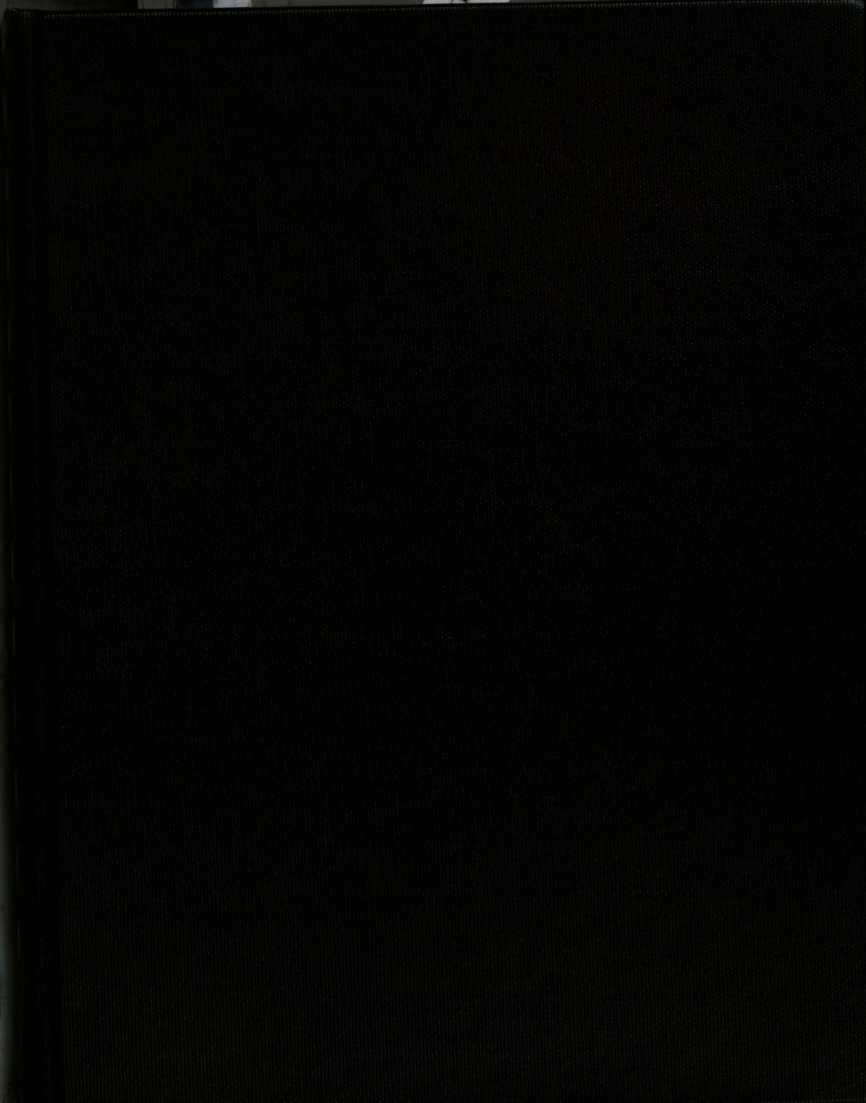






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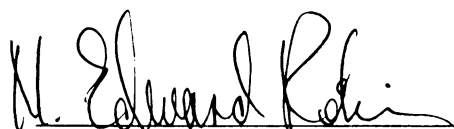
**Prejunctional Modulation of Acetylcholine Release
from Horse Airway Cholinergic Nerves**

presented by

Zhaowen Wang

has been accepted towards fulfillment
of the requirements for

PhD degree in Physiology



Major professor

Date 7/22/93

**PREJUNCTIONAL MODULATION OF ACETYLCHOLINE RELEASE
FROM HORSE AIRWAY CHOLINERGIC NERVES**

By

Zhaowen Wang

A DISSERTATION

**Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of**

DOCTOR OF PHILOSOPHY

Department of Physiology

1993

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ABSTRACT

PREJUNCTIONAL MODULATION OF ACETYLCHOLINE RELEASE FROM HORSE AIRWAY CHOLINERGIC NERVES

By

Zhaowen Wang

The modulatory effects of some prejunctional receptors on acetylcholine (ACh) release from equine airway cholinergic nerves were studied. Acetylcholine release was induced by electrical field stimulation (EFS) of isolated airway smooth muscle preparations suspended in 2-ml tissues baths. Prejunctional modulatory effects were evaluated either by direct measurements of ACh release with HPLC plus electrochemical detection or by comparing the contractile responses of airway smooth muscle to EFS and ACh. Acetylcholine release was frequency-, voltage-, and pulse duration-dependent. Changes in muscle preload had no influence on ACh release. Muscarinic antagonists augmented ACh release, indicating the presence of muscarinic autoreceptors. Because the M_3 antagonist hexahydrosiladifenidol inhibited muscle contraction more potently than ACh release, autoreceptors are not M_3 . Autoreceptors are unlikely to be M_1 because the augmenting effect of the M_1 -receptor antagonist pirenzepine on ACh release emerged at a concentration 240 times its K_d for M_1 receptors. Evidence was inadequate to either confirm or deny M_2 receptors as muscarinic autoreceptors. Exogenous PGE_2 was more potent in inhibiting pony trachealis contractions induced by EFS than by ACh, whereas

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cyclooxygenase inhibitors had no significant effect on the response of either to EFS or to ACh, suggesting that exogenous PGE₂ but not endogenous prostanoids inhibits cholinergic neurotransmission in pony trachea. In the horse, however, neither exogenous PGE₂ nor endogenous prostanoids had any inhibitory effect on ACh release. The α_2 -adrenoceptor agonists UK 14,304 and clonidine inhibited ACh release, and their effects were antagonized by the α_2 -antagonists idazoxan and yohimbine, indicating the presence of inhibitory α_2 -adrenoceptors on horse airway cholinergic nerves. In horses with heaves, a type of chronic obstructive pulmonary disease, ACh release from cholinergic nerves innervating the trachealis was not different from that in normal horses and prejunctional muscarinic autoreceptors were functionally normal. The responses to PGE₂ and the cyclooxygenase inhibitor were similar to those of normal horses. However, clonidine had less inhibitory effect on ACh release in the trachea and was without an effect in the bronchi, suggesting a dysfunction of the prejunctional α_2 -inhibitory mechanism in horses with heaves.

Dedicat

Dedicated to my wife Mingfu Yu and my son Sijie Wang for their love and support.

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I am indebted to many people who have helped me over the past few years. Their support has contributed tremendously to the fulfillment of my research.

I feel lucky to have worked on my Ph.D. dissertation under the supervision of Dr. N. Edward Robinson. My experience with him has been pleasant and rewarding. He is an excellent scientist and a great teacher. He encouraged me to pursue what I am interested in and provided me with excellent guidance. My future career will benefit greatly from the training that I have received in his lab.

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Owing to

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Owing to the nature of my study, I often had to ask Dr. Galligan questions about pharmacology. His answers have been very valuable to me.

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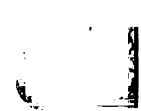
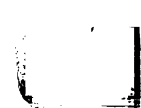


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LIST OF ABBREVIATIONS

4-DAMP	4-diphenylacetoxy-N-methylpiperidine
α -MeHA	α -methylhistamine
$\Delta P_{pl_{max}}$	maximal change in intrapleural pressure
ACh	acetylcholine
AChE	acetylcholinesterase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
C_{dyn}	dynamic compliance
cGMP	cyclic guanosine monophosphate
cTXA ₂	carbocyclic thromboxane A ₂
DMPP	dimethylphenylpiperazinium
EC ₅₀	molar concentration of an antagonist required to reach 50% of its maximal augmenting effect
EFS	electrical field stimulation
EJPs	excitatory junction potentials
EPSPs	excitatory postsynaptic potentials

HACU	high-affinity choline uptake
HHSiD	hexahydrosiladifenidol
HPLC	high-performance liquid chromatography
MAO	monoamine oxidase
NANC	nonadrenergic noncholinergic
NE	norepinephrine
NO	nitric oxide
PGEs	prostaglandins of the E series
PHI	peptide histidine isoleucine
PHM	peptide histidine methionine
R_L	pulmonary resistance
VIP	vasoactive intestinal peptide

LIST OF DRUGS USED AND THEIR ACTIONS

acetylcholine (ACh)	a non-selective muscarinic receptor agonist
AF-DX 116	a muscarinic M ₂ -receptor antagonist
aspirin	a cyclooxygenase inhibitor
atropine	a non-selective muscarinic receptor antagonist
clonidine	an α_2 -adrenoceptor agonist
guanethidine	a sympathetic nerve blocker
hexahydrosiladifenidol	a muscarinic M ₃ -receptor antagonist
hexamethonium	a ganglionic blocker
idazoxan	an α_2 -adrenoceptor antagonist
indomethacin	a cyclooxygenase inhibitor
meclofenamate	a cyclooxygenase inhibitor
neostigmine	an acetylcholinesterase inhibitor
PGE ₂	a cyclooxygenase product
pirenzepine	a muscarinic M ₁ -receptor antagonist
prazosin	an α_1 -adrenoceptor antagonist
tetrodotoxin	a neural blocker
UK 14,304	an α_2 -adrenoceptor agonist
yohimbine	an α_2 -adrenoceptor antagonist

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INTRODUCTION

The cholinergic innervation is the predominant neural pathway in mammalian airways. Its activation releases the neurotransmitter acetylcholine (ACh), which causes airway smooth muscle contractions by acting on muscarinic receptors on the muscle cell membranes. Acetylcholine release from the airway cholinergic nerves may be modulated by the activation of a number of receptors located on the nerve terminals. These prejunctional receptors probably play an important role in the regulation of airway cholinergic neurotransmission. There is evidence to suggest that certain prejunctional receptors are dysfunctional in humans with asthma and in guinea pigs infected with parainfluenza virus or challenged by antigen. An abnormality of prejunctional receptors may lead to increased ACh release and, therefore, be a mechanism of the airway obstruction or hyperresponsiveness present under these conditions.

