inhibitory innervation in the trachea of control horses and an equal importance of each inhibitory system at this level. In this respect, the horse is like the cat, guinea pig, baboon, rabbit, human, and cow, which have both adrenergic and non-adrenergic inhibitory innervation in the trachealis, but unlike the dog, which has only sympathetic innervation (Coleman and Levy 1974, Colburn and Tomita 1973, Diamond and O'Donnell 1980, Middendorf and Russell 1980, Richardson and Beland 1976).

At the level of the third-generation airways, EFS produced much less relaxation than in the trachea. Furthermore, propranolol did not reduce the inhibition caused by EFS in the controls, which suggests that a nonadrenergic inhibitory system predominates at this level. No neurally mediated relaxation was seen in the airways of heavey horses. The lack of inhibitory innervation at the level of the third-generation airways of the heavey horse may be similar to the absence of VIP immunoreactive nerves reported in human ashmatics (Ollerenshaw et al. 1989). However, if the neurotransmitter of the NANC nervous system is a peptide, then a functional defect in this system may develop in the heavey horses as a result of inflammation. Increased degradation of peptide transmitters by inflammatory cell peptidases could disrupt the modulating action of the nonadrenergic inhibitory system.

The contractile response of the tissues to EFS was dependent on frequency, voltage, and pulse duration. The contractions were blocked by atropine, which demonstrates the presence of cholinergic excitatory nerves. Abolition of contractions by tetrodotoxin demonstrated that they were mediated by release of neurotransmitters rather than by direct effects on smooth muscle cells. The optimal stimulus parameters agreed with those described in a number of other species (Russell 1978, Olson et al. 1988, Carlyle 1964).

The EFS response of the tracheal smooth muscle from heavey horses expressed as a percentage of the tension achieved with ACh (10^{-3}M) was greater than that observed in the control group. This enhanced response to EFS was most apparent at the lower voltages and stimulus frequencies. The increased response to EFS in the heavey horses at the level of the trachea is not likely to be due to a lack of inhibitory innervation, because no differences were found between groups in the relaxation in response to EFS. Evidence of both sympathetic and non-adrenergic inhibitory innervation was found in the trachea of heavey horses. Lack of presynaptic inhibition or prejunctional enhancement of ACh release would explain the enhanced response in the trachea to EFS of smooth muscle from heavey horses. Alternatively, the exaggerated response could be a postsynaptic mechanism activated during EFS, which causes an enhanced contraction in response to neurally released ACh.

In the third-generation bronchi, as in the trachea of the heavey horses, the contractile response to EFS was enhanced. In the third-generation airways

of the heavey group, EFS resulted in 100% of the ACh maximal contraction. The enhanced response at high stimulus frequencies and the greater maximal response to EFS in the heavey group at this level could in part be a result of the lack of inhibitory innervation in the diseased third-generation airways. Additionally, the enhanced response of the third-generation airway could be a result of more extensive excitatory innervation in the heavey horse or a lack of presynaptic inhibition by mediators such as PGE_2.

In vivo, a large part of the airway obstruction observed in the horses with recurrent obstructive pulmonary disease is mediated via muscarinic mechanisms.[3] Administration of atropine causes a major decrease in resistance and a much smaller increase in C_{dyn}, suggesting that the muscarinic activity is concentrated in the larger airways. It is not known if the increased muscarinic activity in vivo is due to increased release of ACh in response to cholinergic nerve activation or to an increased response of smooth muscle to ACh. The present study shows no evidence of increased muscle responsiveness to ACh but rather suggests differences between control and heavey horses at the level of the trachealis and third-generation airways in response to nerve activation.

Table 2. Maximal response to electrical field stimulation expressed as a percentage of the maximal response to exogenous ACh (10^{-3}M).

	Trachea	Third-generation bronchi
Control	98.6 ± 1.2%	69.3 ± 8.2%*
Heavey	110.6 ± 7.9%	105.4 ± 14.7%

* = Significant difference between third-generation bronchi of control and heavey animals. Values are means ± S.E.

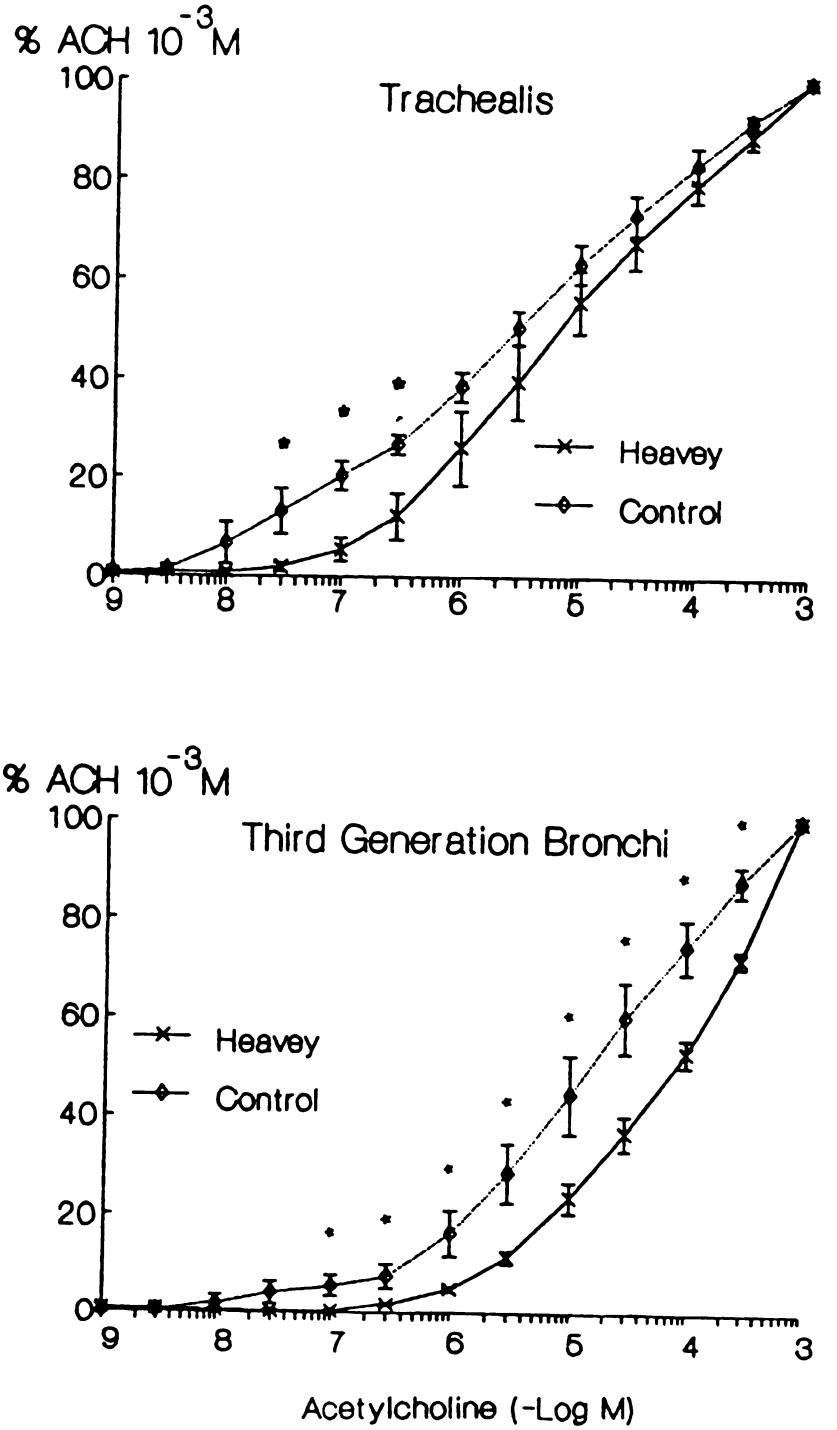

Figure 3-1. Mean acetylcholine concentration-response curves for trachealis strips and third-generation bronchi of control and heavey horses. Force developed expressed as per cent of maximal contraction with acetylcholine (10^{-3}M) (± SE). n = 5 for each curve. * = Significant difference between control and heavey horses at the designated ACh concentration.

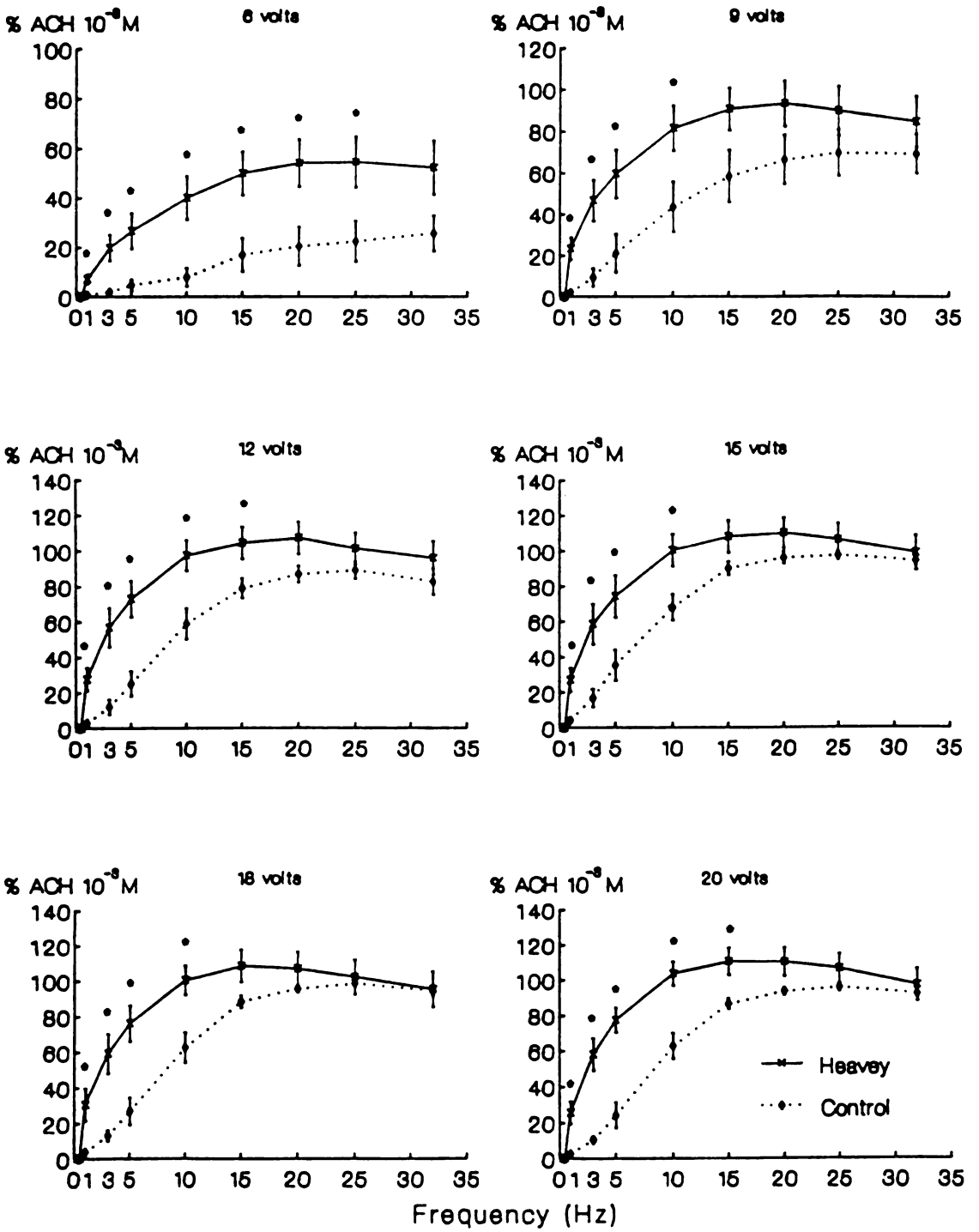

Figure 3-2. Mean frequency-response curves of trachealis strips of control and heavey horses expressed as percent of the maximal response to acetylcholine (10^{-3}M) (± SE) at 6, 9, 12, 15, 18, 20 v. n = 5 for each curve. * = Significant difference between control and heavey horses at the designated frequency.

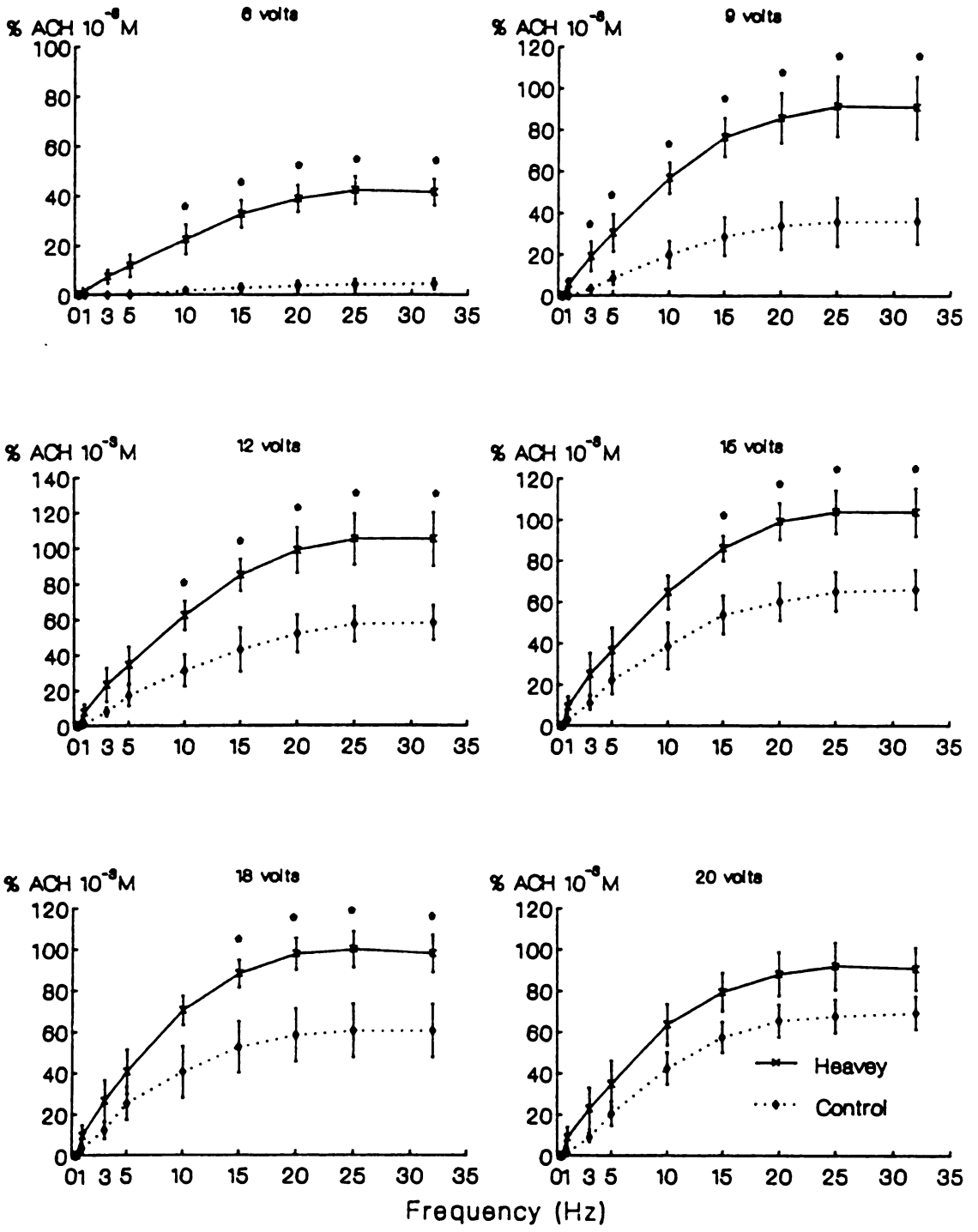

Figure 3-3. Mean frequency-response curves of third-generation bronchi of control and heavey horses expressed as a percent of the maximal response to acetylcholine (10^{-3}M) (± SE) at 6, 9, 12, 15, 18, 20v. n = 5 for each curve. * = Significant difference between control and heavey horses.

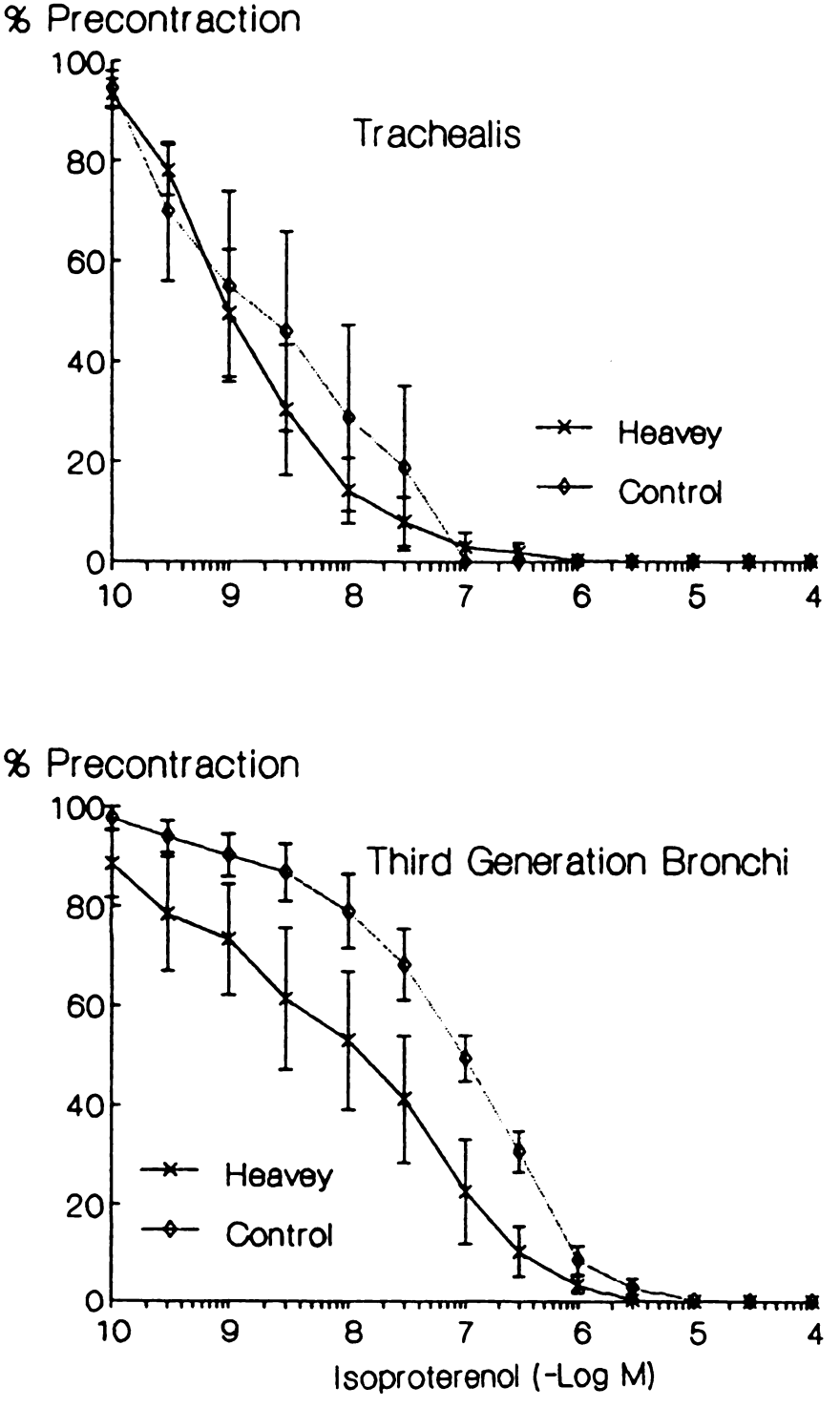

Figure 3-4. Mean isoproterenol concentration-response curve of trachealis strips and third-generation bronchi of control and heavey horses. All tissues were pretreated with 10^{-6}M phentolamine, 3 x 10^{-6}M indomethacin, and 10^{-6}M atropine and precontracted with histamine. Responses are plotted as percentages of maximal force developed by histamine precontraction during each trial. n = 5 for each curve.

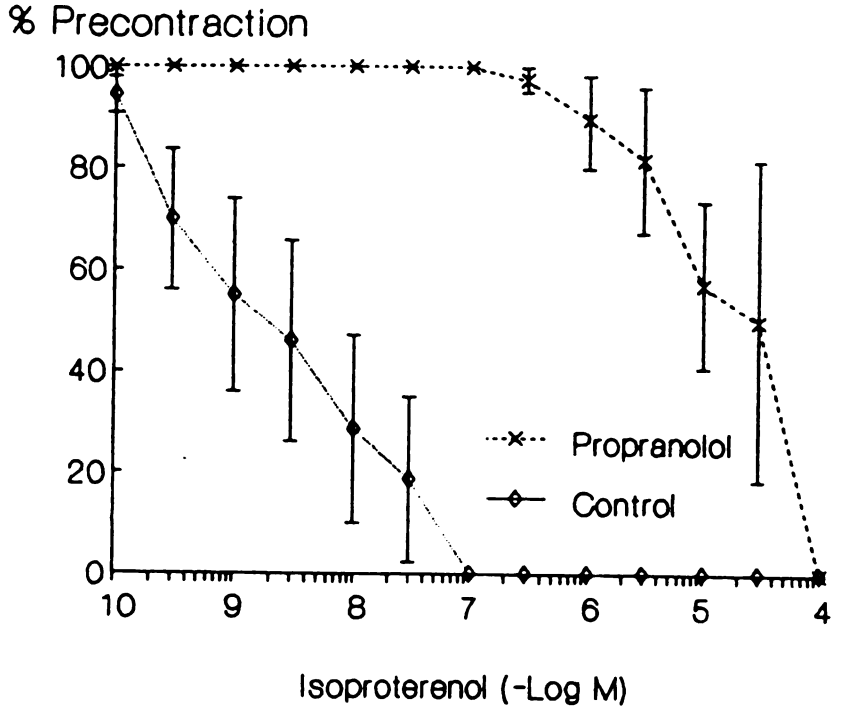

Figure 3-5. Comparison of inhibitory effect of isoproterenol in the presence and absence of propranolol (10^{-6}M). All tissues were pretreated with 10^{-6}M phentolamine, 3 x 10^{-6M} indomethacin, and 10^{-6}M atropine, and precontracted with histamine. Responses are plotted as percentages of maximal force developed by histamine precontraction during each trial. n = 5 for each curve.

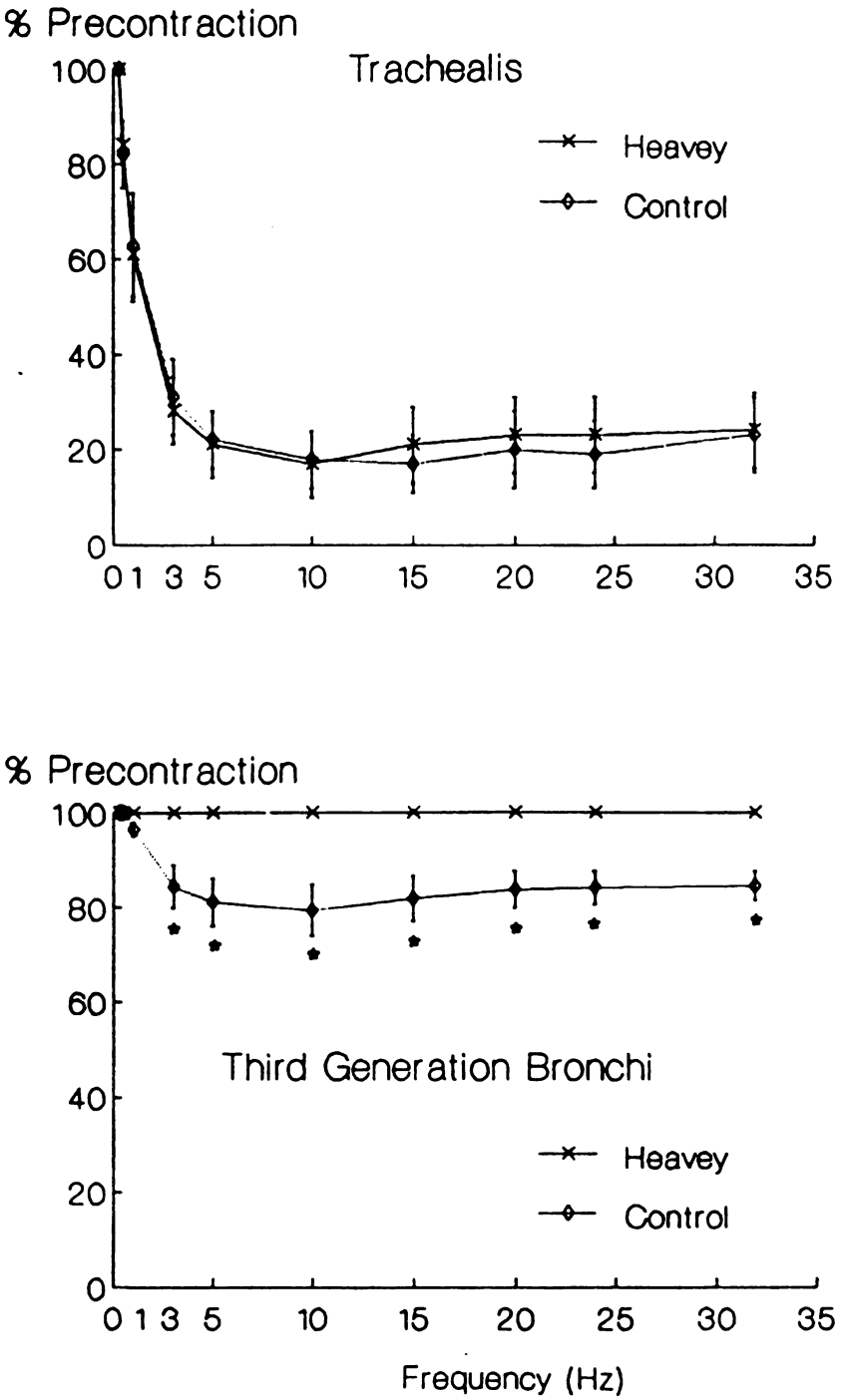

Figure 3-6. Inhibitory effect of electric stimulation on trachealis strips and third-generation bronchi of control and heavey horses. All tissues were precontracted with histamine following pretreatment with 10^{-6}M phentolamine, 3 x 10^{-6}M indomethacin, and 10^{-6}M atropine. Stimulus parameters: 18 v, 0.5 ms duration, and variable frequency as indicated. n = 5 for each curve.

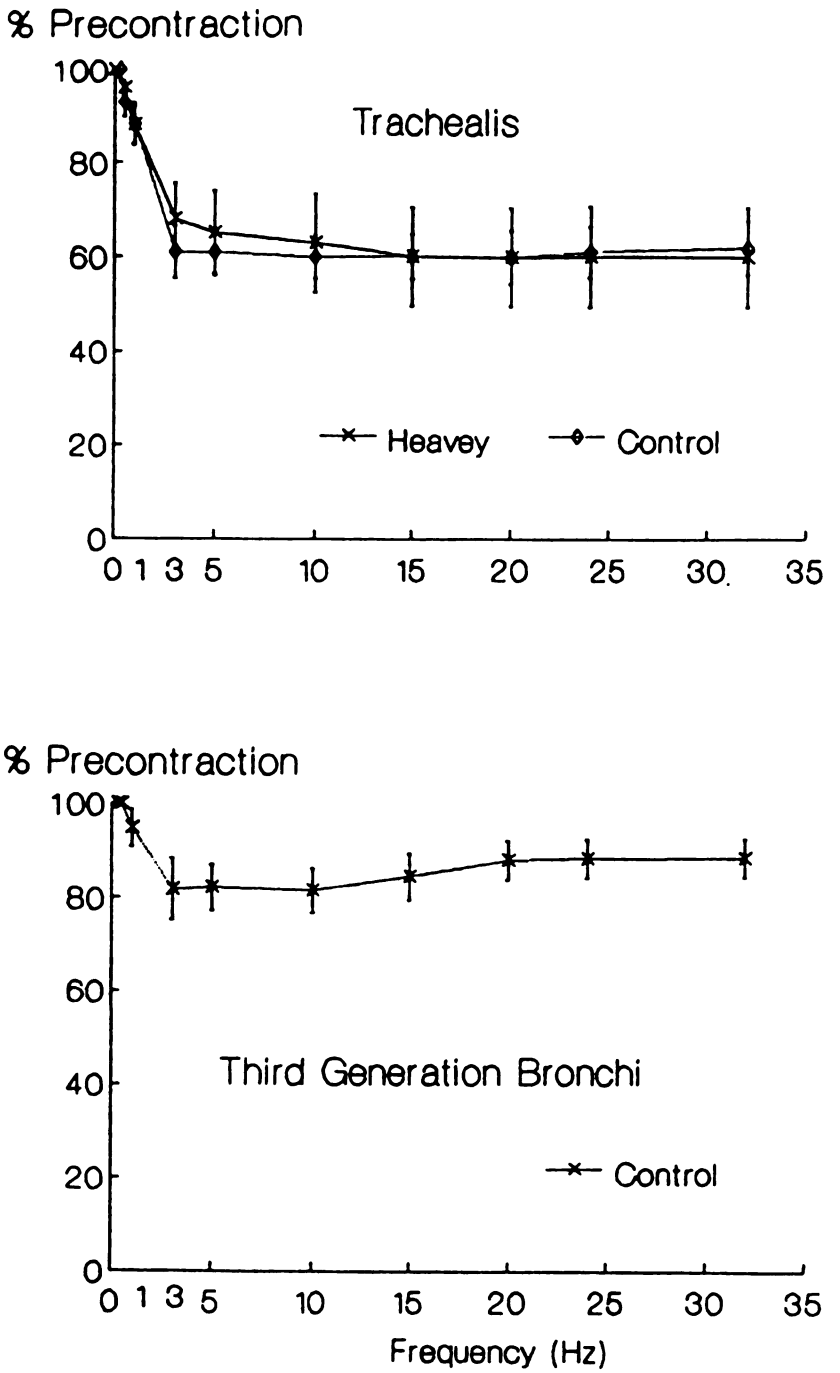

Figure 3-7. Inhibitory effect of electric stimulation on trachealis strips and third-generation bronchi of control and heavey horses. All tissues were precontracted with histamine following pretreatment with 10^{-6}M phentolamine, 3 x 10^{-6}M indomethacin, 10^{-6}M atropine, and propranolol (10^{-6}M). Stimulus parameters: 18v, 0.5 ms duration, and variable frequency as indicated. n = 5 for each curve.

CHAPTER 4

MODULATION OF THE MUSCARINIC RESPONSES OF EQUINE AIRWAY SMOOTH MUSCLE BY AIRWAY EPITHELIUM

Introduction

In the heavey population, in vitro EFS studies of the trachealis muscle and third-generation bronchi demonstrated a hyperresponsiveness compared to the control tissues. The final common pathway of this response was the parasympathetic nerves and the muscarinic receptor, because all contractions were abolished by tetrodotoxin and atropine. The hyperresponsiveness to EFS, coupled with the hyporesponsiveness to exogenous ACh in heavey animals, suggests an increased release of ACh from parasympathetic terminals at each stimulus frequency or decreased degradation of ACh. Either there is a lack of processes that normally inhibit ACh release or processes that facilitate ACh release are present. Prostaglandins, particularly of the E series, have been shown to exert an inhibitory effect on the release of ACh from the varicosities in parasympathetic nerve terminals (Inoue et al. 1984, Inoue and Ito 1985, Shore et al. 1987). Gray and co-workers (1992b) have recently shown that PGE_2 is the major metabolite produced by equine tracheal epithelium. Epithelium and prostanoids may play a role in modulating the underlying

smooth muscle of the control and heavey tissues. The function of the epithelium and prostanoids may be different in the two groups.