Most of our knowledge about prejunctional modulation of airway cholinergic neurotransmission comes from comparison of the effect of a factor on the contractile response of airway smooth muscle to exogenous ACh and to nerve activation that releases endogenous ACh. A greater effect on the response to nerve activation than to exogenous ACh would be interpreted as prejunctional modulation. To use this approach, it is necessary to assume that exogenous and endogenous ACh behave similarly. However, because the amount of neurally released ACh is much less than the exogenously

administered ACh, contractions induced by nerve activation are more likely to be influenced by modulators of muscle contractions than those induced by exogenous ACh. Besides, various neurotransmitters may be released from all kinds of nerves during electrical field stimulation (EFS) and may therefore affect the response to EFS but not to exogenous ACh. In addition, if a factor has an effect on gap junctions, it may influence the response to EFS but not to exogenous ACh. Furthermore, if a potential neuromodulator has a direct effect on airway smooth muscle, it would be difficult to differentiate its prejunctional effect from the postjunctional effect. For these reasons, the comparison of responses to EFS and ACh may not always provide the correct conclusion about ACh release. Knowledge about prejunctional modulation would be greatly extended by direct measurement of ACh release from airway cholinergic nerves.

Our laboratory has a long-term interest in studying heaves, a type of chronic obstructive pulmonary disease that affects horses and ponies. Affected equids develop airway obstruction when stabled and fed hay and enter remission at pasture. During airway obstruction, horses have increased pulmonary resistance (R_L), increased maximal change in intrapleural pressure ($\Delta P_{pl_{max}}$) during tidal breathing, and decreased dynamic compliance (C_{dyn}). The disease bears several major similarities to human asthma such as allergic etiology, spontaneous occurrence, and bronchiolitis. Therefore, information about the mechanism of this disease may help us to understand the mechanism of human asthma.

In heavy horses, intravenous administration of muscarinic antagonists such as atropine alleviate the airway obstruction markedly, indicating the involvement of a muscarinic mechanism. In tissue baths, isolated airway smooth muscle preparations from

heavy horses are hyporesponsive to exogenous ACh but hyperresponsive to EFS compared with those of the control horses, suggesting that ACh release from airway cholinergic nerves is probably increased in the heavy horse. One possible mechanism for this increased ACh release is altered prejunctional modulation.

During my Ph.D. dissertation work, I succeeded in adapting an ACh analysis technique to measure ACh release from the horse airway cholinergic nerves. Using this technique and the more traditional approach of comparing the responses to EFS and ACh, I studied the prejunctional modulatory effect of muscarinic autoreceptors, α_2 -adrenoceptors, and prostanoid receptors on ACh release from airway cholinergic nerves in control and heavy horses. The dissertation is presented as a series of chapters that can be read independently. Chapter 1 is a literature review on airway innervation, prejunctional modulation of airway cholinergic neurotransmission, and heaves. Chapters 2–7 are descriptions of my experiments, and Chapter 8 is a summary of the major results and conclusions.

CHAPTER 1

LITERATURE REVIEW

Pulmonary efferent innervation

Introduction

Three neural pathways may be found innervating the lungs: cholinergic, adrenergic, and nonadrenergic noncholinergic. These neural pathways form two plexuses in the lung: peribronchial and periarterial plexuses. The peribronchial plexus further *divides* into extrachondrial and subchondrial plexuses according to their relation to the cartilage (Laitinen and Laitinen 1991). This chapter discusses the structure and function of pulmonary efferent innervation. Where possible, studies on equine airways have been emphasized.

Parasympathetic (cholinergic) innervation

The parasympathetic pathway originates at the vagal nuclei of the brain stem and passes to the lung in the vagus (Barnes 1986). These efferent (preganglionic or presynaptic) fibers synapse with postganglionic neurons in ganglia located adjacent to effector cells. The preganglionic nerve terminals release ACh, which acts on nicotinic receptors on the plasma membrane of the postganglionic neurons. The axons of the postganglionic neurons innervate airway smooth muscle, submucous glands, and,

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possibly, blood vessels. Activation of airway cholinergic nerves causes airway smooth muscle contraction, mucus secretion, and vasodilation.

In airways, the parasympathetic innervation is the predominant neural pathway in all species. Results obtained with histochemical staining of acetylcholinesterase (AChE) suggest that cholinergic nerves are distributed in airways from trachea down to terminal bronchioles (Partanen et al. 1982; Sheppard et al. 1983). The density of cholinergic innervation is greatest in proximal airways and diminishes peripherally (Barnes 1986); so does the density of muscarinic receptors on the airway smooth muscle cell membrane (Barnes et al. 1983). Stimulation of the parasympathetic nerves causes constriction from the trachea to the small airways with resting diameters of 1 mm but less significant contraction in airways smaller than 0.5 mm in diameter (Nadel et al. 1971).

By taking advantage of the universal distribution of AChE in neurons, Baker et al. (1986) have provided a detailed picture about the architecture of nerves and ganglia in ferret trachea by the use of AChE histochemistry. The most distinctive feature of the neuronal architecture of the trachea is the longitudinal nerve trunks located at the border of the trachealis muscle and cartilaginous rings. Branches of the longitudinal nerve trunks divide and anastomose, thus forming three plexuses: one superficial plexus on each side of the dorsal tracheal membrane and one deep muscle plexus. There are numerous ganglia containing one to four ganglion cell bodies located in the superficial plexuses and fewer ganglia containing 10–38 cell bodies dispersed along the longitudinal nerve trunks.

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The autonomic neuroeffector junction is not a "synapse" where there are both pre- and post-synaptic specialized structures, but a "junction" where transmitter is released from varicosities in extensive branching terminal nerve fibers. The distance between varicosities and effector cells varies from 20 nm to 2 μ m (Burnstock 1988). Acetylcholine acts on M_3 receptors on the smooth muscle cell membrane (Brichant et al. 1990; Minette and Barnes 1990; Yu et al. 1992a) and activates the membrane-bound enzyme phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from intracellular stores and induces airway smooth muscle contraction. Contraction of airway smooth muscle appears to be independent of changes in membrane potential or the presence of extracellular Ca^{2+} (Hughes et al. 1990).

Acetylcholine is formed from acetyl coenzyme A and choline under the catalysis of choline acetyltransferase (Tucek 1988) and stored in vesicles in the cholinergic nerve terminals. When the neuron is stimulated, it may develop an action potential that opens voltage-dependent Ca^{2+} channels. Influx of Ca^{2+} causes the vesicles to fuse with the plasma membrane of the nerve terminals and release their contents (Kandel 1985). The released ACh can act on nicotinic or muscarinic receptors on the effector cells and the action is quickly terminated due to the breakdown of ACh to choline and acetate by cholinesterase.

Acetyl coenzyme A is an intermediary product in the metabolism of carbohydrates, fatty acids, and several amino acids and is therefore present in all types of animal cells. Although there is evidence that choline can be synthesized *de novo* in nerve terminals (Blusztajn and Wurtman 1981), it is not clear to what extent, if any, this

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choline contributes to the intraterminal pool of free choline and the subsequent synthesis of ACh. Choline can be brought into the cytoplasm through a high-affinity choline uptake (HACU) system. The process is energy-, sodium-, and voltage-dependent. The uptake is enhanced when cholinergic terminals are depolarized. The HACU system is perhaps the main source of choline for the synthesis of releasable ACh. Most of the substrate for this system comes from recapture of choline produced by the hydrolysis of released ACh (Ducis 1988).

Choline acetyltransferase and AChE are perhaps synthesized in the nerve-cell body and are carried to the nerve endings by the axoplasmic current streaming down each fiber. At the endings they accumulate. Therefore, although the two enzymes and ACh are found in significant amounts along the whole length of every cholinergic axon, the concentration is highest in the nerve terminals (MacIntosh 1959).

The presence of a functional cholinergic innervation in airway smooth muscle can be demonstrated by either direct vagal stimulation or EFS of isolated tissues. If the contraction can be blocked by tetrodotoxin and atropine, it is due to activation of cholinergic nerves. Contractions induced by vagal stimulation may be abolished by the ganglionic blocker hexamethonium, whereas those induced by EFS are unaffected because EFS directly activates the postganglionic neurons. Dimethylphenylpiperazinium (DMPP) induces airway smooth muscle contraction by activating the nicotinic receptors of the postganglionic neurons. Therefore, the effect of this drug is independent of the preganglionic neurons.

In the horse, EFS induces airway smooth muscle contractions, which can be blocked by tetrodotoxin or atropine, indicating the presence of cholinergic innervation.

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The cholinergic innervation is present from the trachea down to peripheral airways with a resting outside diameter of 4-5 mm (Broadstone et al. 1991; LeBlanc et al. 1991; Yu et al. in press b). No experiments have been conducted with smaller airways. Although inhibitory sympathetic and NANC innervation can also be demonstrated in the trachea after blockade of the cholinergic function, the contractile response to EFS is not affected by blockers of the inhibitory nerves (Yu et al. in press b), indicating that the cholinergic function is predominant.

There appears to be no basal cholinergic tone in the airways of normal equids because administration of atropine has no influence on airway resistance (Broadstone et al. 1988). However, there may be a certain degree of cholinergic tone in some other species such as humans (Vincent et al. 1970; de Troyer et al. 1979) and cats (Olsen et al. 1965; Widdicombe et al. 1961). An increase in airway cholinergic activity could be an important mechanism in the development of airway obstruction and hyperresponsiveness (De Jongste et al. 1991). This may result from three mechanisms: 1) increased central efferent firing; 2) reflex activation of cholinergic pathway due to stimulation of sensory receptors; 3) abnormality in prejunctional receptors that regulate ACh release.

Ganglia, clusters of neuronal cell bodies, are not just simple relay stations between the pre- and post-ganglionic neurons. Their functions also may include the following:

Filtering function—Not all the action potentials of the preganglionic neurons are relayed to the postganglionic neurons. In the airway ganglia, there is a type of neuron called the AH cell, which is characterized by a single action potential followed by a large and long-lasting after-hyperpolarization. This post-action potential after-hyperpolarization

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effectively prevents some of the excitatory postsynaptic potentials (EPSPs) induced by activation of presynaptic neurons from developing into action potentials (Coburn 1987).

Site of neuromodulation—Adrenergic nerve terminals are seen in close apposition to neurons in the ganglia (Jacobowitz et al. 1973), raising the possibility that sympathetic input may influence airway cholinergic activity. In support of this possibility, Baker et al. observed that the adrenergic nerve neurotransmitter norepinephrine inhibits cholinergic neurotransmission in airway ganglia (Baker et al. 1983). Excitatory muscarinic receptors (M_1 subtype) may be also present in the ganglia, and activation of these receptors facilitates airway cholinergic neurotransmission (Bloom et al. 1988; Barnes 1989c).

A simple integration center—The complexity of airway ganglia suggests that they may serve as simple integration centers for processing inputs from cholinergic excitatory, sympathetic and nonadrenergic inhibitory, and afferent nerves (Barnes 1986). There is no evidence yet that peripheral airway ganglion neurons may generate signals independent of input from the central nervous system (Coburn 1987), although such is the case in the myenteric plexus (Wood 1984), where central input has only a modulatory function.

Adrenergic (sympathetic) innervation

The sympathetic nerve supply to the lung originates from the lateral horn of the upper six thoracic segments of spinal cord. The preganglionic fibers leave the spinal cord through the ventral roots and synapse in the middle and inferior cervical ganglia and the upper four thoracic paravertebral ganglia. Postganglionic fibers then enter the lungs

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at the hilum and intermingle with the cholinergic nerves to form plexuses (Barnes 1986; Berne and Levy 1988a). The preganglionic nerve terminals release ACh, which acts on nicotinic receptors on the plasma membrane of the postganglionic neurons. The postganglionic nerve terminals release norepinephrine (NE).

The postganglionic sympathetic nerve terminals innervate blood vessels, airway submucous glands, and airway smooth muscle. Activation of the adrenergic nerves causes vasoconstriction, mucus secretion, and airway smooth muscle relaxation. Vasoconstriction is due to the activation of α -adrenergic receptors, whereas relaxation of airway smooth muscle results from binding of NE to β -receptors. In the dog tracheal smooth muscle, it appears that relaxation to exogenous β -agonists is mediated by β_2 -receptors, but relaxation to sympathetic nerve stimulation is mediated by β_1 -receptors (Barnes 1989a). α -adrenoceptors mediating smooth muscle contractions have also been demonstrated in the airways of some species. However, their function can be observed only under certain conditions such as following β -adrenergic blockade (Leff et al. 1986).

The synthesis of NE is a multiple-step process. Tyrosine is converted to L-dopa by tyrosine hydroxylase. L-dopa is converted to dopamine by a specific decarboxylase. Dopamine β -hydroxylase converts dopamine to NE (Berne and Levy 1988b). Norepinephrine is stored in vesicles in the nerve terminal and released upon nerve stimulation. The released NE acts on α - or β -receptors on the effector cells. Its action is quickly terminated due to three mechanisms: 1) the majority of released NE is removed by reuptake into the nerves, where it is either reincorporated into vesicular stores or degraded by monoamine oxidase (MAO); 2) some NE is taken up by smooth muscle cells and inactivated by intracellular MAO or catechol-O-methyltransferase; 3) some NE leaks

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The distribution of adrenergic nerve terminals in airway smooth muscle is generally sparse; however, there is considerable variation between species. There are abundant adrenergic fibers in the bronchial muscles of the cat (Dahlstrom et al. 1966), goat, sheep, pig, calf (Mann 1971), and dog (Knight et al. 1981), whereas few are found in the bronchial muscles of the rabbit (Mann 1971) and man (Pack and Richardson 1984). In horse airways, the inhibitory function of sympathetic nerves can be demonstrated in upper trachea but not in lower trachea and bronchi (Yu et al. in press b). Adrenergic nerves have been found in close association with airway parasympathetic ganglia (Jacobowitz et al. 1973), which is perhaps important for modulation of the cholinergic activity.

Adrenergic innervation is the major neural pathway of blood vessels in the lungs. In airways, it is not as important as in the blood vessels. Adrenergic nerves oppose the function of cholinergic nerves. In species with abundant direct adrenergic innervation in the airway smooth muscle, the adrenergic nerves may inhibit muscle contractions directly. In species where direct innervation of adrenergic nerves to airway smooth muscle is scanty, adrenergic nerves may still modulate airway smooth muscle contractions by acting on airway cholinergic nerves.

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Nonadrenergic noncholinergic innervation

In the airways, there are neurally mediated responses that cannot be abolished by either adrenergic or cholinergic blockers, indicating the presence of NANC innervation. Both inhibitory and excitatory NANC innervations may be demonstrated in the airways.

The preganglionic fibers of inhibitory NANC innervation run in the vagus and synapse in the peripheral airway ganglia with postganglionic neurons. Similar to the parasympathetic and sympathetic nervous system, the neurotransmitter of preganglionic neurons of the inhibitory NANC system is ACh, which activates postganglionic neurons by acting on nicotinic receptors in the ganglia. It is not known whether the neurotransmitter of the postganglionic neurons is released from specialized inhibitory neurons or from neurons that also contain ACh, although ultrastructural studies suggest that vasoactive intestinal peptide (VIP), a possible neurotransmitter of the inhibitory NANC nerves, may coexist in the same nerve terminals as ACh (Laitinen et al. 1985b).

Inhibitory NANC innervation has been demonstrated in the airways of several species including humans (Richardson and Beland 1976; Taylor et al. 1984), pigs (Mitchell et al. 1990), horses (Broadstone et al. 1991; Yu et al. in press b), and guinea pigs (Taylor et al. 1984; Grundström et al. 1981b). The importance of inhibitory NANC innervation in the control of airway smooth muscle tension varies between species. In human (Richardson and Beland 1976), pig (Mitchell et al. 1990), and horse (Yu et al. in press b) airways, it is the primary inhibitory nervous system. In contrast, dog airways lack an NANC innervation (Russell 1980).

There is no general agreement about the neurotransmitters of the inhibitory NANC nerves. Nitric oxide (NO) (Yu et al. in press b; Kannan and Johnson 1992; Li

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and Rand 1991; Tucker et al. 1990) and neuropeptides such as VIP, peptide histidine isoleucine (PHI), and peptide histidine methionine (PHM) (Barnes 1986) are among possible candidates.

Vasoactive intestinal peptide is a 28 amino acid peptide (Barnes 1986). It is a potent relaxant of airway smooth muscle (Palmer et al. 1986). Vasoactive intestinal peptide has been localized to neurons and nerve terminals in airway smooth muscle and submucosal glands (Laitinen et al. 1985b; Dey et al. 1981). Electrical field stimulation of tracheal preparations releases VIP into the bathing medium, and this is blocked by tetrodotoxin (Cameron et al. 1983; Matsuzaki et al. 1980), indicating that VIP is released from nerves. Peptide histidine isoleucine and PHM are also potent relaxants of airway smooth muscle (Palmer et al. 1986; Lundberg et al. 1984a; Christofides et al. 1984). They are structurally related to VIP.

Nitric oxide is widely known as the endothelium-derived relaxing factor in blood vessels. It has a powerful ability to relax smooth muscle. Nitric oxide appears to be the neurotransmitter or mediator of the inhibitory NANC nerves in the airways of pigs (Kannan and Johnson 1992), horses (Yu et al. in press b), and guinea pigs (Li and Rand 1991; Tucker et al. 1990). Conflicting data have been reported in cat airways: in one study conducted with isolated trachealis, nitric oxide was proposed as the primary, if not the only, mediator (Fisher et al. 1993), but in another study, nitric oxide did not mediate the inhibitory NANC relaxation in vivo or in vitro (Diamond et al. 1992).

Vasoactive intestinal peptide and NO relax smooth muscle through different effector pathways. Nitric oxide stimulates soluble guanylate cyclase to increase cyclic guanosine monophosphate (cGMP), whereas VIP stimulates adenylate cyclase to promote

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the accumulation of cyclic adenosine monophosphate (cAMP). The mechanisms by which the cyclic nucleotides produce smooth muscle relaxation are not fully understood. The accumulation of either cAMP or cGMP is thought to reduce intracellular Ca^{2+} concentration (Said 1992).

In horse airway tissues incubated with atropine and propranolol and precontracted with histamine, EFS produces frequency-dependent relaxation and the magnitude of the relaxation decreases from trachea to central bronchi and is absent in peripheral airways. The degree of relaxation in bronchi is not simply a function of bronchial size or generation. N^{G} -nitro-L-arginine, a nitric oxide synthase inhibitor, eliminates the relaxation. This effect is reversed by L-arginine, the NO precursor, but not by D-arginine. These results suggest that inhibitory NANC nerves supply only the trachea and central bronchi and that NO mediates inhibitory NANC function (Yu et al. in press b).

A dysfunction of the inhibitory NANC nerves is probably related to asthma and some other types of airway obstruction. Immunoreactive VIP is present in lung tissue of normal humans but absent in that of asthmatics (Ollerenshaw et al. 1989). Functional inhibitory NANC innervation can be demonstrated in the central bronchi of normal horses but not of horses with airway obstruction (Broadstone et al. 1991). It remains to be determined if the dysfunction of inhibitory NANC nerves in the heavy horses is a cause or a result of airway inflammation and obstruction.

Excitatory NANC innervation has also been demonstrated in the airways of a few species. The neurotransmitter of the excitatory NANC nerves is probably a tachykinin such as substance P or neurokinin A. Substance P appears to be the most likely

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candidate. For example, EFS of guinea pig bronchi produces a component of bronchoconstriction that cannot be prevented by atropine (Lundberg et al. 1983c; Andersson and Grundström 1983; Grundström et al. 1981). This response is mimicked by substance P and antagonized by substance P antagonists, suggesting that substance P is the neurotransmitter of the excitatory NANC nerves. Substance P is localized to nerves in the airways of several species (Lundberg et al. 1984b; Polak and Bloom 1982; Wharton et al. 1979). The nerves that contain substance P appear to be afferent nerves (Barnes 1986). Damage to airway epithelium may expose sensory nerve terminals which, upon stimulation by inflammatory mediators, may lead to the release of substance P through an axon reflex (Barnes 1986). Capsaicin releases substance P from sensory nerves. Acute treatment with capsaicin induces airway smooth muscle contraction (Lundberg et al. 1983b), whereas chronic treatment with capsaicin eliminates the function of excitatory NANC nerves due to the depletion of substance P (Lundberg et al. 1983a). Removal of epithelium increases the constrictor effect of substance P, probably due to the predominant localization of neural endopeptidase, the major degrading enzyme for tachykinins, in airway epithelium (Barnes 1991).