Mechanical removal of the airway epithelial layer augments histamine-, methacholine-, and ACh-induced contractile responses of isolated airway smooth muscle from the dog (Flavahan et al. 1985), ox (Barnes et al. 1985), guinea pig (Hay et al. 1986), and rabbit (Raeburn et al. 1986). These findings suggest that an inhibitory factor, released from airway epithelial cells, may interact with the underlying smooth muscle to modulate its reactivity. Decreased production or release or increased breakdown of such a factor could increase the responsiveness of airway smooth muscle to contractile stimuli and thus contribute to the hyperresponsiveness seen in diseases such as equine recurrent obstructive pulmonary disease and asthma. One of the characteristic features of both bronchial asthma in humans and recurrent obstructive pulmonary disease in horses is loss of, or damage to, airway epithelial cells (Laitinen et al. 1985a, Kaup et al. 1990a, 1990b). Therefore, I tested the hypothesis that epithelium from normal horses modulates the response of airway smooth muscle to contractile stimuli. I further hypothesized that this inhibitory factor is absent or diminished in the epithelium from heavey horses. In several species, the in vitro effects of epithelial removal can be reproduced by treating tissues with indomethacin, a cyclooxygenase inhibitor (Barnett et al. 1988, Butler et al. 1987, Tschirhart et al. 1987). PGE_2 is a major AA metabolite produced by intact epithelium (Jacoby et al. 1987, Kazura et al. 1987, Widdicombe et al. 1989) in response to a variety of stimuli. Gray and co-workers (1992b) have shown that the epithelium of the horse is a major site of PGE_2 production. They have also shown

that the epithelium from control animals tended to produce greater quantities of PGE_2 than did epithelial strips from principal animals during acute exacerbations of disease, and that the connective tissue stroma beneath the epithelium from control horses produced greater quantities of PGE_2 than did epithelium-removed strips from heavey horses (Gray et al. 1992a). Therefore, I further hypothesized that if this inhibitory factor is a cyclooxygenase metabolite, then a cyclooxygenase inhibitor such as indomethacin should reproduce the effects of epithelium removal.

Therefore, the main purpose of this study was to examine the influence of the epithelium on the reactivity of the smooth muscle of the trachea of normal horses and horses with recurrent airway obstruction. In addition, the effects of the cyclooxygenase inhibitor indomethacin on responses of preparations with and without epithelium were also examined.

Methods

Experimental protocol

Introduction

Experimental protocols were performed on tracheal strips taken from two groups of mixed-breed adult horses. The control horses had no history of respiratory disease, showed no clinical signs of respiratory tract disease, and did not develop clinical signs of airway obstruction when housed in a barn, bedded on straw, and fed hay. The horses with a history of developing recurrent airway obstruction when exposed to hay and entering remission when pastured and denied access to a hay, straw, or barn environment were studied during an attack of airway obstruction

precipitated by housing in a barn environment. These "heavey" horses were studied when they developed clinical signs of airway obstruction, including recruitment of abdominal muscles on expiration, hypoxemia (PaO_2 less than 75 mmHg), and increases in the ΔPpl_{max} during tidal breathing (greater than 15 cm H_2O). These parameters were used to define the acute state of airway obstruction of heavey horses in previous studies (Derksen et al. 1985a, Armstrong et al. 1986, Scott et al. 1988a, 1988b). At this time, neutrophil numbers are significantly increased in broncho-alveolar lavage fluid samples (Derksen et al. 1985b).

Six horses with a history of heaves (principals) were paired with six horses with no history of heaves (controls). Prior to the experiments, horses were kept at pasture. Baseline pulmonary function measurements were performed and horses were then placed in adjoining stalls in the barn, bedded on straw, and fed hay. When the principal horse developed clinical signs of airway obstruction, pulmonary function tests were performed on both horses. If the results of these tests met the predetermined criteria for airway obstruction, the principal animal was euthanized and tracheal tissues collected for the in vitro studies. The control horse was kept in the barn for an additional 48 hours and pulmonary function measurements repeated. If these indicated no evidence of airway obstruction, the control animal was euthanized and tracheal tissue was collected.

Pulmonary function measurements

Pleural pressure was measured by means of an esophageal balloon (10 cm long, 3.5 cm perimeter, 0.06 cm wall thickness) sealed over the distal end of a

polypropylene catheter (3mm i.d., 4.4 mm o.d., 240 cm long). The balloon was passed into the distal third of the esophagus and connected to a pressure transducer (Validyne Model DP/45-35). The pressure transducer was calibrated before each study against a water manometer. The position of the esophageal balloon was adjusted to obtain the ΔPpl_{max} during tidal breathing. A face mask containing a pneumotachograph (No. 5 Fleisch, Dynasciences, Blue Bell, PA) was placed over the external nares and a rubber shroud and tape were used to seal the mask to the face. The pneumotachograph was connected to a pressure transducer (Validyne DP/45-22) that provided a signal proportional to flow. The flow signal was passed to a lung function computer, which integrated the signal to provide V_T. The pneumotachograph computer system was calibrated using a syringe with which 2 liters of air was passed through the pneumotachograph. Flow, V_T, and the ΔPpl during breathing were processed by the lung function computer and breath-by breath values were printed out on the physiograph trace. These values were also used for a breath-by-breath calculation of R_L, C_{dyn}, f, and $\dot{V}_E$. Resistance and C_{dyn} were calculated by the technique of Amdur and Mead (Amdur and Mead 1958). Arterial blood samples for arterial oxygen tension estimation were obtained by percutaneous puncture of the carotid artery in the lower neck using a #20 needle and syringe. Correct placement of the needle was confirmed by observing high pressure flow of blood from the needle before the syringe was connected and the sample collected.

Animal and tissue preparation

Immediately following euthanasia with an overdose of concentrated pentobarbital sodium (85 mg/kg, iv), tracheal rings 11-16 cranial to the carina were excised and placed in cold (4°C) Krebs-Ringers solution that had been aerated with 5% CO_2 in oxygen. The tissue was then transported to the laboratory. Rectangular strips of trachealis tissue were cut approximately 2 mm x 20 mm using a template so that the muscle fibers were oriented along the long axis of the tissue. The epithelium and mucosa were left intact in half the preparations. To remove the epithelium and mucosa in the other preparations Allis tissue forceps were used to gently tease the epithelium and underlying mucosa off the smooth muscle strips. The tracheal smooth muscle strips were suspended by 3-0 silk in 30-ml organ baths filled with Krebs-Ringers solution (composition in mM: NaCl 110.5, KCl 3.4, $NaHCO_3$ 25.7, dextrose 5.5, KH_2PO_4 1.2, $MgSO_4$, $7H_2O$ 0.4, and $CaCl_2 \cdot 2H_2O$ 2.4) bubbled with 95% O_2 - 5% CO_2 and maintained at 37.4°C. The trachealis strips were placed into the tissue baths within 45 minutes after killing the horse. One end of the trachealis muscle strip was tied to the bottom of a stationary plexiglass tissue holder. The other end was tied to a force transducer (Grass FTO3) mounted on micromanipulators so that the tissue length could be changed when studying the length-tension relationships of the airway smooth muscle. A continuous recording of isometric force generation was obtained by connecting the force transducer to a polygraph (Grass Model 7D). Two rectangular platinum electrodes (dimensions: 8 mm x 40 mm) were placed parallel to the tissue for electrical stimulation of the preparations. Electrical impulses were produced by a Grass S88 stimulator and passed through a stimulus power

booster (Stimu-Splitter II, Med Lab Instruments, Loveland, Colorado). The electrical impulses consisted of square waves. The voltage and duration were monitored on an oscilloscope (Model OS-7020 Goldstar Precision Co., Ltd., Cerritos, CA).

Tissues were equilibrated for approximately 90 minutes with an initial passive tension of 2 g applied and maintained. Following equilibration, the length of each tissue was increased progressively in 0.5 mm to 1.0 mm increments until the AT produced by a standard submaximal electrical stimulus (10 v, 15 Hz, 0.5 ms pulse duration, 30-second stimulation period) reached AT_{max}. Active tension was determined by subtracting passive tension from total tension. Tissue length was considered optimal when the AT in response to the stimulus no longer increased when the tissue was lengthened. The length at which AT_{max} occurred was held constant throughout each experiment. After completion of a protocol, tissues were removed from the tissue bath and fixed in 10% phosphate-buffered formalin. Sections were then prepared, stained with hematoxylin and eosin, and examined for the presence or absence of epithelium.

In this study a total of 12 horses was used. Six control horses and six horses with recurrent airway obstruction were used for the EFS and ACh protocol. The following drugs were used: ACh chloride and indomethacin. Solutions were prepared fresh daily. Acetylcholine was dissolved in deionized water and indomethacin was dissolved in equal molar concentrations of sodium carbonate and deionized water. Solution concentrations were prepared so that all drugs were added in 100-μl aliquots. Drug concentrations are reported as final bath concentrations.

Experimental protocol

Electrical field stimulation and ACh dose-response curves to ACh were recorded in control and heavey horses: 1) with and without epithelium and 2) with and without epithelium in the presence of 10^{-6}M indomethacin. Tissue strips were incubated with indomethacin for one hour prior to stimulation with EFS. The tissues were stimulated at frequencies of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 3, 5, 10, 15, 20, 25, and 32 Hz and voltages of 6, 12, and 18 volts. The sequence of voltages was random. These voltages and frequencies were applied at a stimulus duration of 0.5 ms. At least 5 minutes elapsed between each stimulation. A 30-minute rinse and recovery period was allowed between the generation of the voltage and frequency-response curves and the cumulative ACh response curves. The 10^{-6}M concentration of indomethacin was maintained in the specified tissue baths. Acetylcholine was added in 1/2 log increments and the cumulative concentration response curves to ACh (10^{-9}M to 10^{-3}M) were recorded. Contractions produced by each dose were allowed to reach a plateau before adding the next higher concentration.

Data analysis

Responses to electrical stimulus frequency and concentration of agonist were calculated as a percentage of the maximum contraction produced by ACh (10^{-3}M). The values for the ED_{50} were obtained graphically from a plot of log concentration versus the percent of maximum response produced by ACh for each animal. The mean ED_{50} results of a group were obtained by averaging the ED_{50} results of individual animals.

Statistical analysis

The results are expressed as means ± S.E. Differences between means of two groups of horses at a specific agonist or a specific stimulation frequency were compared by the unpaired Student's t-test. Differences between treatments or conditions in the same group were compared using a paired student's t-test at a particular frequency or specific agonist concentration. When $p \leq 0.05$, means were considered significantly different.

Results

Pulmonary function measurements

In the principal horses, R_L, *f*, ΔPpl during tidal breathing, and arterial carbon dioxide tension were significantly increased, and C_{dyn} and arterial oxygen tension significantly decreased at period B relative to period A (Table 3). No similar changes were present in the control horses between period A and period B. At period A, the ΔPpl was significantly greater in the principals relative to the controls, but within normal values. At period B, R_L and the ΔPpl were significantly increased, and C_{dyn} and arterial oxygen tension significantly decreased in principals relative to controls.

In vitro results

The maximal response to ACh was similar in the epithelial-mucosa removed and intact tissues, suggesting that the integrity of the smooth muscle was not affected by epithelial-mucosa removal (Table 4). The AT_{max} at 10^{-3}M ACh was not significantly different between the control and heavey horses. Neither did the

addition of indomethacin alter the maximal response to 10^{-3}M ACh (Table 4) in either group.

Indomethacin induced a transient contractile response in the controls with and without epithelium. In epithelium intact, the magnitude of the response was 10.9 ± 1.23 grams of AT (or approximately 33% of the contraction seen with ACh 10^{-3}M) above the resting baseline tension. In the controls with epithelium-mucosa removed, the AT resulting from indomethacin was 12.3 ± 2.0 grams of AT, not significantly different from the controls with epithelium. The addition of indomethacin 10^{-6}M to the heavey tissue increased the AT by an average of 0.4 ± 0.09 grams in epithelium intact and 0.5 ± 0.14 grams in epithelium-mucosa removed tissues. The AT recorded from the heavey tissue in response to the addition of indomethacin was significantly less than the control tissues.

Electrical field stimulation

In comparing the EFS data of the control group to the heavey group with epithelium-mucosa intact, the controls were more responsive than the heavey group (Figure 4-1). Epithelium-mucosal removal had little effect in the response of the trachealis tissues of both the control and heavey group to EFS. Only at voltages of 12 and 18 and frequencies of 0.025-0.1 Hz did epithelium-mucosal removal cause the tissue to become significantly more responsive compared to the epithelium-intact strips in the control group (Figure 4-2). In the heavey group, a significant difference was noted only at 6 volts (0.5-3 Hz) between the epithelium-intact and epithelium-removed groups (Figure 4-3). With the epithelium-mucosa removed, the control

group compared to the heavey group was more responsive at voltages of 12 and 18 and frequencies of 0.02-1Hz (Figure 4-4).

Indomethacin increased the responsiveness of epithelium-intact tracheal strips from controls at all voltages studied; however, significant differences were seen only at lower frequencies (0.1-1Hz) (Figure 4-5). With the removal of the epithelium-mucosa in the controls, indomethacin increased the responsiveness and shifted the curves at all voltages, especially at the lower frequencies of stimulation (Figure 4-6). Similarly, in the heavey group, indomethacin increased the responsiveness of epithelium intact strips but only at the higher range of frequencies (Figure 4-7). Indomethacin's effect on epithelium-mucosal removed heavey tissues (Figure 4-8) was similar to the controls with epithelium-mucosa removed.

In the presence of indomethacin, the epithelium-intact tissues of the control group were more responsive than the heavey group (Figure 4-9). However, in the presence of indomethacin, the epithelium-mucosa removed strips of both groups showed no difference to EFS (Figure 4-10). Thus, the differences between the heavey and control groups was abolished by epithelium-mucosa removal plus the addition of indomethacin, with this effect being primarily due to indomethacin.

Response to acetylcholine

Epithelium-mucosal removal in the control group significantly increased the responsiveness of the tracheal smooth muscle to ACh, causing a leftward displacement of the concentration-effect curve (Figure 4-11). The ED_{50} expressed as the -log was 5.68 ± 0.29 in the control group with epithelium and 6.91 ± 0.14 in the control

with epithelium removed, a significant difference (Table 4). However, in the heavey group, epithelium removal had no significant effect on the responsiveness of the tracheal strips to ACh (Figure 4-11, Table 4).

Indomethacin increased the responsiveness of the epithelium-intact tracheal strips from the control horses, with intact epithelium-mucosa shifting the curve to the left (Figure 4-12, Table 4). However, indomethacin did not increase the responsiveness of the control tissues with the epithelium-mucosa removed (Figure 4-12, Table 4). In the heavey trachealis tissues, indomethacin increased the responsiveness of both epithelium-intact and epithelium-removed tracheal strips (Figure 4-13, Table 4).

With the epithelium mucosa intact, there was no difference in the response to ACh between the control and heavey group; however, comparing the two groups with the epithelium-mucosa removed showed that the control group was significantly more responsive to ACh than the heavey group (Figure 4-14, Table 4).

In comparing the addition of indomethacin in the controls with epithelium and the controls without epithelium, the ACh dose-response curves are the same (Figure 4-15) demonstrating that epithelium-mucosa removal in the control horses has the same effect as adding the cyclooxygenase inhibitor indomethacin. The addition of indomethacin to the epithelial-mucosa removed strips of the control group shifts the curve only slightly (Figure 4-16) compared to the curve seen in Figure 4-15 without the addition of indomethacin.

Finally, in comparing the ACh concentration-response curves in the heavey group with the addition of indomethacin to epithelium-intact and epithelium-removed

tracheal strips, the addition of indomethacin shifts the curve leftward similarly in the presence or absence of epithelium (Figure 4-17).

Discussion

The changes in lung function observed in the principal horses at period B are consistent with those described in Chapter 2 and those previously described (Derksen et al. 1985a, Armstrong et al. 1986, Scott et al. 1988a, 1988b). Barn housing caused hypoxemia and airway obstruction (increased R_L and decreased C_{dyn}) in the principal group and no changes in lung function in the control horses.

In this study the in-vitro responsiveness of the trachealis smooth muscle to EFS produced a frequency-dependent contractile response. The heavey group was hyporesponsive compared to the control group at the same stimulus parameters. In an earlier study, the trachealis smooth muscle from the heavey group was significantly hyperresponsive compared to the controls (Chapter 3). In comparing the frequency-response curves of these studies, the frequency response of the heavey group is similar in both studies; however, the control group was more responsive and its response curve was shifted to the left in the present study. The reason for this shift is not known. However, the present study was conducted after housing the control animals in a barn environment with their heavey counterparts, whereas in the original study the controls were removed from pasture prior to killing the horse and collecting tissues. This exposure to a barn environment does not alter pulmonary function measurements but perhaps alters the responsiveness of the controls to EFS. The results of this study with the heavey group hyporesponsive to EFS compared to the

control group were similar to the results of the more peripheral bronchi studies of LeBlanc et al. (1991). LeBlanc et al. found that the peripheral bronchi from the heavey group were hyporesponsive to both EFS and ACh when compared to the airways of the controls.

The removal of the epithelium from tracheal smooth muscle of both the control and heavey horses did not significantly alter the sensitivity of the cumulative frequency-response curves generated at the given voltages, except at lower frequencies. Similar findings that epithelium removal caused no difference in the electrical stimulation-induced contractions at frequencies greater than 1 Hz have been observed in the guinea pig (Holroyde 1986) and ox (Barnes 1985). Therefore, the results of the present study and others (Holroyde 1986, Barnes 1985) suggest that the muscle contractility is changed little by the presence or absence of epithelium, because the response to EFS was similar. These results might also suggest that the release of the relaxing factor(s) from the epithelium cannot be induced by EFS, except at lower frequencies. It has been established that most neurotransmitters have a greater inhibitory effect at lower frequencies (Barnes 1992).

The difference in responses to EFS between heavey and control tissues was not epithelially mediated. This conclusion was reached by comparing epithelial-mucosa removed strips from both groups and finding that the diseased airways remained hyporesponsive. The finding that indomethacin, a cyclooxygenase inhibitor, increased the EFS responsiveness of all the preparations (epithelium-mucosa intact and removed) in both groups suggests that cyclooxygenase products modulate the control of equine tracheal smooth muscle to EFS. Previous in vitro data has

indicated that various exogenous cyclooxygenase products either potentiate or depress the response of tracheal muscle to various stimuli. If indomethacin decreases the concentration of both potentiating and inhibiting cyclooxygenase products, the increased response to EFS after treatment with the cyclooxygenase blocker suggests that the endogenous cyclooxygenase products that suppress neuroeffector activity predominate over those that potentiate activity in the horse airway smooth muscle.

The differences between the responses of heavey and control groups to EFS was abolished by epithelium-mucosal removal plus the addition of indomethacin. This effect was primarily due to indomethacin, since epithelial-mucosal removal in the two groups had similar effects and the heavey tissues remained hyporesponsive. The difference in response to EFS between the two groups was therefore a result of differences in cyclooxygenase products produced from a non-epithelial source. This suggests that the control group produced more bronchodilatory prostanoids than the heavey group.

The significant increase in responsiveness to ACh of the tracheal smooth muscle of the control group when the epithelium mucosa was removed suggests that a major inhibitory factor is located in the epithelium. Therefore, removal of the epithelium from normal horse airways causes changes in the contractile response of isolated airway smooth muscle similar to those reported from various species (Flavahan et al. 1985, Barnes et al. 1985, Hay et al. 1986, Raeburn et al. 1986). These findings suggest that an inhibitory factor, released from airway epithelial cells, may interact with the underlying smooth muscle to modulate its reactivity. Decreased production or release of such an inhibitory factor could increase the

responsiveness of airway smooth muscle to contractile stimuli and thus contribute to the hyperresponsiveness seen in diseases such as recurrent airway obstruction in horses and asthma. In some species this epithelial-derived relaxant factor appears to be a cyclooxygenase metabolite, because the effects of epithelial removal can be reproduced by treating tissues with indomethacin, a cyclooxygenase inhibitor (Barnett et al. 1988, Butler et al. 1987, Folkerts et al. 1989, Tschirhart et al. 1987). Indeed, in the tissues of the control horses epithelium removal had the same effect as adding the cyclooxygenase inhibitor indomethacin. These observations that indomethacin increased the responsiveness to ACh of the epithelium intact strips but not the epithelium mucosa removed strips of the control animals supports the hypothesis that in the control horses the major inhibitory factor is a prostanoid located in the epithelium. A logical explanation for these observations is that the addition of exogenous ACh to the tissue bath induces the tracheal epithelium to release an inhibitory factor(s), which reduces the contractile response of the smooth muscle to ACh.

The epithelial-mucosa intact tracheal strips of the heavey group contracted similarly to ACh as the control epithelium-intact tracheal strips. However, epithelium-mucosa removal in the heavey group did not increase the responsiveness of the tracheal strips to ACh as it did in the controls. This result suggests that the epithelium of the airways is capable of modulating the responsiveness of the airway smooth muscle in normal equine tissues. In the heavey group the epithelium does not appear to have a modulatory role; however, it might be that that the epithelium's modulatory role is masked by the release of other inhibitory modulators from tissues

other than the epithelium. In the heavey group, the addition of indomethacin shifted the ACh concentration-response curves similarly in the presence or absence of epithelium, suggesting a non-epithelial source of inhibitory prostanoid. Comparison of the response of both groups with the epithelium-mucosa removed demonstrates the smooth muscle of the heavey group is hyporesponsive to ACh. In the heavey group, the addition of indomethacin made the muscle more responsive to ACh and shifted the dose response leftward in both the presence and absence of epithelium. This similar shift in the epithelial-intact and epithelium-mucosa removed heavey tissues suggests a non-epithelial source of inhibitory prostanoid. Therefore, in the heavey group, even if the epithelium is producing an inhibitory factor, the effect is being hidden by the non-epithelial source of an inhibitory factor. As with the differences in response to EFS, differences in response to ACh were eliminated by epithelial removal and indomethacin treatment, suggesting that differences in prostanoid production can explain the differences observed between the two groups.

In summary, the control group was more responsive than the heavey group to EFS, and epithelial-mucosal removal had no effect on the response of either group. Thus, the difference in response to EFS in heavey and control tissues was not epithelially mediated. In contrast, removal of the epithelium-mucosa increases the sensitivity of the tissues to ACh in the control group but not in the heavey group, suggesting that the epithelium in normal equine airways modulates the responsiveness of the airway smooth muscle. I have implicated epithelium-derived prostaglandins in this modulatory function to ACh in controls since the cyclooxygenase blocker mimics epithelium removal. The differences between heavey and control groups in

the response to both ACh and EFS were both abolished by cyclooxygenase blockade, suggesting that non-epithelially derived prostanoids are primarily responsible for the differences between the heavey and control groups. Gray and co-workers (Gray et al. 1989) pretreated both control and heavey animals with flunixin meglumine, a non-steroidal cyclooxygenase blocker, to determine if the cyclooxygenase blocker would alter the disease process. Treatment with flunixin produced no alteration in pulmonary function of the two groups. The in vivo failure of this cyclooxygenase blocker to alter the disease process suggests that, although differences in prostanoid products may explain the differences in response to EFS and ACh in vitro, additional mediators and mechanisms are involved in the pathogenesis of the disease.

Table 3. Pulmonary function measurements in principal and control horses at pasture (Period A) and during (Period B) airway obstruction (n = 6).

	Period A						Period B					
	P_R	C_{dyn}	R_L	f	PaO_2	$PaCO_2$	P_R	C_{dyn}	R_L	f	PaO_2	$PaCO_2$
Principal	7.6 ± 0.3+	1.23 ± 0.18	0.91 ± 0.09	12.0 ± 1.8	94.8 ± 2.7	38.2 ± 0.9	36.1 ± 4.9*+	0.31 ± 0.06*+	2.43 ± 0.26*+	17.6 ± 2.0*	60.6 ± 3.8*+	45.9 ± 1.4*+
Control	6.3 ± 0.4	1.37 ± 0.28	0.63 ± 0.11	17.2 ± 1.05	95.3 ± 2.8	40.5 ± 1.1	6.7 ± 0.4	1.26 ± 0.15	0.87 ± 0.10	13.9 ± 2.2	95.1 ± 1.7	42.6 ± 1.0

PaO_2 = arterial oxygen tension, $PaCO_2$ = arterial carbon dioxide tension, C_{dyn} = dynamic compliance, R_L = pulmonary resistance, P_R = change in pleural pressure, f = frequency.
Values are means ± SEM.
* = significant difference from period A value ($p < 0.05$)
+ = significant difference from control value ($p < 0.05$)

Table 4. Effects of epithelium removal and indomethacin on responsiveness of equine tracheal strips to acetylcholine (10^{-9} to 10^{-3}M).

	-log ED		Maximal Response (Grams)	
	Control	**Heavey**	**Control**	**Heavey**
Epith/Mucosa Intact	5.68 ± 0.29	5.22 ± 0.38	33.5 ± 4.1	38.6 ± 2.8
Epith/Mucosa Removed	6.91 ± 0.14*	5.68 ± 0.17+	40.9 ± 3.4	32.3 ± 2.9
Epith/Mucosa Intact + Indomethacin	7.18 ± 0.37*	6.66 ± 0.28*†	39.1 ± 6.2	35.0 ± 2.6
Epith/Mucosa Removed + Indomethacin	7.67 ± 0.13*	6.17 ± 0.12*†	39.8 ± 3.9	40.7 ± 4.2

Values are means ± SE
* Significantly different from Epith/Mucosa Intact strips within group
† Significantly different from Epith/Mucosa Removed strips within group
+ Significantly different from control response with Epith/Mucosa removed

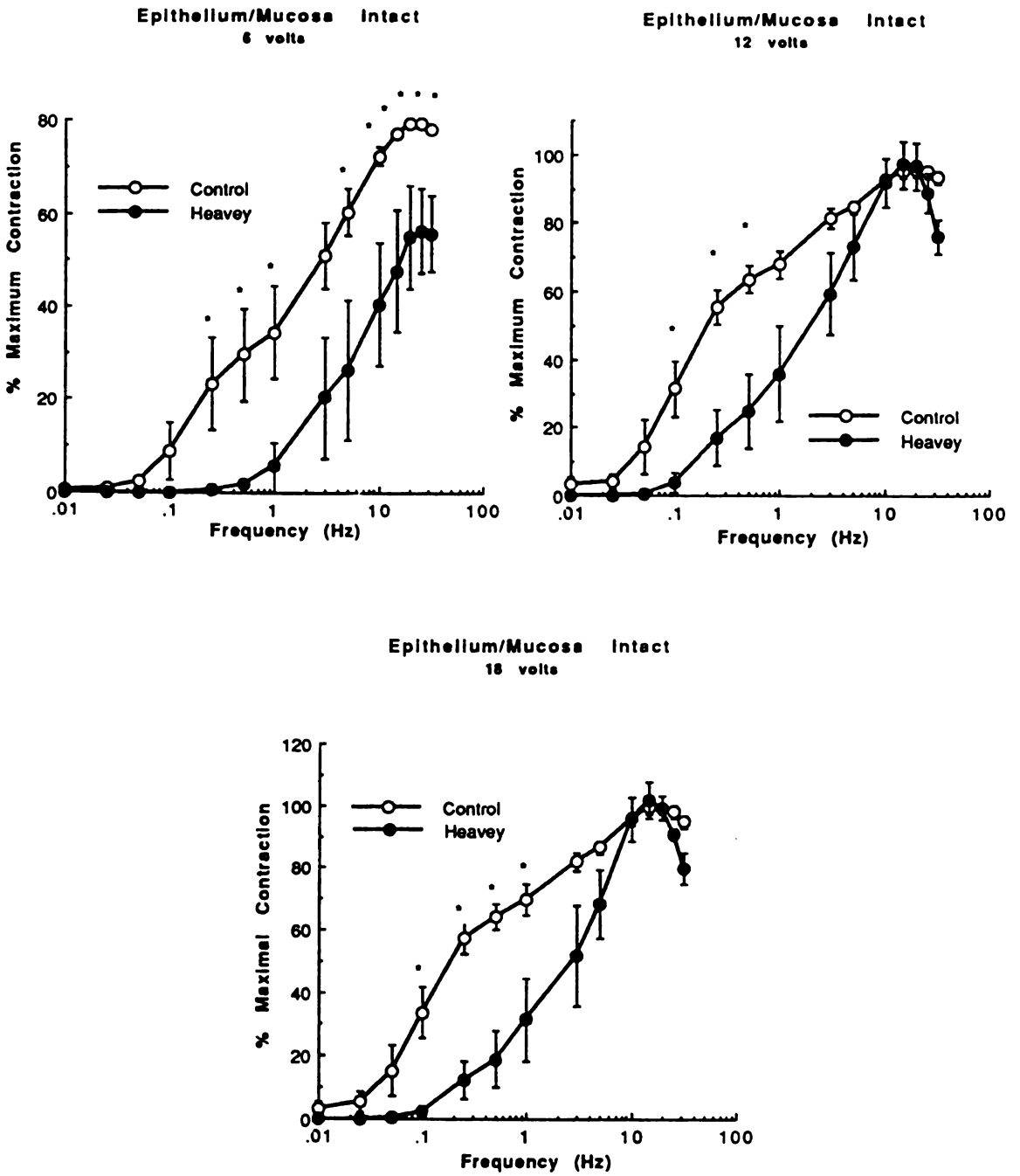

Figure 4-1. Mean (± S.E.) frequency-response curves of trachealis strips of control and heavey horses at 6, 12, 18 volts. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). The epithelium-mucosa is intact. * = Significant difference between control and heavey groups. n = 6 horses for each curve.