Prejunctional modulation of airway cholinergic neurotransmission and its role in airway obstruction

Control of airway smooth muscle tension in humans and most animal species is dominated by excitatory neural inputs transmitted by cholinergic motor nerves (Barnes 1986). The fact that muscarinic antagonists such as atropine frequently alleviate airway obstruction in some airway diseases suggests that activation of muscarinic receptors by

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ACh released from airway cholinergic nerves plays an important role in the development of airway obstruction (De Jongste et al. 1991). Acetylcholine release from airway cholinergic nerves may be modulated by a number of factors, including ACh, prostanoids, catecholamines, histamine, adenosine, serotonin, neuropeptides, and nitric oxide. Some of these factors are often found at abnormal levels in asthma and some other types of airway obstruction; therefore, they may contribute to the development of airway obstruction by augmenting ACh release.

Prejunctional receptors are frequently classified as auto- or heteroreceptors depending on the origin of the modulator. Autoreceptors respond to the neuron's own transmitter. They are involved in negative or positive feedback modulation, whereas heteroreceptors respond to agonists other than the neuron's own transmitter. Heteroreceptors are further divided according to the agonists with which they interact. In some nomenclatures, the term "homoreceptor" is also included. However, it is not uniformly defined (Vizi et al. 1991; Kalsner 1990a).

In this review, I summarize the current knowledge about modulation of airway cholinergic neurotransmission and discuss the possible relationship between defective regulation of airway cholinergic neurotransmission and airway obstruction.

Modulation by prejunctional muscarinic receptors

Muscarinic receptors are not homogenous. As many as five different receptor proteins have been cloned. By the use of selective muscarinic antagonists, the existence of at least three functionally different subtypes has been recognized. Muscarinic receptors with a high affinity for pirenzepine are called M₁ receptors, those with a high

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affinity for methoctramine and AF-DX 116 are called M_2 receptors, and those with a high affinity for 4-diphenyl acetoxy N-methyl piperidine (4-DAMP) and hexahydro-siladifenidol are called M_3 receptors (Minette and Barnes 1990). Most evidence suggests that, in the airways, functional M_3 receptors are present on smooth muscle cells, while functional M_1 and M_2 receptors are present on prejunctional sites. Activation of M_3 receptors leads to muscle contraction, while activation of M_1 and M_2 receptors modulates ACh release from cholinergic nerves (Minette and Barnes 1990; Barnes 1989b).

M_1 receptors may be present in airway parasympathetic ganglia. In atopic subjects, pirenzepine inhibits bronchoconstriction induced through reflex activation of cholinergic nerves by sulfur dioxide but not that induced by methacholine, whereas the nonspecific muscarinic antagonist ipratropium was able to block both responses. These results suggest that pirenzepine is not acting directly on airway smooth muscle but on some part of the cholinergic reflex pathway (Lammers et al. 1989). This is likely to be the parasympathetic ganglia. Autoradiographic studies demonstrated a high density of muscarinic receptors in human airway parasympathetic ganglia (van Koppen et al. 1988). In in vitro studies with rabbit airways, pirenzepine has been shown to be more effective in blocking contractions induced by pre- than post-ganglionic vagal nerve stimulation, suggesting also that the M_1 receptors are most likely on parasympathetic ganglia (Bloom et al. 1988).

Acetylcholine may exert a negative feedback effect on its own release by acting on the autoreceptors on the airway cholinergic nerves. The muscarinic agonists oxotremorine, carbachol, and pilocarpine concentration-dependently inhibit the electrically evoked ACh release in guinea pig trachea (D'Agostino et al. 1990). The

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non-specific muscarinic antagonist atropine (Baker and Brown 1991; D'Agostino et al. 1990) and antagonists with relatively high affinity for the M_2 receptors, such as methoctramine (Baker and Brown 1991), AF-DX116 (Kilbinger et al. 1991; Loenders et al. 1992a) and AQ-RA741 (Kilbinger et al. 1991), facilitate ACh release from airway cholinergic nerves in guinea pig, dog, and rabbit trachea. In guinea pigs, gallamine potentiates the airway constriction induced by either preganglionic or postganglionic stimulation, but it does not influence the airway constriction produced by intravenous injection of ACh. The selective M_2 receptor agonist pilocarpine has the opposite effect to gallamine. The inhibitory effect of pilocarpine is antagonized by gallamine. Because gallamine and pilocarpine affect the responses to postganglionic stimulation but not to ACh, the inhibitory muscarinic receptors must be located on the postganglionic neurons of the airway parasympathetic nerves (Faulkner et al. 1986). Similar results have been obtained with feline bronchial smooth muscle (Blaber et al. 1985).

Not all the evidence is in favor of the notion that muscarinic autoreceptors on airway cholinergic nerves belong to the M_2 subtype. MacLagan and Faulkner (1989) and Kagaya et al. (1992) were unable to demonstrate the presence of prejunctional M_2 receptors in rabbit and canine airways by comparing the effects of muscarinic receptor agents on bronchoconstriction induced by vagal stimulation and intravenous ACh. This is in contrast to the evidence obtained by direct measurements of ACh release (Loenders et al. 1992a; Baker and Brown 1991). Some investigators have proposed that M_1 (Janssen and Daniel 1990b) or M_3 (Deckers et al. 1989b) rather than M_2 receptors are inhibitory muscarinic receptors on airway cholinergic nerves.

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Alterations in muscarinic receptors could occur in diseases with airway obstruction or hyperresponsiveness. It has been reported that pilocarpine inhibits reflex bronchoconstriction induced by sulfur dioxide (SO₂) in normal but not in asthmatic subjects, suggesting that feedback inhibitory muscarinic receptors may be present on cholinergic nerves in normal airways and that there may be a dysfunction of this feedback mechanism in asthmatic airways (Minette et al. 1989). Ayala and Ahmed (1989) reported that prior muscarinic stimulation with methacholine has a protective effect on histamine-induced bronchoconstriction in normal but not asthmatic subjects. The absence of this inhibitory effect in asthmatic patients may represent loss of a protective muscarinic receptor mechanism. Fryer et al. (1990) reported that pulmonary infection with parainfluenza virus reduces the agonist affinity for muscarinic (probably M₂ subtype) receptors in guinea pig lung and this decreased agonist affinity may be responsible for the vagally induced bronchoconstriction seen in viral respiratory infections. Fryer and Wills-Karp (1991) observed that ovalbumin challenge causes dysfunction of M₂ muscarinic receptors in guinea pig pulmonary parasympathetic nerves.

The reason why muscarinic autoreceptors may be dysfunctional in asthma and some other types of airway hyperresponsiveness is not known. There is evidence that, in guinea pigs, the dysfunction of muscarinic receptors during viral infection is due to the neuraminidase activity of the virus and that induced by ovalbumin challenge may be related to the eosinophil major basic protein, which is an endogenous allosteric M₂ receptor antagonist (Jacoby et al. 1992). It is possible that inflammation may also damage other subtypes of muscarinic receptors in the lung such as the M₃ receptors on

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airway smooth muscle cells. However, because these receptors are in excess ("spare receptors"), little change can be detected.

Modulation by sympathetic nerves and circulating catecholamines

Airway cholinergic neurotransmission can be inhibited by activation of certain types of prejunctional adrenergic receptors. This effect has been demonstrated in human, dog, and guinea pig airways.

Inhibitory adrenoceptors on human airway cholinergic nerves probably belong to the α_2 and/or β_2 subtypes. In experiments with human bronchi, Grundström and Andersson (1985) observed that exogenous norepinephrine (in the presence of propranolol and cocaine to block β -receptors and neuronal uptake of norepinephrine, respectively) inhibits EFS-evoked bronchial contractions that are atropine-sensitive. The inhibition was antagonized by the α_2 -adrenoceptor antagonist yohimbine, whereas the α_1 -adrenoceptor antagonist prazosin was ineffective. Contractions evoked by exogenous ACh were unaffected by the addition of norepinephrine. The results suggest that human bronchial cholinergic neurotransmission can be inhibited by stimulation of prejunctional α_2 -adrenoceptors. Rhoden et al. (1988) reported that isoproterenol, epinephrine, and norepinephrine (in that order of potency) produced concentration-dependent inhibition of human bronchial contractions induced by either EFS or ACh, and the inhibitory effect was 10-100 fold stronger on EFS-induced contractions. The inhibitory effects of isoproterenol on responses to EFS were prevented by propranolol and ICI 118551 (a β_2 -antagonist) but not by betaxolol (a β_1 -antagonist). These experiments suggest modulation of cholinergic neurotransmission by prejunctional β_2 -receptors.

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In dog airways, inhibitory β receptors seem to be important in modulation of cholinergic neurotransmission. Janssen and Daniel (1990a) reported that isoproterenol or salbutamol inhibits the contractions and amplitude of excitatory junction potentials (EJPs) of canine bronchial smooth muscle induced by EFS but, when used at concentrations sufficient to eliminate EJPs, has little or no effect on resting membrane potential or on carbachol-induced depolarization. These inhibitory effects are blocked by propranolol or timolol, as well as by the selective antagonists ICI 89,406 (β_1 -selective) and ICI 118,551 (β_2 -selective). They conclude that catecholamines act on prejunctional β_1 and β_2 receptors, leading to inhibition of cholinergic neurotransmission in canine bronchi. Ito (1988) reported that procaterol, a β_2 -adrenoceptor stimulant, dose-dependently reduces the amplitudes of the twitch contractions and the EJPs evoked by EFS in the dog trachea with no effect on the postjunctional response of smooth muscle cells to exogenous ACh. Pretreatment with ICI-118,551 reduced the inhibitory action of procaterol on the amplitude of the twitch contractions. This study indicates that prejunctional β_2 receptors are important in modulation of cholinergic neurotransmission in canine trachea. But Martin and Collier (1986), using a radioenzymatic method to directly measure ACh release, reported that ACh release from canine isolated airway is not modulated by norepinephrine. Failure to observe an effect of norepinephrine is probably due to the fact that norepinephrine is mainly an α -receptor agonist, whereas the inhibitory adrenoceptors on canine airway cholinergic nerves belong to the β type.

In a study with guinea pig tracheal rings, Grundström et al. (1981a) reported that in the presence of β -blocking drugs, norepinephrine and epinephrine dose-dependently inhibited contractions induced by cholinergic nerve activation. By contrast, contractions

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induced by exogenous ACh were unaffected. In order to characterize the norepinephrine receptor, the effects of α_1 and α_2 blockers were evaluated using Schild plot. The results indicate that, in guinea pig trachea, norepinephrine inhibits cholinergic neurotransmission by acting on prejunctional α_2 receptors. The results of two other studies with guinea pig trachea tissue also suggest that α_2 receptors are the inhibitory receptors on guinea pig tracheal cholinergic nerves (Jacobsson et al. 1991; Kamikawa and Shimo 1990).

There are two possible sources of the physiologic agonists for these prejunctional inhibitory adrenoceptors. One source is NE released by airway sympathetic nerves. 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. In humans, few, if any, adrenergic fibers have been demonstrated in smooth muscle of intrapulmonary airways. Although there appears to be no direct functional sympathetic innervation of human airway smooth muscle, it is possible that adrenergic nerves may influence bronchomotor tone indirectly, e.g., by modulating cholinergic neurotransmission. Adrenergic nerves have been demonstrated within human airway ganglia. Norepinephrine inhibits the firing of airway ganglion cells in cat and ferret. In dogs, electrical stimulation of thoracic sympathetic nerves causes bronchodilation, which is abolished by β -antagonists and unaffected by adrenalectomy. The magnitude of bronchodilation induced by sympathetic nerve stimulation is dependent on the degree of preexisting vagal tone (Barnes 1986). These lines of evidence suggest that NE released by the sympathetic nerves may modulate airway cholinergic neurotransmission.

The other possible source is circulating catecholamines. Norepinephrine in plasma is derived almost entirely from overspill of sympathetic nerve activity, while

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epinephrine is secreted by the adrenal medulla. Infusions of NE in normal subjects have no significant effects on cardiovascular or airway function at concentrations that are within the physiologic range, suggesting that NE does not function as a circulating hormone in humans. By contrast, epinephrine has potent metabolic effects within its physiologic range and is a potent bronchodilator in normal and asthmatic subjects. Therefore, epinephrine is probably the catecholamine hormone that is important in modulating airway cholinergic neurotransmission, and such an effect may be more likely if cholinergic neurotransmission is increased (Barnes 1986).

There is increasing evidence suggesting that the secretion of epinephrine may be abnormal in asthma. Exercise causes a rise in circulating catecholamines in normal subjects, but in asthmatics who perform an exercise test sufficient to induce bronchoconstriction, there is a blunted rise in NE and no rise in epinephrine (compared with a 3-fold rise in normal subjects who perform the same task) (Barnes et al. 1981; Warren et al. 1982). It has been demonstrated that antigen challenge results in marked β -adrenoceptor desensitization in guinea pig trachea, as indicated by the reduced relaxing effect of epinephrine and decreased binding of ^{125}I -cyanopindolol (Daffonchio et al. 1990). Although this observation is mainly a reflection of β receptors on airway smooth muscle, it is possible that prejunctional β -receptors could be impaired through a similar mechanism.

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Modulation by prostanoids

Airway cholinergic neurotransmission may be modulated by prostanoids. Prostaglandins of the E series (PGEs), particularly PGE₂, appear to be the most important prostanoids in this respect.

It is fairly well established that PGEs inhibit canine airway cholinergic neurotransmission in spite of the existence of some conflicting data. Deckers et al. (1989a) reported that the cyclooxygenase inhibitor indomethacin enhances ACh release from canine bronchial tissue in response to EFS and this effect is reversed by the addition of PGE₂. Waters et al. (1984) observed that contractile responses of canine tracheal smooth muscle to EFS diminished over a two-hour period of incubation in tissue bath. Addition of indomethacin reversed this inhibition of contractile response. Measured PGE₂ increased in the tissue bath over two hours in control tissues. Addition of PGE₂ to the tissue produced similar inhibition of contractile responses to EFS in a concentration-dependent manner. In contrast, incubation alone, treatment with indomethacin, or addition of PGE₂ had little, if any, effect on contraction induced by exogenous ACh. Ito and Tajima (1981a) observed that the EJPs induced by cholinergic nerve activation in canine tracheal smooth muscle showed gradual and continuous reduction in amplitude during prolonged exposure in Krebs solution (1–2 hours). Indomethacin pretreatment prevented this reduction. Low concentrations (10⁻⁷–10⁻⁸ M) of PGE₁ or PGE₂ markedly suppressed the amplitude of EJP in indomethacin-pretreated tissue. However, Daniel et al. (1987) were unable to identify an inhibitory effect of endogenous cyclooxygenase products on canine airway cholinergic neurotransmission.

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Shore et al. (1987) observed only a trivial inhibitory effect of PGE₂ (10⁻⁸ M) on ACh release from cholinergic nerves innervating dog trachealis.

Evidence about the effect of PGE₂ on airway cholinergic neurotransmission in other species is very limited. In the horse, meclofenamate, a cyclooxygenase inhibitor, augments the EFS-induced contractions of trachealis strips and bronchial rings to a greater extent than the exogenous ACh-induced contractions, suggesting that endogenous prostanoids inhibit airway cholinergic neurotransmission (Yu et al. in press a). In human bronchi, low concentrations of PGE₂ suppressed the amplitude of twitch contractions and EJPs evoked by EFS but had no effect on the contractile response to exogenous ACh. This observation suggests that PGE₂ has an inhibitory effect on cholinergic neurotransmission in human bronchi. However, Black et al. (1989) were unable to demonstrate such an effect of PGE₂ in human bronchial tissues.

There is only limited information about the effects of other prostanoids on airway cholinergic neurotransmission. Exogenous carbocyclic thromboxane A₂ (cTXA₂) inhibits the excitatory neuro-effector transmission in the dog trachea, presumably by inhibiting the release of ACh from the vagal nerve terminals (Inoue and Ito 1985). Thromboxane A₂-mimetic, U46619, enhanced the contractile response of canine bronchial rings to EFS but not to methacholine (an ACh analogue) and may therefore increase the prejunctional release of ACh (Chung et al. 1985). Tamaoki et al. (1987) observed that prostaglandin D₂ increased the contractile response of canine bronchial rings to EFS but did not affect the response to administered ACh, suggesting that PGD₂ potentiates ACh release. However, Deckers et al. (1989a) did not detect a modulatory effect of PGD₂ on ACh output from canine bronchial tissue by direct measurement of ACh release. This

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discrepancy may be due to the very low concentrations of PGD₂ applied in their study. An inhibitory effect of PGI₂ on airway cholinergic neurotransmission has also been observed (Shore et al. 1987; Inoue and Ito 1985).

Approximately 5–10% of asthmatics experience profound bronchoconstriction 30 minutes to several hours after taking aspirin or other nonsteroidal antiinflammatory drugs (Robinson and Holgate 1991). A few days after indomethacin injection, some dogs begin coughing and wheezing (Ito and Tajima 1981b). Both of these effects might be due to a decrease in certain inhibitory prostanoids following cyclooxygenase inhibition. In asthmatics, histological lesions are common with airway epithelium (Dunnill 1960; Laitinen et al. 1985a), which is thought to be an important source of PGE₂ in the airways (Ozaki et al. 1987). In horses with heaves, a chronic obstructive pulmonary disease, the airway subepithelial tissue produces significantly less PGE₂ in response to stimulation by A23187 and bradykinin than that of the control animals (Gray et al. 1992a). In humans with atopic asthma, bronchial washings contained increased concentrations of PGD₂, its metabolite 9 α , 11 β -PGF₂, and TXB₂ (Robinson and Holgate 1991). These lines of evidence suggest that alterations in prostanoid metabolism are likely to influence airway resistance due to changes in ACh release.

Modulation by histamine

Histamine is a biogenic amine that is found in large quantities in lung mast cells. It is an important mediator of allergic bronchoconstriction and airway inflammation. Plasma histamine level is elevated in asthmatics. Histamine induces bronchoconstriction through numerous mechanisms. When inhaled or intravenously injected, it induces

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bronchospasm and airway microvascular leakage. In vitro, it increases the secretion of mucus glycoproteins and has chemotactic activity for eosinophils (Chand et al. 1990; Chung 1990).

Three types of histamine receptors have been recognized: H_1 , H_2 , and H_3 . All probably modulate cholinergic neurotransmission. In isolated canine trachea, histamine potentiates the tracheal contraction induced by EFS. Direct measurement of ACh release by the radioimmunoassay method showed that the ACh release induced by EFS is augmented by histamine. The augmenting effect is blocked by the H_1 receptor antagonist chlorpheniramine (Tsuchiya et al. 1990). These findings suggest that histamine, through H_1 -receptors, may prejunctionally augment the release of ACh.

In another study, the histamine H_3 -receptor agonist (R)- α -methylhistamine (α -MeHA) caused a dose-dependent inhibition of vagally mediated contraction of a guinea pig tracheal tube preparation but did not alter tracheal contraction induced by exogenously applied ACh. Blockade of H_1 - and H_2 -histamine receptors, and α - and β -adrenoceptors did not prevent the inhibitory effect of α -MeHA, whereas the specific H_3 -receptor antagonist thioperamide prevented the effect of α -MeHA. In the presence of H_1 - and H_2 -receptor antagonists, histamine also inhibited vagally mediated tracheal contraction (Ichinose et al. 1989). These results suggest that prejunctional H_3 -receptors inhibit cholinergic neurotransmission in the guinea pig airways. There is evidence that inhibitory histamine H_3 -receptors are also present on postganglionic cholinergic nerves in human airways (Ichinose and Barnes 1989).