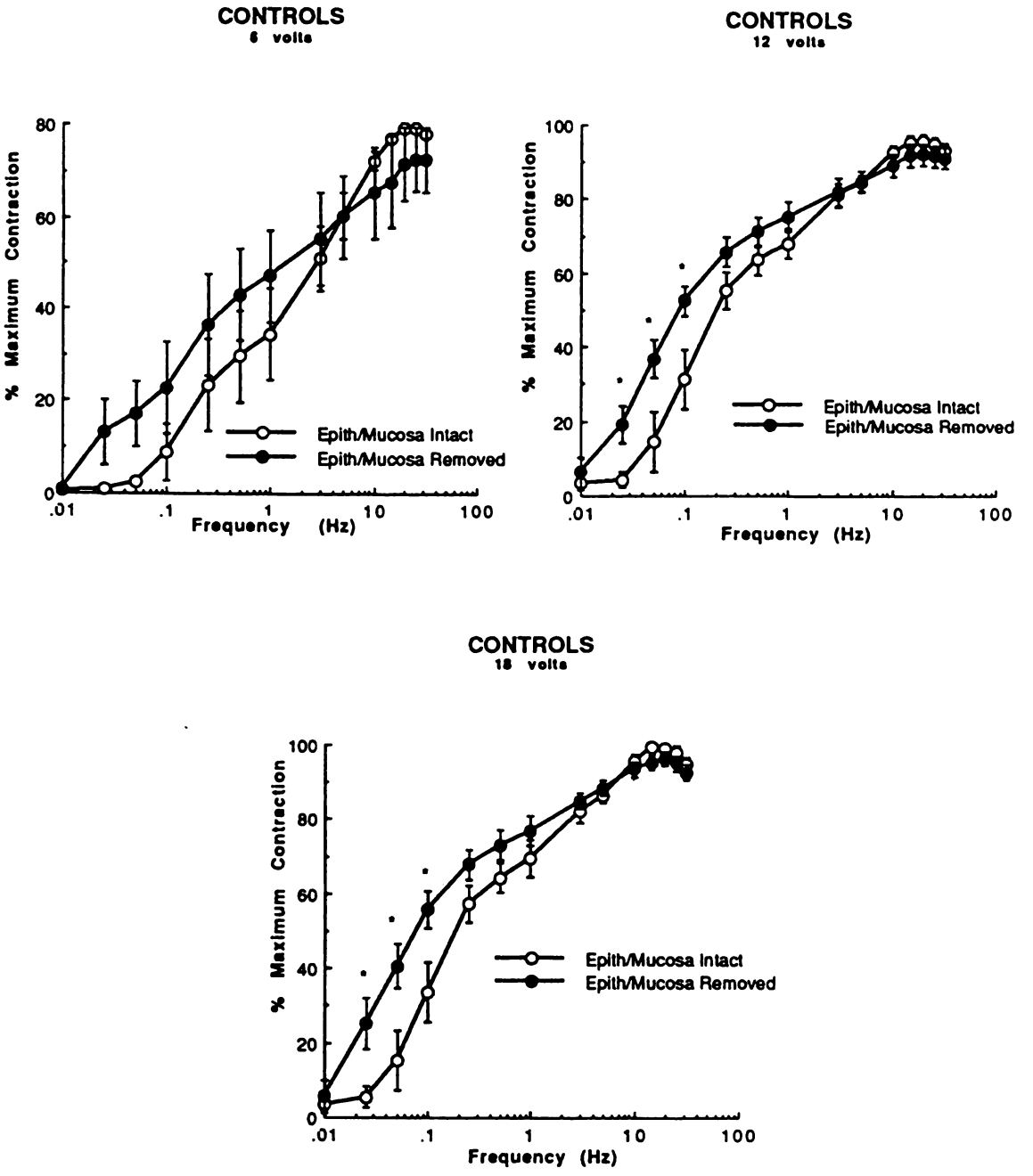

Figure 4-2. Mean (± S.E.) frequency-response curves of trachealis strips of control horses with the epithelium-mucosa intact and the epithelium-mucosa removed in the absence of indomethacin at 6, 12, 18 volts. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between epithelium-mucosa intact and the epithelium-mucosa removed strips. n = 6 horses for each curve.

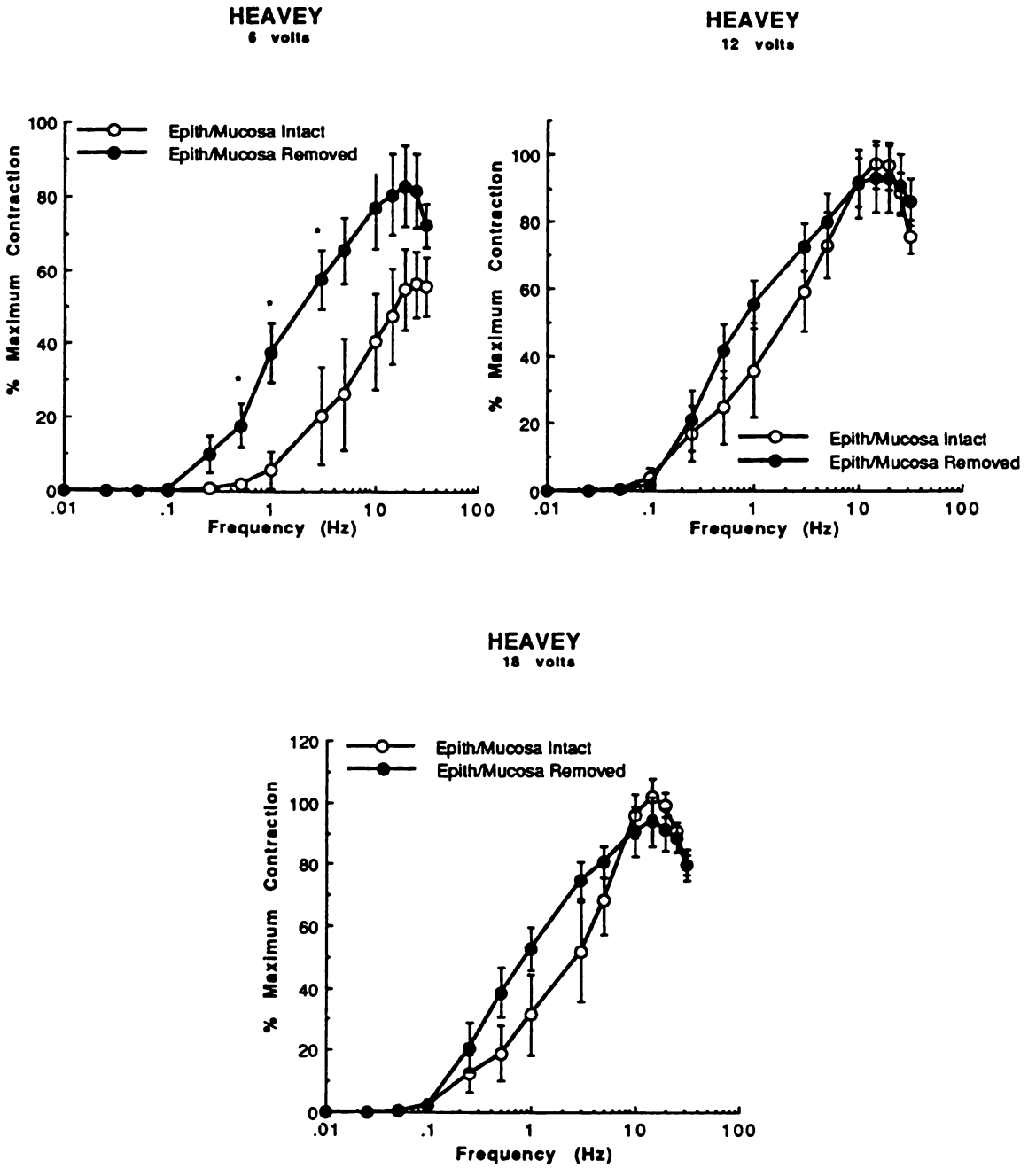

Figure 4-3. Mean (± S.E.) frequency-response curves of trachealis strips of heavey horses with the epithelium-mucosa intact and the epithelium-mucosa removed in the absence of indomethacin at 6, 12, 18 volts. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between epithelium-mucosa intact and the epithelium-mucosa removed strips. n = 6 horses for each curve.

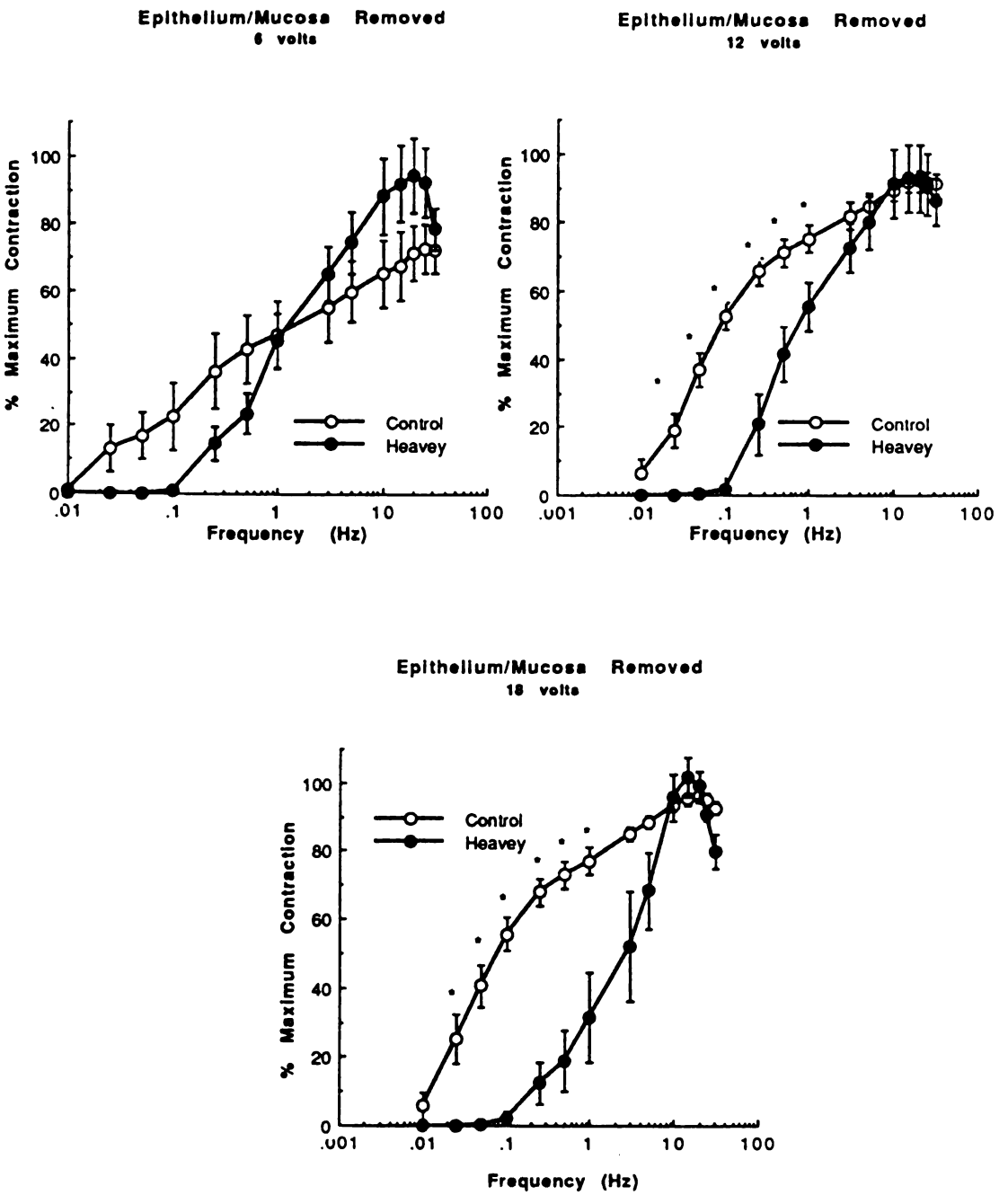

Figure 4-4. Mean (± S.E.) frequency-response curves of trachealis strips of control and heavey horses expressed as percent of the maximal response to acetylcholine 10^{-3}M at 6, 12, 18 volts. The epithelium-mucosa is removed. * = Significant difference between control and heavey groups. n = 6 horses for each curve.

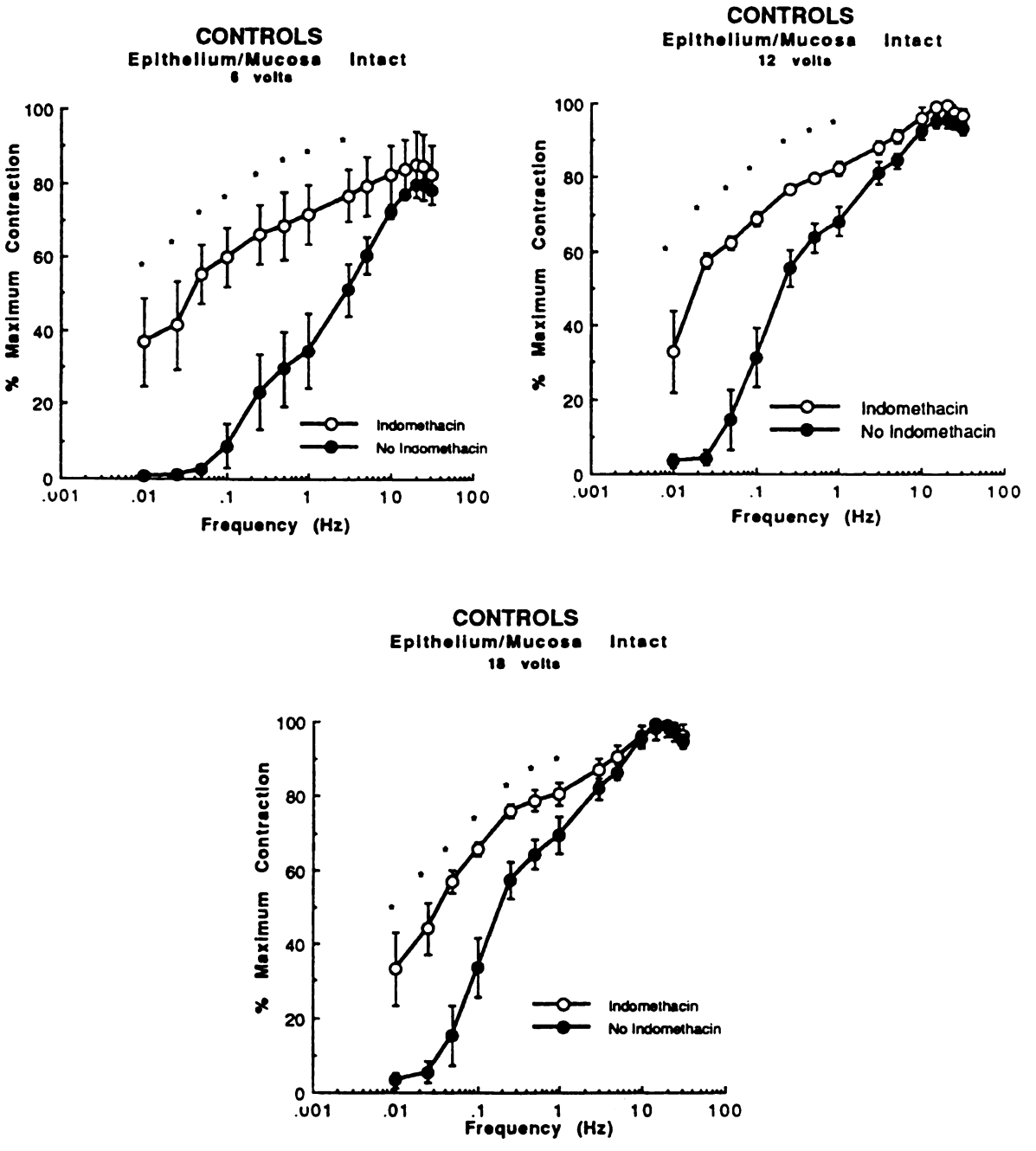

Figure 4-5. Mean (± S.E.) frequency-response curves of trachealis strips of control horses in the presence and absence of indomethacin at 6, 12, 18 volts. The epithelium-mucosa is intact. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between indomethacin and no indomethacin treatment. n = 6 horses for each curve.