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The effect of H₂ receptors on cholinergic neurotransmission in the airways is not documented. But there is evidence that H₂-receptor activation results in depressed ACh release from the superior cervical ganglion of the rat (Snow and Weinreich 1987).

Although all of the three types of histamine receptors are probably involved in the regulation of ACh release from cholinergic nerves, the relative importance of each type of receptor may vary between cholinergic nerves innervating different organs and between different species. Because of the scantiness of evidence, it is too early to precisely speculate on their role in controlling ACh release from airway cholinergic nerves under physiological and pathological conditions.

Modulation by adenosine and adenosine nucleotides

There are two types of purinergic receptors. P₁ receptors ("adenosine receptors") are most sensitive to adenosine and adenosine monophosphate (AMP). P₂ receptors ("ATP receptors") are most sensitive to adenosine di- and triphosphate (ADP and ATP) (Steinberg 1990; Burnstock 1988). The P₁ receptors are further subclassified as A₁ and A₂ receptors, originally based on the change in cAMP levels after receptor activation by adenosine. In cells that possess A₁ receptors, adenosine decreases intracellular cAMP by interacting with the G_i regulatory protein; conversely, adenosine increases intracellular cAMP levels in cells bearing A₂ adenosine receptors, which stimulate G_s (Steinberg 1990).

Numerous lines of evidence indicate that adenosine modulates neurotransmission. Most of the results were obtained from the central nervous system and peripheral tissues other than the airways (Fredholm and Hedqvist 1980). There is only limited information

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about the modulatory effect of adenosine on airway cholinergic nerves. Aas et al. reported that adenosine inhibits potassium-induced release of [^3H]ACh from rat bronchial smooth muscle. This effect is antagonized by the P_1 receptor antagonist 8-phenyltheophylline (Aas 1990; Aas and Fonnum 1986). The A_2 receptor agonist NECA facilitates the ACh release and A_1 receptor agonist R-PIA does not affect it, indicating that the inhibition of [^3H]ACh release by adenosine in the airway smooth muscle has to be due to stimulation of a prejunctional receptor different from the A_2 receptor (Aas 1990). It is unclear whether this inhibitory effect of adenosine on ACh release from airway cholinergic nerves is also present in other animal species and human beings.

Adenosine and adenosine nucleotides are released from both nerve endings and postjunctional structures as a consequence of nerve activity and the latter source seems to be the more important. Like adenosine, adenosine nucleotides (ATP, ADP, and AMP) also inhibit neurotransmission and they are probably as potent as adenosine (Fredholm and Hedqvist 1980). Adenosine triphosphate is probably a transmitter coreleased with NE from sympathetic nerves (Burnstock 1988). Evidence obtained in some non-airway smooth muscles suggests that adenosine is probably an inhibitory neurotransmitter of the NANC system (Small et al. 1990).

Very little is known about the mechanism by which adenosine inhibits transmitter release. It seems clear that the presynaptic effects of adenosine are not mediated via cyclic AMP (Fredholm and Hedqvist 1980).

The significance of the inhibitory effect of adenosine on ACh release from airway cholinergic nerves in airway hyperresponsiveness and disease is unknown. There are hints that adenosine and purinoceptors may be of importance in the airways. Inhalation



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of aerosolized adenosine causes a concentration-related bronchoconstriction in asthmatic patients but not in normal subjects (Cushley et al. 1983; Holgate et al. 1984; Marmo et al. 1985). Theophylline, a competitive antagonist of cell surface purinoceptors in concentrations within the therapeutic range of asthma therapy, inhibits bronchoconstriction induced by adenosine (Cushley et al. 1984; Mann and Holgate 1985). Adenosine also enhances antigen-induced bronchoconstriction and histamine release in rat isolated lungs (Post et al. 1990).

Modulation by vasoactive intestinal peptide and nitric oxide

Both VIP and NO appear to be neurotransmitters or mediators of the inhibitory NANC nerves in the lung although their relative importance varies between species. Vasoactive intestinal peptide and NO not only have a direct relaxant effect on airway smooth muscle (Palmer et al. 1986; Munakata et al. 1990) but also may inhibit airway cholinergic neurotransmission.

Vasoactive intestinal peptide inhibits cholinergic neurotransmission in the airways of ferrets, guinea pigs, and cats (Sekizawa et al. 1988; Martin et al. 1990; Xie et al. 1991). Sekizawa et al. (1988) observed that in isolated ferret tracheal segments, low concentrations of VIP (up to 10^{-9} M) potentiate the response to EFS and higher concentrations of VIP reduce the response. Contractions produced by ACh were not affected by VIP at concentrations of 10^{-9} and 2×10^{-7} M. They concluded that VIP modulates contractions produced by EFS via presynaptic cholinergic mechanisms. Martin et al. (1990) reported that, in an isolated innervated guinea pig tracheal preparation, exogenous VIP dose-dependently inhibits the contractile response to vagal

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nerve stimulation (NS) and EFS. Vasoactive intestinal peptide (2.5×10^{-8} , 2.5×10^{-7}) inhibits contractions induced by NS significantly more than EFS at 1 and 5 but not 20 Hz. In contrast, it does not significantly reduce responses to ACh. They concluded that in the isolated guinea pig tracheal preparation, VIP can modulate cholinergic neurotransmission by actions that are predominantly on postganglionic nerves, but it has also a small additional effect on ganglionic transmission. Xie et al. (1991) observed that VIP antagonists [AC-Tyr¹, D-Phe²]-GRF(1-29)-NH₂ and [4-Cl-D-Phe⁶, Leu¹⁷]-VIP dose-dependently enhance the amplitude of EJPs evoked by a single stimulus in the cat but not dog trachea. Neither VIP antagonist has any effect on the post-junctional response of smooth muscle cells to exogenously applied ACh in the dog or cat trachea. Their results indicate that both VIP antagonists have a prejunctional action accelerating the excitatory neuroeffector transmission, presumably by enhancing transmitter release from the vagus nerves in the cat, but not in the dog trachea. An inhibitory effect of endogenous VIP on airway cholinergic neurotransmission has also been proposed based on the observation that α -chymotrypsin, a proteolytic enzyme which degrades VIP, enhances the contractile response of guinea pig tracheal smooth muscle to EFS without affecting the response to exogenous ACh (Belvisi et al. 1993).

A modulatory effect of nitric oxide on airway cholinergic neurotransmission has been demonstrated in guinea pigs. The nitric oxide synthase inhibitor L-N^G-Nitro-arginine methyl ester (L-NAME) enhances the contractile responses of guinea pig trachea to EFS but not to exogenous ACh. The effects of L-NAME are reversed by L-arginine but not D-arginine (Belvisi et al. 1991; Belvisi et al. 1993). These results suggest that nitric oxide released by nerve stimulation inhibits cholinergic neurotransmission in guinea

pig trachea. This action of nitric oxide is in contrast to that observed in the rat basal forebrain where nitric oxide enhances rather than inhibits the release of ACh from neurons (Prast and Philippu 1992).

Inhibitory NANC nerves have been demonstrated in vitro in the airways of several species, including humans, horses, guinea pigs, pigs and cats. It appears to be the sole inhibitory neural pathway in human airway smooth muscle (Barnes 1986). The demonstration of the inhibitory effects of endogenous VIP and NO on airway cholinergic neurotransmission suggests that VIP and NO may act as functional "brakes" on cholinergic bronchoconstriction. In diseases with airway obstruction or hyperresponsiveness, the inhibitory NANC innervation is frequently abnormal. For example, Broadstone et al. (1991) reported that a functional NANC inhibitory innervation is present in the bronchi of the control but not heavy horse. Ollerenshaw et al. (1989) reported that immunoreactive VIP is present in lung tissue of normal humans but absent in that of asthmatic patients. Miura et al. (1992) observed that the airway dilatory response to activation of inhibitory NANC nerves was significantly inhibited in antigen-sensitized cats. These observations suggest that an increased ACh release due to abnormalities in the inhibitory NANC innervation is perhaps a mechanism in the development of airway obstruction.

Modulation by other factors

Besides the above-mentioned factors, there are others that have also been reported to be modulatory on airway cholinergic neurotransmission. Opioids (Belvisi et al. 1992), 5-hydroxytryptamine (Hahn et al. 1978), substance P (Tanaka and Grunstein 1990),

somatostatin (Sekizawa et al. 1989), and L-glutamate (Aas 1990) potentiate airway cholinergic neurotransmission. Neuropeptide Y (Grundemar et al. 1988), L-aspartate, D-glutamate, L- α -amino adipate (Aas 1990), and furosemide (a diuretic with bronchodilating effect) (Yu et al. 1992b) inhibit airway cholinergic neurotransmission. The modulatory effect of these factors is not discussed in detail in this review because the evidence is very limited.

The majority of our information about modulation of airway cholinergic neurotransmission has been gathered in the past five years. Most evidence is obtained by comparing the contractile responses of airway smooth muscle to exogenous ACh and to electrical activation of nerves, which releases endogenous ACh. As indicated in the introduction of my dissertation, such an approach has its limitations. Direct measurement of ACh release from airway cholinergic nerves is the most reliable way to monitor changes in airway cholinergic activity. Although this had been considered technically difficult (Barnes 1991), a few groups of investigators have succeeded in measuring ACh release over the last few years. The techniques used by these investigators to directly measure ACh release include HPLC coupled with electrochemical detection (Deckers et al. 1989a; Baker and Brown 1991), a radioenzymatic method (Martin and Collier 1986; Shore et al. 1987), radioimmunoassay (Tsuchiya et al. 1990), and monitoring tritium outflow from airway smooth muscle after preincubation with [^{3} H]choline (D'Agostino et al. 1990; Kilbinger et al. 1991). Although each of these techniques has its advantages and disadvantages, the HPLC plus electrochemical detection technique and the tritiated technique seem to be gaining popularity in studies of ACh release for their overall specificity, sensitivity, and simplicity. A judicious use of these techniques would greatly

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expand our knowledge about prejunctional modulation of airway cholinergic neurotransmission.

Heaves

In horses and ponies, there is a syndrome colloquially called "heaves" that is characterized by recurrent airway obstruction. Clinical signs of heaves include dyspnea, cough, exercise intolerance, and intermittent or chronic purulent nasal discharge. During airway obstruction, animals have increased R_L , increased $\Delta P_{pl_{max}}$ during tidal breathing, decreased C_{dyn} , and hypoxemia. Synonyms for heaves include chronic obstructive pulmonary disease, recurrent airway obstruction, broken wind, chronic bronchitis, chronic bronchiolitis, and equine pulmonary emphysema.

The occurrence of heaves is sporadic and the size of the population affected varies widely in different regions. It is common in temperate parts of the world where horses and ponies are stabled for a large part of their lifetime but rare in warmer parts where the animals are kept on pasture year-round. Affected animals experience acute exacerbations of heaves when stabled and fed poor-quality hay, and enter remission at pasture. Normal equids do not develop heaves in the same environment.

Etiology

Heaves apparently has an allergic etiology. Although several factors, such as viral infection (Gerber 1973) and pneumotoxin ingestion (Breeze et al. 1978), have been proposed as the cause of heaves, exposure to environmental antigens in the barn (Breeze 1979) is most frequently associated with this syndrome.

Lowell (1964) was probably the first person to report a relationship between the acute exacerbations of heaves and the feeding of hay. He suggested that the syndrome was precipitated by the inhalation of "heaves producing factors" in hay. A variety of allergens can be found in hay (Halliwell et al. 1979; Woods et al. 1993). However, the two most commonly implicated antigens are *Micropolyspora faeni* and *Aspergillus fumigatus*, and the greatest number of heavey cases are associated with *M. faeni*. Antibodies against these two antigens are frequently detected in the serum of affected horses (Lawson et al. 1979). Many affected horses have a positive skin test in response to these antigens (McPherson et al. 1979a). *M. faeni* is also associated with farmer's lung in humans (Fink 1976), a form of hypersensitivity pneumonitis. Factors that increase environmental contamination with these antigens, such as inadequate ventilation or feeding poor-quality hay, are associated with an increased incidence of heaves (McPherson et al. 1979b).

However, an allergic response to environmental antigens is unlikely to be the sole cause of the disease. Positive skin tests and serum antibodies against *M. faeni* and *A. fumigatus* are not restricted to animals with heaves, while animals without positive skin test or the antibodies develop clinical signs characteristic of heaves (Lawson et al. 1979; McPherson et al. 1979a).

Pathology

The lungs of heavey horses euthanized during an acute exacerbation of the syndrome hardly collapse upon opening of the thoracic cavity and have a much larger volume than lungs of normal horses. These changes are probably results of gas-trapping

due to airway obstruction. Excessive mucus is often present throughout the bronchial tree.

Information about pulmonary histological changes in heavey horses is limited. Published descriptions of the pulmonary lesions vary considerably because heaves is not a specific disease identity but a syndrome that occurs in a variety of diseases. Major morphologic changes, which are most apparent in airways less than 2 mm in diameter, include chronic bronchiolitis marked by epithelial hyperplasia and metaplasia; plugs of mucus and/or neutrophils in the lumina of small airways; peribronchiolar fibrosis and cellular infiltration by lymphocytes, plasma cells and mast cells; acinar overinflation; eosinophil infiltration of bronchiolar walls and lumina; and alveolar emphysema (Breeze 1979; Thurlbeck and Lowell 1964). These lesions are not all found in every case, nor are they always present to the same degree.

Examination of the bronchoalveolar lavage fluid reveals that there is a neutrophil influx into the airways during acute exacerbation of heaves. Exposure of normal animals to the barn environment does not change airway cell populations (Derksen et al. 1985c).

Pulmonary function

Pulmonary resistance, $\Delta P_{pl_{max}}$ during tidal breathing, and C_{dyn} are the three most commonly used parameters for determining lung function in heaves. Heavey horses in clinical remission have a R_L similar to that of normal horses. When horses are brought from pasture to a barn, airway obstruction occurs in heavey but not normal horses. During airway obstruction, R_L and $\Delta P_{pl_{max}}$ increase, and C_{dyn} decreases. These

parameters of lung function return to remission levels in 1-2 weeks when heavy horses are brought back to pasture (Derksen et al. 1985a).

The increase in $\Delta P_{pl_{max}}$ is a result of both a decrease in intrapleural pressure during inspiration and an increase in intrapleural pressure during expiration. The increased $\Delta P_{pl_{max}}$ is necessary to generate airflow through the partially obstructed airways. Gray et al. (1992a) reported that $\Delta P_{pl_{max}}$ (mean $\pm$ SE) is 6.3 ± 0.4 and 7.6 ± 0.3 cm H₂O in normal and heavy horses kept at pasture, respectively. When horses are stabled, $\Delta P_{pl_{max}}$ rises to 36.1 ± 4.9 cm H₂O in heavy horses but remains constant in normal horses (6.7 ± 0.4 cm H₂O).

Pulmonary resistance is the sum of airway resistance and lung-tissue resistance, and is determined from the simultaneous measurements of the flow and the pressure difference between the airway opening and the pleural cavity. Because the cross-sectional area of airways increases as the airways bifurcate, more than 80% of the airway resistance is localized in the trachea and larger bronchial branches, whereas the resistance of the bronchioles constitute less than 20% of the total resistance (Macklem and Mead 1967). Therefore, the R_L value reflects mainly the resistance of larger airways. Results of a previous study indicate that, during acute exacerbation of heaves, the R_L is 2.42 ± 0.26 cm H₂O/L/s, which is more than twice the value during clinical remission (0.91 ± 0.09 cm H₂O/L/s). The R_L does not change significantly when normal horses are transferred from pasture to a barn (0.63 ± 0.11 versus 0.87 ± 0.10 cm H₂O/L/s) (Gray et al. 1992a).

Lung compliance is defined as the lung volume change per unit of pressure change across the lung. It can be determined either during breath holding (static

compliance) or during a normal breathing cycle (C_{dyn}). Static compliance defines the static volume-pressure relationships of the lungs whereas C_{dyn} is the ratio of tidal volume to the $\Delta P_{pl_{max}}$ occurring between the beginning and end of a tidal volume. In normal lungs, static and dynamic compliance should have the same value at normal respiratory frequencies. In lungs with small airway obstruction, C_{dyn} is less than static compliance, particularly at rapid respiratory frequencies (Robinson 1979). Dynamic compliance decreases in heavy horses during periods of airway obstruction (1.23 ± 0.18 versus 0.31 ± 0.06 L/cm H₂O during remission and airway obstruction, respectively) (Gray et al. 1992a).

During acute exacerbations of heaves, diffuse airway obstruction also leads to ventilation and perfusion mismatching, which mainly impedes oxygen uptake in the lung and has little influence on CO₂ removal due to the difference in the characteristics of the O₂ and CO₂ dissociation curves. As a result, heavy horses are hypoxemic with only mild hypercapnia or no hypercapnia (Gray et al. 1992a).

Mechanism of airway obstruction

The airway obstruction of heavy horses is due to a combination of excessive mucus and exudates in the airway lumen, edema of the airway wall, and bronchospasm. The proportion of airway obstruction contributed by bronchospasm varies widely not only between different horses but also between different days in the same horse. Nevertheless, bronchospasm is generally responsible for a major proportion of the obstruction as indicated by the marked and immediate reduction in R_L caused by bronchodilators (Broadstone et al. 1988; Broadstone et al. 1992). The mechanism of bronchospasm in

heavy horses is unclear. Some factors that are probably related to the bronchospasm are discussed below:

Airway hyperresponsiveness—Airway responsiveness is the ease with which a stimulus induces airway narrowing. During airway obstruction, airways of heavy horses and ponies are hyperresponsive to a variety of stimuli including aerosol and intravenous histamine, aerosol methacholine, aerosol citric acid, and aerosol water (Armstrong et al. 1986; Derksen et al. 1985a; Derksen et al. 1985b; Obel and Schmitterlow 1948). This nonspecific hyperresponsiveness wanes when animals are brought back to the pasture. The hyperresponsiveness makes it possible for a small amount of mediators or neurotransmitters to induce airway obstruction. In vitro, however, airway smooth muscle preparations from heavy horses are hypo- rather than hyperresponsive to exogenous ACh compared with those from control horses (Broadstone et al. 1991). Therefore, the in vivo hyperresponsiveness of heavy horse airways probably results from changes other than that of the smooth muscle cells. Some of the possible mechanisms for the in vivo hyperresponsiveness follow. 1) The neurohumoral environment may facilitate airway smooth muscle contraction in the lungs of heavy horses. 2) There may be an increased thickness of airway wall due to edema or infiltration of inflammatory cells. When airway smooth muscle contracts, the outside diameter of the airway decreases, but the wall cross-sectional area does not change. As a result, the lumen of the airway narrows to a much greater extent when airway walls thickened (Moreno et al. 1986). 3) The lung parenchyma tethers the airways so that the airways do not close. This phenomenon, called "interdependence," pulls open the airways as the lung inflates (Ding et al. 1987). Peribronchial edema and infiltration of inflammatory cells may unlink the

interdependence between the airway and the surrounding parenchyma (Macklem 1989) and, therefore, increase the responsiveness of airway smooth muscle to bronchoconstrictors.

Pulmonary neutrophilia—In ponies with a history of heaves, barn exposure results in increased neutrophils in bronchoalveolar lavage fluid. This phenomenon does not occur in normal ponies (Derksen et al. 1985c). Evidence obtained in other species indicates that neutrophils produce mediators, such as platelet activating factor (Borgent and Samuelsson 1979), prostaglandins (Doldstein et al. 1977), free radicals (Kanazawa et al. 1991) and leukotrienes (Wang et al. 1986), that can induce bronchoconstriction. Furthermore, the oxygen radicals produced by neutrophils may lead to airway hyperresponsiveness (Lansing et al. 1993). Therefore, pulmonary neutrophilia could be a contributing factor to the development of airway obstruction in heavy horses. However, although aerosol challenge with *M. faeni* induces pulmonary neutrophilia in both control ponies and ponies with a history of heaves, it affects pulmonary function only in the latter (Derksen et al. 1987). The results suggest that it is not merely the presence of neutrophils that alters lung function. What is probably more important is the state of activation of the neutrophils and the mediators being released (Robinson 1989). In asthmatics, pulmonary neutrophils are probably in an activated state (Kay 1988).