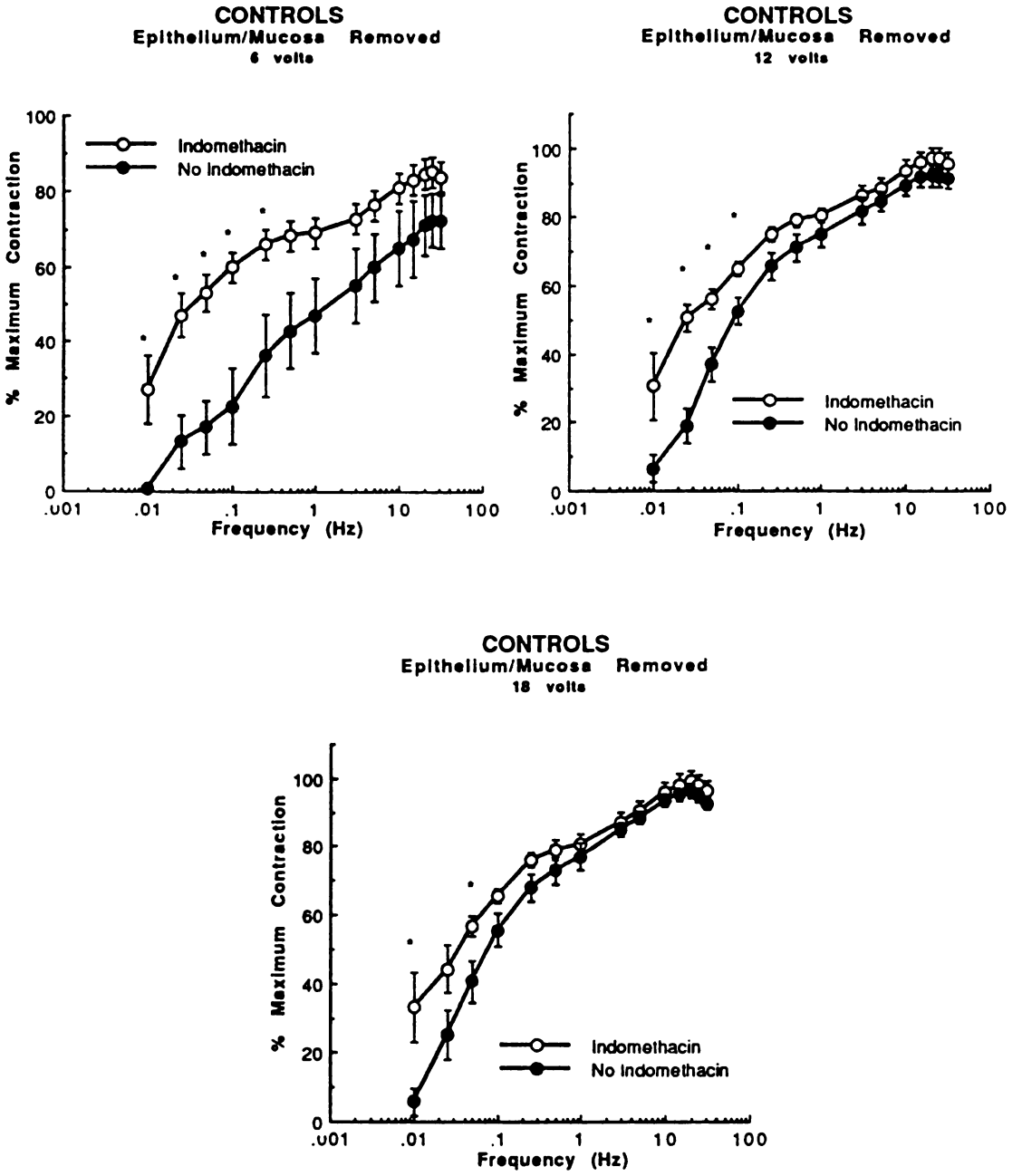

Figure 4-6. Mean (± S.E.) frequency-response curves of trachealis strips of control horses in the presence and absence of indomethacin at 6, 12, 18 volts. The epithelium-mucosa is removed. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between indomethacin and no indomethacin treatment. n = 6 horses for each curve.

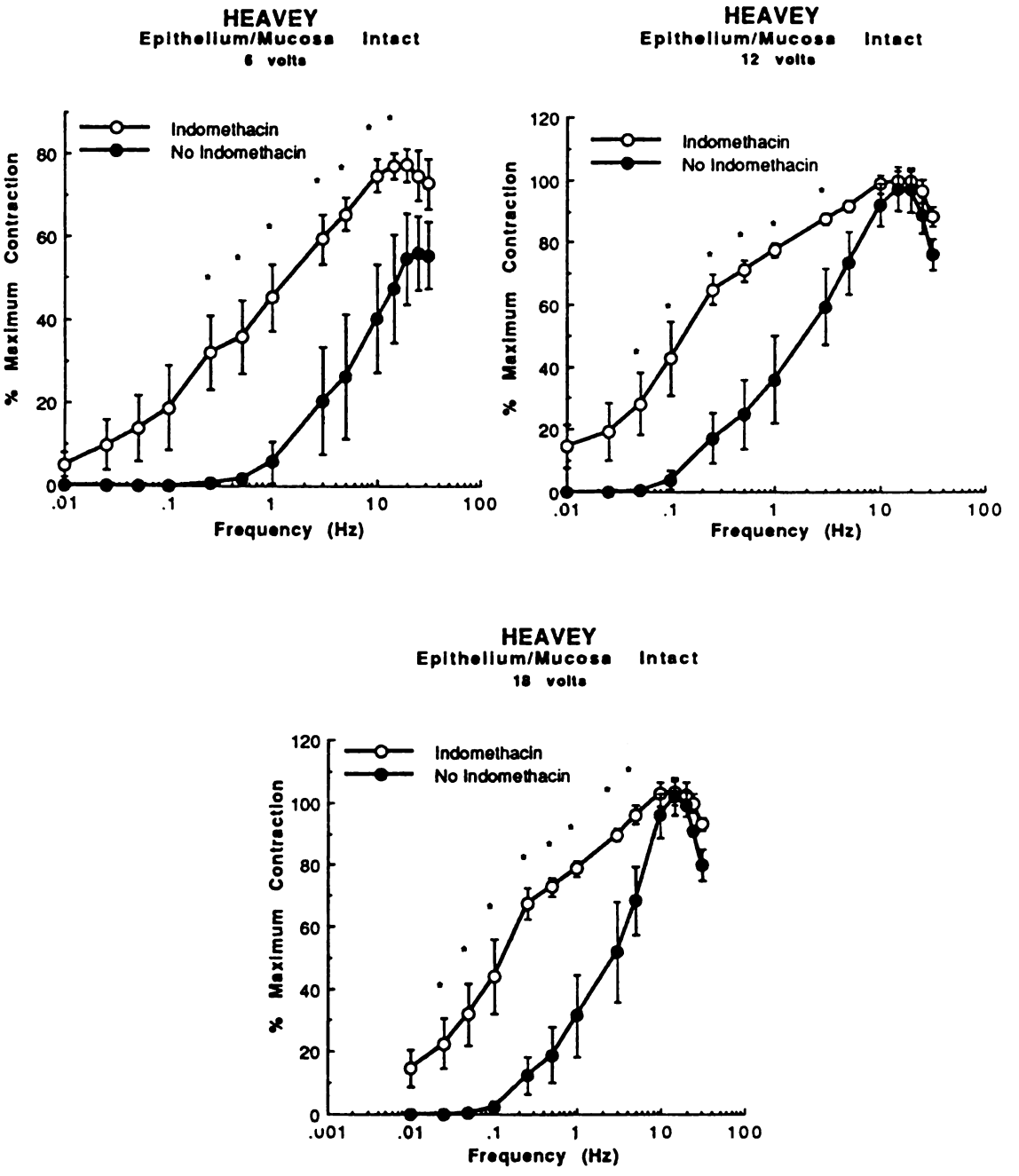

Figure 4-7. Mean (± S.E.) frequency-response curves of trachealis strips of heavey horses in the presence and absence of indomethacin at 6, 12, 18 volts. The epithelium is intact. * = Significant difference between indomethacin and no indomethacin treatment. n = 6 horses for each curve. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M).

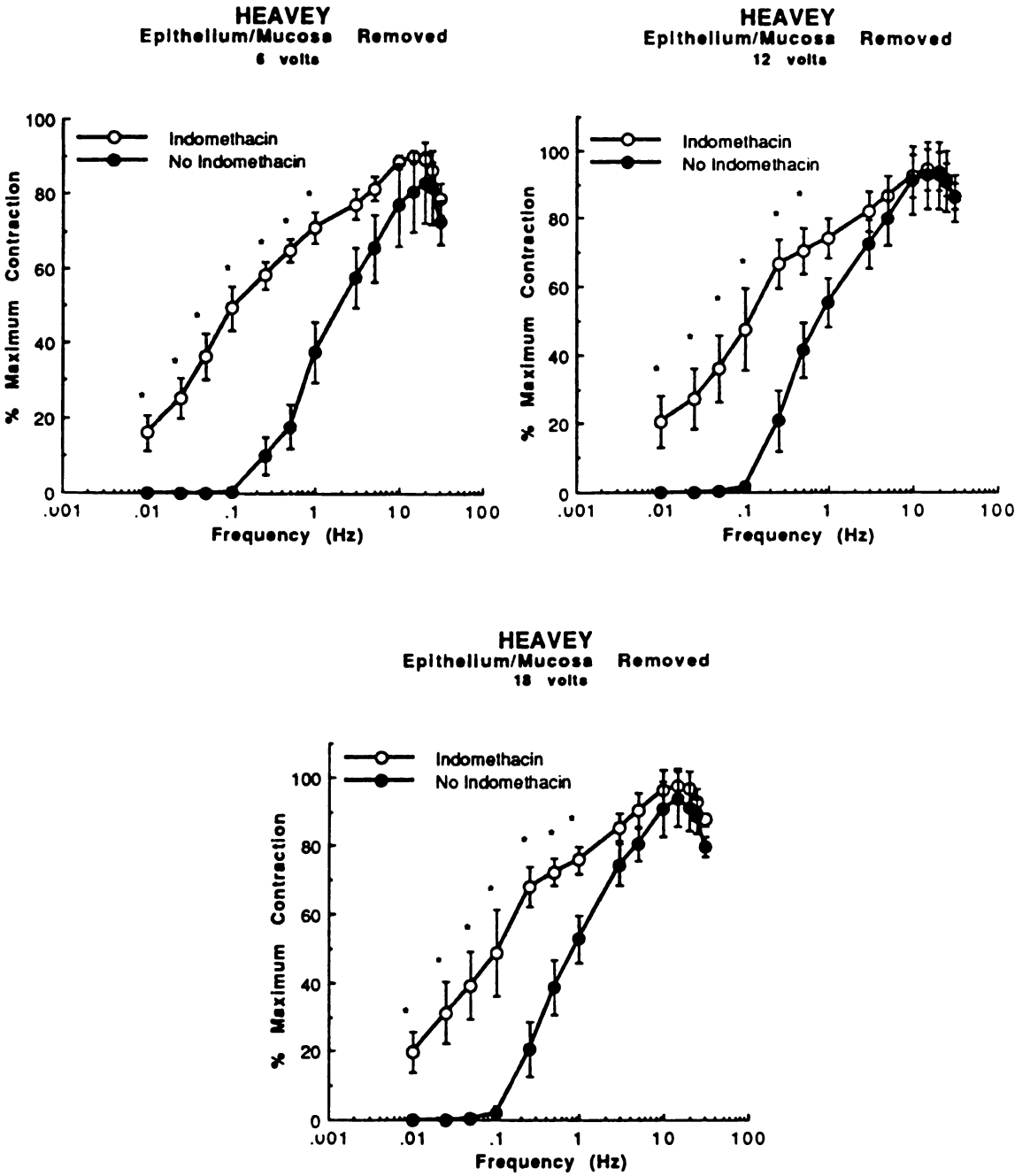

Figure 4-8. Mean (± S.E.) frequency-response curves of trachealis strips of heavey horses in the presence and absence of indomethacin at 6, 12, 18 volts. The epithelium-mucosa is removed. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between indomethacin and no indomethacin treatment. n = 6 horses for each curve.

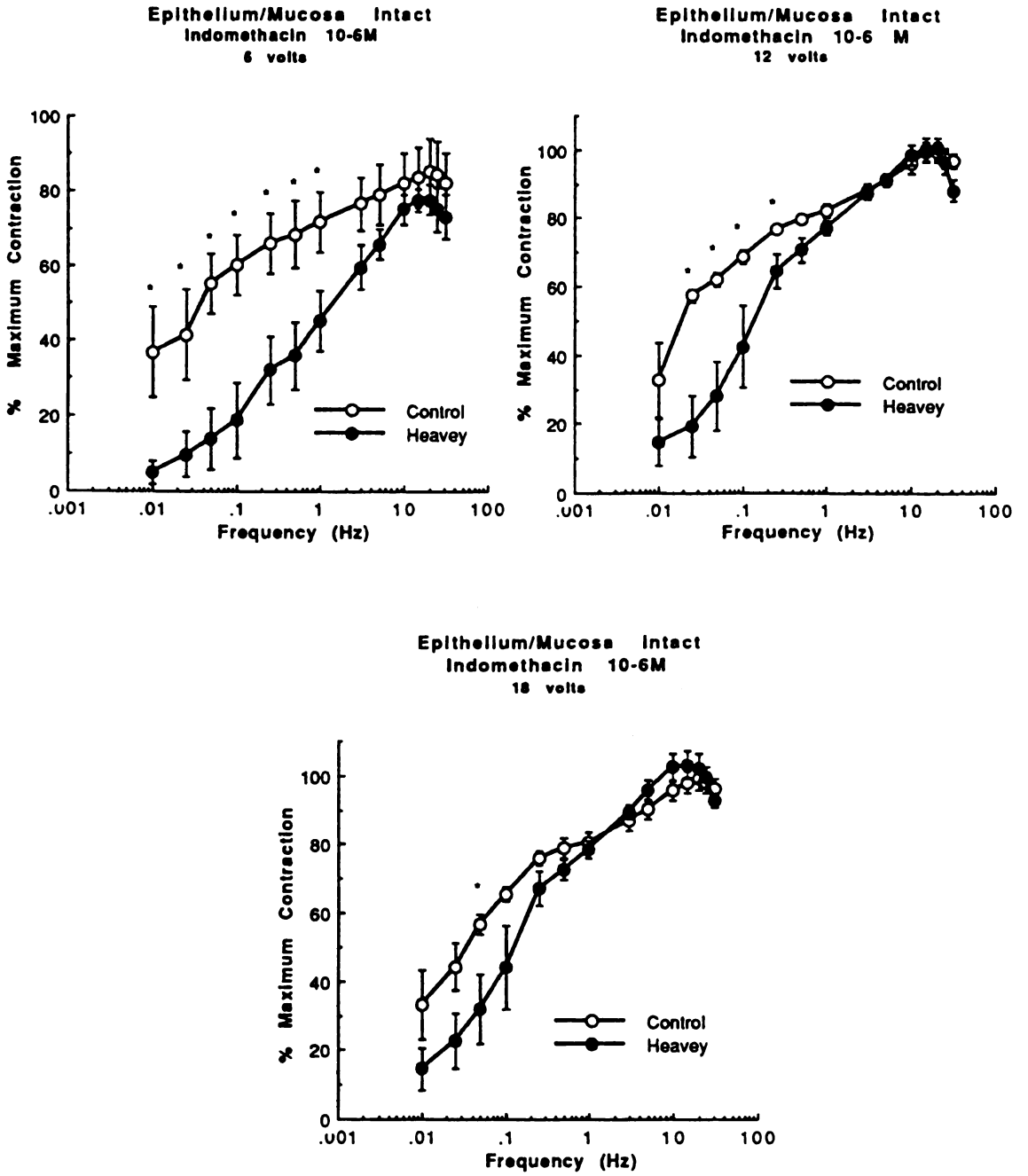

Figure 4-9. Mean (± S.E.) frequency-response curves of trachealis strips of control and heavey horses at 6, 12, 18 volts with the epithelium-mucosa intact and in the presence of indomethacin. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between control and heavey groups. n = 6 horses for each curve.

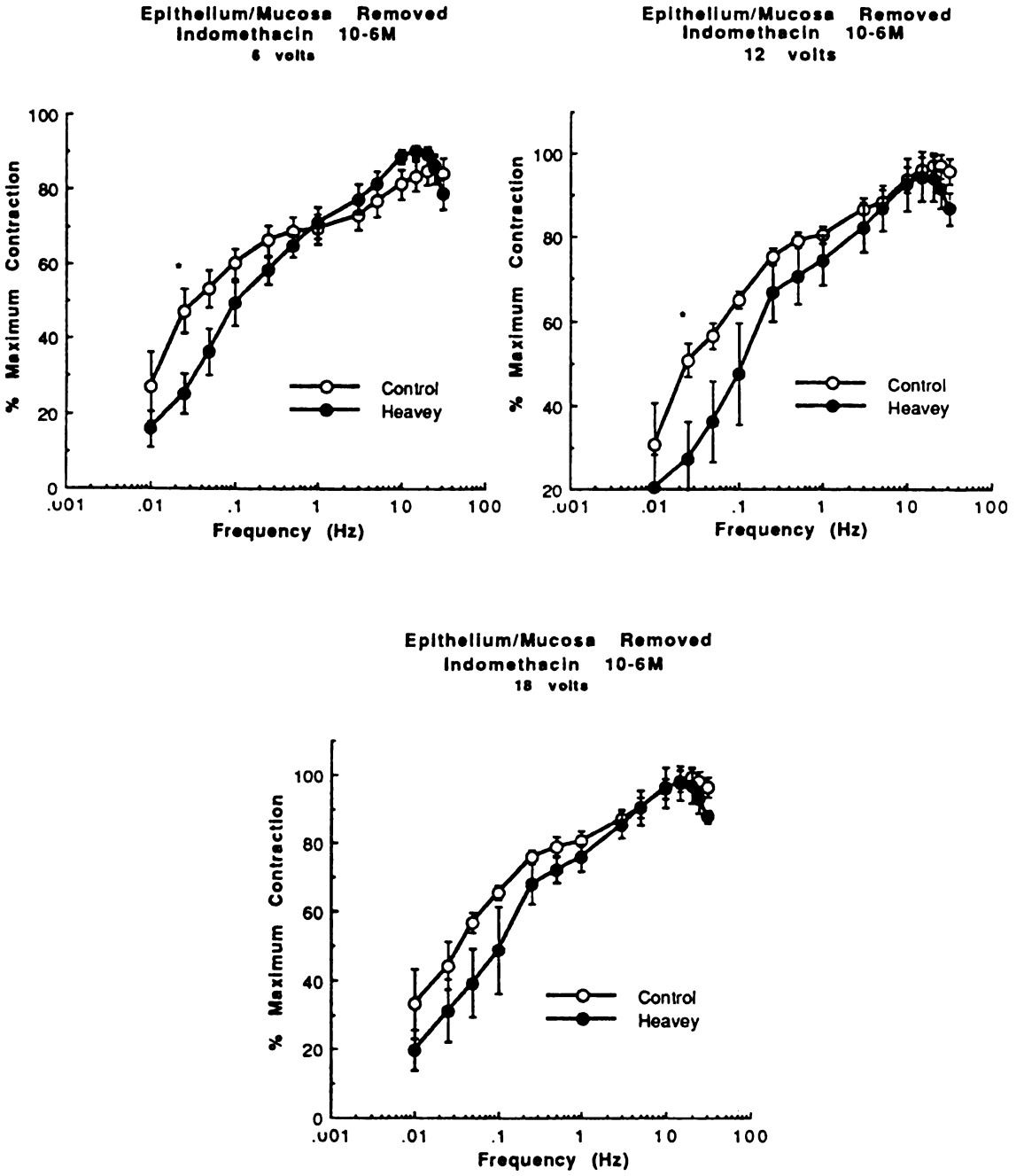

Figure 4-10. Mean (± S.E.) frequency-response curves of trachealis strips of control and heavey horses at 6, 12, 18 volts with the epithelium-mucosa removed and in the presence of indomethacin. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between control and heavey groups. n = 6 horses for each curve.

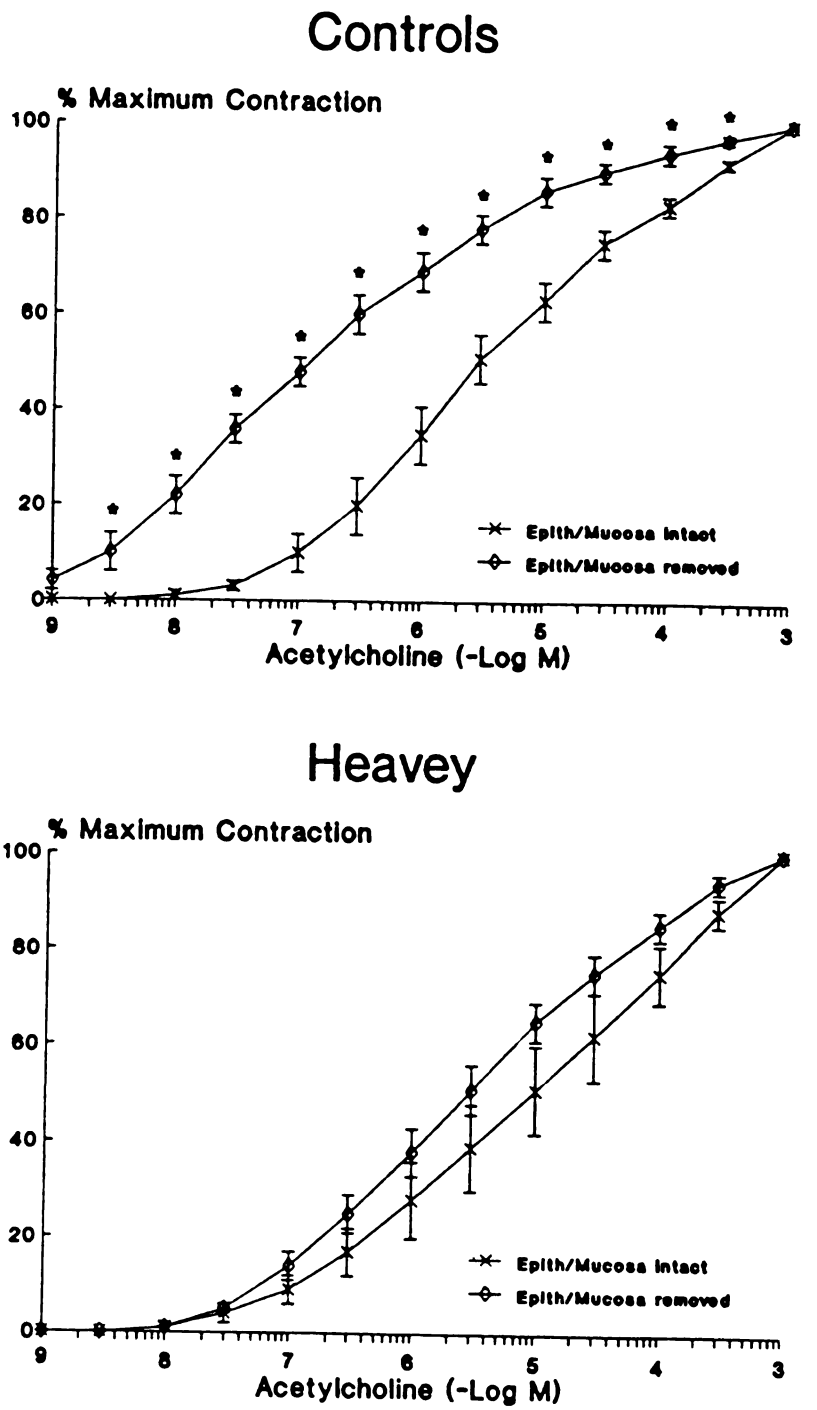

Figure 4-11. Mean (± S.E.) acetylcholine concentration-response curves for trachealis strips of control horses and heavey horses with the epithelium-mucosa intact and the epithelium-mucosa removed. Force expressed as per cent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between epithelium-mucosa intact and epithelium mucosa removed. n = 6 horses for each curve.

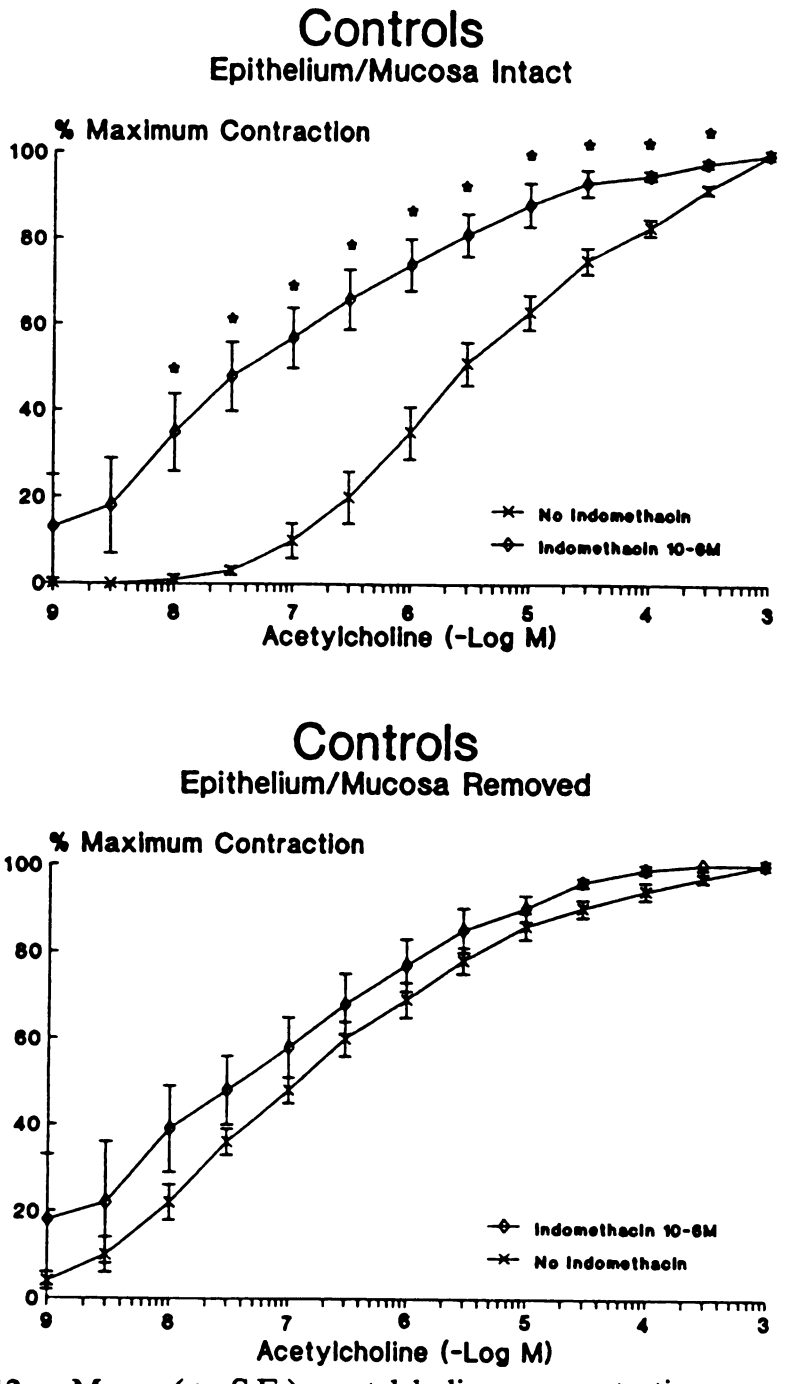

Figure 4-12. Mean (± S.E.) acetylcholine concentration-response curves for trachealis strips of control horses with the epithelium-mucosa intact and the epithelium-mucosa removed in the presence and absence of indomethacin. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between indomethacin and no indomethacin treatment. n = 6 horses for each curve.

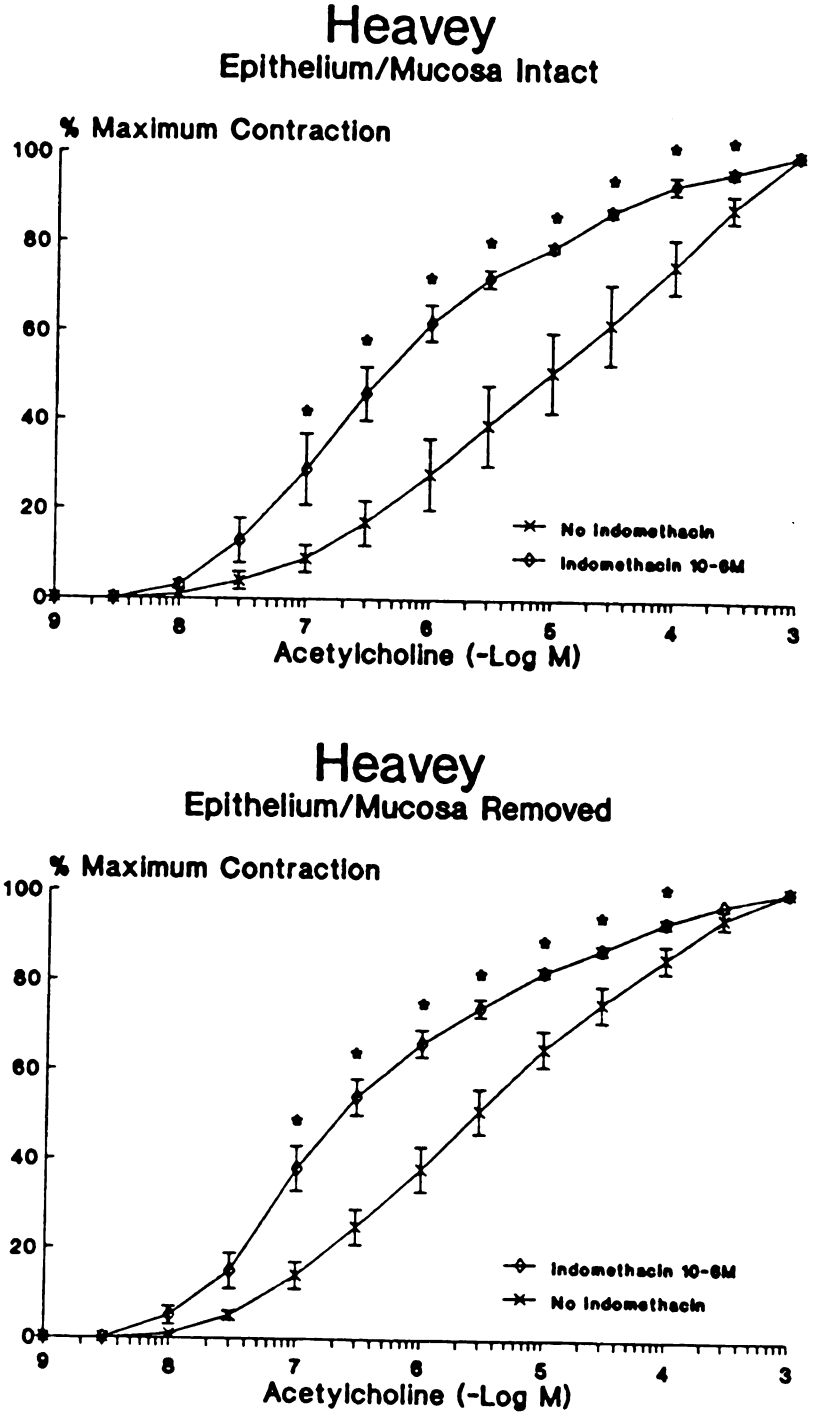

Figure 4-13. Mean (± S.E.) acetylcholine concentration-response curves for trachealis strips of heavey horses with the epithelium-mucosa intact and the epithelium-mucosa removed in the presence and absence of indomethacin. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between indomethacin and no indomethacin treatment. n = 6 horses for each curve.

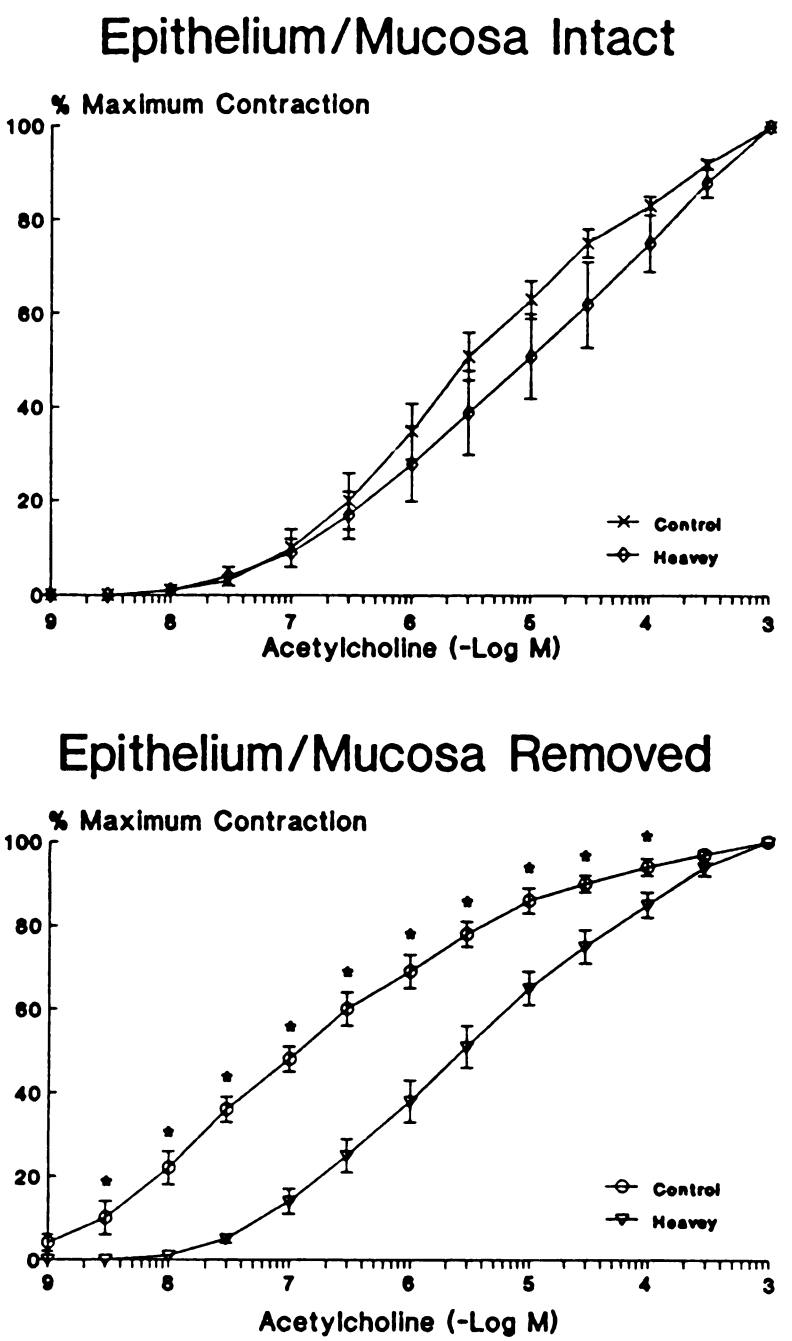

Figure 4-14. Mean (± S.E.) acetylcholine concentration-response curves for trachealis strips of control horses and heavey horses with the epithelium-mucosa intact and the epithelium-mucosa removed. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). * = Significant difference between control and heavey group. n = 6 horses for each curve.

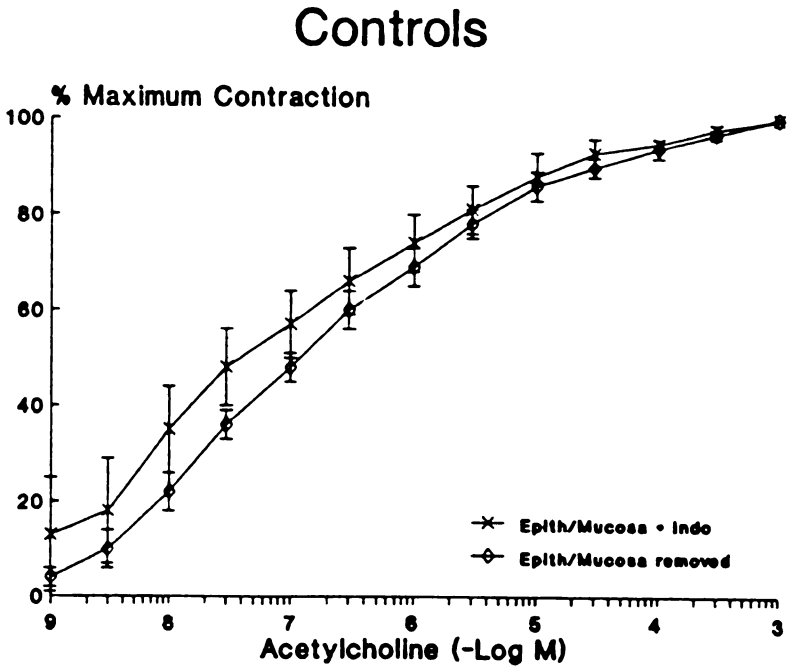

Figure 4-15. Mean (± S.E.) acetylcholine concentration-response curves for trachealis strips of control horses with the epithelium-mucosa intact plus indomethacin and epithelium-mucosa removed strips. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). n = 6 horses for each curve.

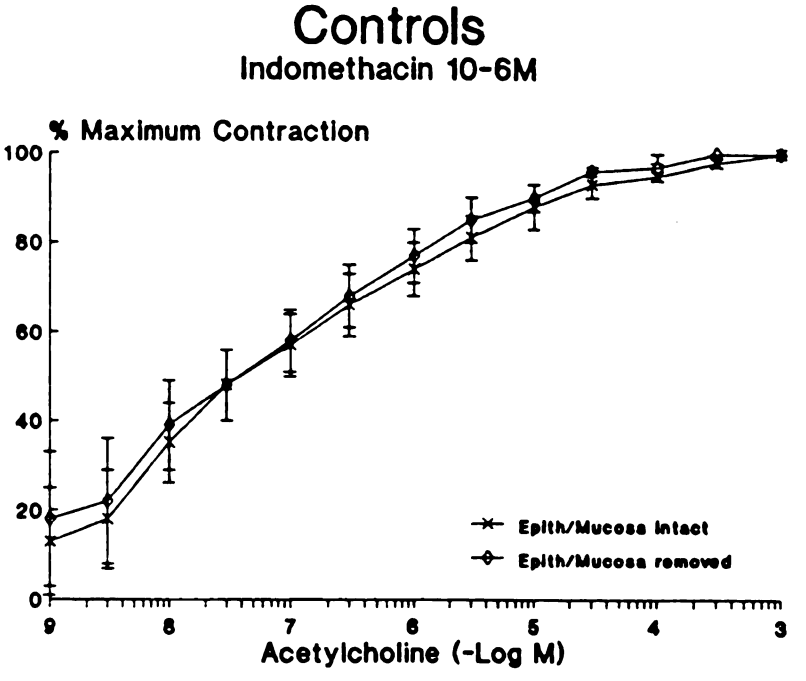

Figure 4-16. Mean (± S.E.) acetylcholine concentration-response curves in the control group with the addition of indomethacin to epithelium-mucosa intact and epithelium-mucosa removed tracheal strips. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). n = 6 horse for each curve.

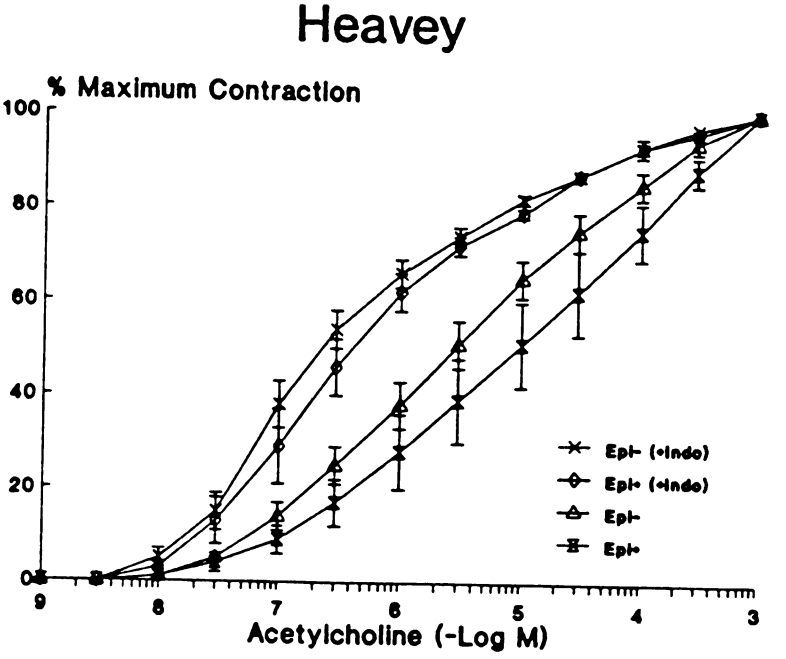

Figure 4-17. Mean (± S.E.) acetylcholine concentration-response curves in the heavey group with the epithelium-mucosa intact and the epithelium-mucosa removed and the addition of indomethacin to epithelium-mucosa intact and epithelium-mucosa removed tracheal strips. Force expressed as a percent of maximal contraction with acetylcholine (10^{-3}M). n = 6 horse for each curve. A combining of the two graphs in Figure 4-13.

CHAPTER 5

SUMMARY AND CONCLUSIONS

In my initial study (Chapter 2), the role of muscarinic receptors in airway obstruction was investigated by the administration of intravenous and aerosol atropine to diseased and control animals at pasture and in the barn. At pasture, atropine, by either route of administration, had no effect on R_L or C_{dyn}, showing that the horse has no resting parasympathetic tone in airway smooth muscle. In the barn, when diseased animals had an increased R_L and decreased C_{dyn}, atropine reduced R_L almost to pasture levels but had a variable effect on C_{dyn}. These observations suggest that the larger airways are primarily innervated by the parasympathetic pathways that were responsible for the smooth muscle contraction that caused airway obstruction in this disease. Atropine did not alleviate airway obstruction in the more peripheral airways, because either there is mucus obstruction, or parasympathetic innervation is less extensive.

The observation that atropine decreases R_L means that muscarinic M_3 receptors on smooth muscle are being activated. This could be the result of reflexes originating in the airways, up-regulation of M_3 receptors, or alterations in the presynaptic regulation of ACh release. Understanding the parasympathetic nerve distribution in normal equine airways and the genesis of the cholinergic component

of heaves required studying airway smooth muscle in vitro. I therefore studied in vitro the smooth muscle of normal and diseased trachealis and third-generation bronchi (Chapter 3).

All tissues from both groups of animals contracted in a dose-dependent fashion in response to increasing concentrations of ACh, but the smooth muscle of heavey animals at all levels of the airway was either hyporesponsive or had the same response to ACh when compared to control animals. Thus, the hyperresponsiveness of heavey ponies to methacholine observed in vivo cannot be explained by hyperresponsiveness of smooth muscle per se. The hyporesponsiveness of "heavey" muscle to ACh also means that the dominant parasympathetic component of airway obstruction in heaves cannot be explained by an exaggerated response to ACh mediator released from cholinergic nerve terminals.

Smooth muscle from the two levels of the airway contracted in a frequency-dependent fashion in response to EFS. In the trachea of control animals, the maximal response to EFS and 10^{-3} ACh was identical, suggesting either that parasympathetic nerves have an extensive distribution or that there is excellent cell-cell communication in the trachealis. In the third-generation bronchi of controls, the maximal response to EFS was 69% of the response to 10^{-3} ACh, suggesting either less extensive distribution of nerves or poorer cell-cell coupling than in the trachea. All EFS-induced contractions were blocked by atropine and tetrodotoxin, proving that they were mediated through nerves and muscarinic receptors.

In the heavey population studied in Chapter 3, the trachealis muscle was hyperresponsive to EFS, contracting to a greater percentage of the 10^{-3} ACh than

control muscle at low frequencies and voltages. In the third-generation bronchi, similar hyperresponsiveness to EFS was observed. Thus, both trachealis and third-generation bronchi had an exaggerated response to EFS in the heavey animals. The final common pathway of this response was the parasympathetic nerves and the muscarinic receptor, because all contractions were abolished by tetrodotoxin and atropine. The hyperresponsiveness to EFS, coupled with hyporesponsiveness to ACh in heavey animals, suggests an increased release of ACh from parasympathetic terminals at each stimulus frequency. Either there is a lack of substances or processes that normally inhibit ACh release or the presence of substances or processes that facilitate ACh release.

I then investigated the systems inhibiting smooth muscle contraction by studying trachealis muscle and third-generation airways in muscle baths. Electrical field stimulation and beta-agonists had no effect on the resting airway smooth muscle of either group of animals, demonstrating that there is no resting tone in equine airway smooth muscle. To demonstrate relaxation, I precontracted the muscle with histamine. It was difficult to obtain stable, reproducible contractions solely with histamine. Indomethacin (10^{-6} M) added to the bath resulted in stable, reproducible, histamine-induced contractions. Isoproterenol was added to the bath in a cumulative manner, causing the trachealis and the third-generation airways to relax back to baseline tension. The curves of normal and diseased ponies were superimposed, indicating no upregulation of beta-adrenergic receptors in heavey ponies, which would account for the airway narrowing that follows beta-adrenergic blockade in heavey animals (Scott et al. 1988b).

To search for inhibitory innervation using EFS, it was necessary to block muscarinic and alpha-adrenergic receptors using atropine and phentolamine, respectively, before contracting the muscle with histamine. Electrical field stimulation of the trachealis caused identical relaxation in both diseased and control animals. The trachea relaxed by 80%, and relaxation was blocked by tetrodotoxin, showing the presence of inhibitory nerves. Pretreatment with the beta-blocker propranolol inhibited EFS-induced relaxation by 50%. I therefore concluded that the trachealis of both control and heavey animals has both sympathetic and NANC inhibitory nervous systems and that there was no difference between the two groups of animals.

In the third-generation airways of control animals, EFS produced much less relaxation than in the trachealis. The muscle relaxed by only 21% from the tension present prior to stimulation. All the relaxation apparently was due to stimulation of the NANC inhibitory system, because propranolol did not inhibit the EFS-induced relaxation. In none of the diseased animals examined did EFS induce relaxation in third-generation airways. Thus, it appears that heavey animals lack an effective NANC inhibitory system in the bronchi.

In the heavey population studied, in vitro EFS studies of the trachealis muscle and third-generation bronchi demonstrated a hyperresponsiveness compared to the control tissues (Chapter 3). Either there is a lack of substances or processes that normally inhibit ACh release or the presence of substances or processes that facilitate ACh release. Prostaglandins, particularly of the E series, have been shown to exert an inhibitory effect on the release of ACh from the varicosities in parasympathetic nerve terminals (Inoue et al. 1984, Inoue and Ito 1985, Shore et al. 1987). Gray et

al. (1992a) have shown a reduced production of PGE_2 by subepithelial tissues in heavey horses.

Therefore, the main purpose of the final study (Chapter 4) was to examine the influence of the epithelium on the reactivity of the smooth muscle of the trachea of normal horses and horses with recurrent airway obstruction. In addition, the effects of the cyclooxygenase inhibitor indomethacin on responses of preparations with and without epithelium were also examined. Surprisingly, in this study the control group was more responsive than the heavey group to EFS. This may have been a result of housing the control animals in a barn environment with their heavey counterparts, whereas in the original study the controls were removed from pasture prior to killing the horse. Epithelial-mucosal removal had no effect on the response of either group to EFS. Thus, the difference in response to EFS in heavey and control tissues was not epithelially mediated. In contrast, removal of the epithelium-mucosa increased the sensitivity of the tissues to ACh in the control group but not in the heavey group, suggesting that the epithelium in normal equine airways modulates the responsiveness of the airway smooth muscle. I have implicated epithelium-derived prostaglandins in this modulatory function to ACh in controls, since the cyclooxygenase blocker mimics epithelium removal. In the heavey group, epithelial removal did not alter the acetylcholine response but indomethacin did. Therefore, a non-epithelial source of prostanoids appeared to be present in the heavey but not the control group. The differences between heavey and control groups in the response to both ACh and EFS were both abolished by cyclooxygenase blockade, suggesting that non-epithelially derived prostanoids are primarily responsible for the in vitro differences between the

heavey and control groups. However, in studies done by Gray and co-workers (1989) treatment with the cyclooxygenase blocker flunixin produced no alteration in the pulmonary function of either the controls or heavey group. This in vivo failure of a cyclooxygenase blocker to alter the disease process suggests that additional mediators and mechanisms are involved in the pathogenesis of this disease.

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