Dysfunction of inhibitory NANC nerves—Inhibitory NANC innervation is the most powerful inhibitory neural pathway in normal horse airways (Yu et al. in press b). There is evidence that this inhibitory nervous system is dysfunctional in heavy horse airways: the inhibitory effect of inhibitory NANC nerves on airway smooth muscle tension can be demonstrated in the central bronchi of normal but not of heavy horses (Broadstone

et al. 1991). A dysfunction of the inhibitory NANC nerves may disturb the balance between excitatory and inhibitory innervations and favor the development of airway obstruction. However, functional inhibitory NANC nerves can be demonstrated only in the trachea and central bronchi of normal horses (Yu et al. in press b). Therefore, it is difficult to explain the diffuse airway obstruction of heavy horses simply by an dysfunction of inhibitory NANC.

Nitric oxide is the neurotransmitter or a necessary mediator of inhibitory NANC nerves in horse airways. A dysfunction of inhibitory NANC nerves may be a reflection of a general deficiency of NO, which normally is widely distributed in the lung. Since NO has an antiinflammatory effect (Kubes et al. 1991), a deficiency of NO may favor the development of airway inflammation and obstruction.

Reduced airway mucosal PGE₂ production—PGE₂ is the major prostanoid released by tracheal epithelium of normal horses (Gray et al. 1992b). In heavy horses, airway mucosal PGE₂ production is reduced (Gray et al. 1992a). PGE₂ has a direct inhibitory effect on airway smooth muscle contractions (Wang et al. 1992). Furthermore, PGE₂ inhibits ACh release from cholinergic nerves in canine airways (Deckers et al. 1989; Shore et al. 1987). Therefore, a lack of PGE₂ production could be a mechanism of airway obstruction in heavy horses.

Increased ACh release from airway cholinergic nerves—Blockade of muscarinic receptors by intravenous administration of atropine has no effect on airway resistance in the control horse or in the heavy horse during clinical remission. However, atropine markedly reduces airway resistance in heavy horses during acute exacerbations of heaves (Broadstone et al. 1988), indicating that a muscarinic mechanism is involved in

the airway obstruction. There are at least three possible mechanisms for the increased response to muscarinic receptor blockade: 1) reflex activation of the cholinergic pathway due to stimulation of sensory receptors in the airways; 2) increased sensitivity of airway smooth muscle to ACh; and 3) increased ACh release due to altered prejunctional regulation of airway cholinergic neurotransmission. Derksen et al. observed that, in ovalbumin-sensitized ponies, ovalbumin challenge of one lung does not lead to airway obstruction of the contralateral lung (Derksen et al. 1982). This observation suggests that reflex mechanism is not important in the development of ovalbumin-induced equine airway obstruction. Since heaves is also an allergic response, reflex mechanism is probably also not important in the development of airway obstruction in heaves. During acute exacerbation of heaves, the airways show a nonspecific hyperresponsiveness, suggesting that an increased sensitivity to ACh is at least in part responsible for the response to atropine. In vitro, however, isolated airway smooth muscle preparations from heavy horses are hyperresponsive to EFS but hyporesponsive to exogenous ACh compared with those from control horses (Broadstone et al. 1991), suggesting that ACh release from airway cholinergic nerves is increased. Therefore, increased ACh release from cholinergic nerves is probably also responsible for the enhanced response to atropine. The increased ACh release is most likely due to changes in prejunctional regulation of airway cholinergic neurotransmission.

Muscarinic autoreceptors are potent inhibitory receptors on airway cholinergic nerves. A dysfunction of these receptors may result in airway obstruction or hyperresponsiveness due to increased ACh release. This possibility is strongly supported by the findings that the muscarinic autoreceptors are dysfunctional in asthmatic patients

(Minette et al. 1989) and in guinea pigs infected with parainfluenza virus (Fryer et al. 1990) or challenged by ovalbumin (Fryer et al. 1991). It remains to be determined if muscarinic autoreceptors are present on equine airway cholinergic nerves and if they are dysfunctional in horses with heaves.

In canine airways, administration of exogenous PGE_2 inhibits ACh release, whereas inhibition of endogenous prostanoid production by a cyclooxygenase inhibitor augments ACh release (Deckers et al. 1989; Shore et al. 1987). It is unknown if PGE_2 receptors are present on equine airway cholinergic nerve terminals and what role they play. If inhibitory prejunctional PGE_2 receptors do exist and contribute to normal regulation of airway cholinergic neurotransmission, the decreased airway mucosal PGE_2 production in heavy horses (Gray et al. 1992a) may lead to increased ACh release.

The presence of inhibitory α_2 -adrenoceptors on cholinergic nerves has been demonstrated in the airways of humans (Grundström et al. 1981a) and guinea pigs (Grundström et al. 1981a; Thompson et al. 1990). It remains to be determined if they play a physiological role in the regulation of airway cholinergic neurotransmission. Possible physiological stimulants include NE released locally by sympathetic nerves and circulating catecholamines. The observation that adrenergic and cholinergic nerves are in close association in guinea pig trachea furnishes the anatomical possibility for the interaction between adrenergic and cholinergic nerves (Jones et al. 1980). Catecholamine levels are normally low in the plasma but increase sharply during exercise. It is unclear if the physiological levels of circulating catecholamines are high enough to modulate airway cholinergic neurotransmission. In ponies with heaves, intravenous administration of xylazine, an α_2 receptor agonist, causes bronchodilation and this effect is reduced by

the α_2 -receptor antagonist yohimbine (Broadstone et al. 1992). Although α_2 -receptors also occur on the airway smooth muscle cells of some species (Barnes et al. 1982; Takaganagi et al. 1990), their activation causes smooth muscle contraction. Therefore, the bronchodilation induced by xylazine is probably due to activation of prejunctional α_2 -receptors on airway cholinergic nerves. However, in vivo conditions are complicated. The bronchodilation induced by xylazine may well be due to its tranquilizing effect in the central nervous system. For these reasons, it is necessary to assess the importance of prejunctional α_2 -adrenoceptors on equine airway cholinergic neurotransmission in vitro.

My dissertation research investigated prejunctional modulation of equine airway cholinergic neurotransmission. Most of the research was directed towards the understanding of the physiological regulation of equine airway cholinergic neurotransmission by the prejunctional muscarinic autoreceptors, α_2 adrenoceptors, and PGE₂ receptors. Some experiments were also conducted with airway smooth muscle preparations from heavy horses to determine if the development of airway obstruction is related to a dysfunction of these prejunctional receptors. My experiments are presented in the following chapters. Chapter 2 describes the technique of measuring ACh release from airway cholinergic nerves, Chapter 3 concerns muscarinic autoreceptors on horse airway cholinergic nerves, Chapters 4 and 5 describe the effect of PGE₂ on airway cholinergic neurotransmission, Chapter 6 investigates modulation of ACh release from airway cholinergic nerves by inhibitory α_2 adrenoceptors, Chapter 7 concerns ACh release from airway cholinergic nerves in horses with airway obstruction, and Chapter 8 summarizes the major results and conclusions.

CHAPTER 2
ACETYLCHOLINE RELEASE
FROM HORSE AIRWAY CHOLINERGIC NERVES:
EFFECTS OF STIMULATION INTENSITY AND MUSCLE PRELOAD

Introduction

Knowledge about modulation of airway cholinergic neurotransmission has usually been inferred by comparing the effect of a factor on the contractile responses of airway smooth muscle to EFS and to administration of ACh. Electrical field stimulation activates nerves within the preparation and releases neurotransmitters, including ACh, whereas administration of ACh causes contraction by activation of muscarinic receptors on the muscle. A greater effect on the contractile response to EFS than to ACh indicates modulation of neurotransmission. Such indirect studies have suggested that airway cholinergic neurotransmission may be modulated by a number of factors, such as muscarinic autoreceptors (Fryer and MacLagan 1984; Minette and Barnes 1988), prostanoids (Waters et al. 1984; Wang et al. 1992), histamine (Ichinose and Barnes 1989; Ichinose et al. 1989), substance P (Tanaka and Grunstein 1990), VIP (Martin et al. 1990; Sekizawa et al. 1988), catecholamines (Grundström and Andersson 1985; Rhoden et al. 1988), and opioids (Belvisi et al. 1992).

Interpretation of the results from indirect studies is often difficult and could even be misleading for the following two reasons: 1) During EFS, sympathetic, and NANC nerves may be activated as well as parasympathetic nerves. Although the function of sympathetic nerves can be removed by drugs such as guanethidine, there are no specific and powerful blockers for the various neurotransmitters of the NANC nerves. These latter neurotransmitters such as substance P, VIP, and NO may modulate the contractile response to activation of cholinergic nerves at both pre- and postjunctional sites. 2) A potential modulator of the airway cholinergic nerves frequently also has a postjunctional effect. When both the frequency-response and ACh dose-response curves are affected, it is difficult to differentiate the prejunctional effect from the postjunctional effect.

To clearly demonstrate the neuromodulatory effect, it is essential to directly measure ACh release from airway cholinergic nerves. This has been considered technically difficult (Barnes 1991), but recently a few laboratories have succeeded in measuring EFS-induced ACh release (Aas 1990; Baker et al. 1992; D'Agostino et al. 1990; Deckers et al. 1989a; Martin and Collier 1986; Tsuchiya et al. 1990). The prejunctional effect of neuromodulators is present or most apparent only within a certain range of frequency (Duckles and Budai 1990; Knoll and Vizi 1971), voltage (Cherubini et al. 1985), and pulse duration (Kalsner 1990b). However, the majority of studies have been conducted with a single set of EFS parameters. There has been no systematic study of the effect of stimulation parameters on ACh release from airway cholinergic nerves. Basic information about the effect of alterations in stimulation parameters such as frequency, voltage, and pulse duration on ACh release not only indicates the sensitivity



of the technique to detect changes in ACh output but also provides guidelines for selecting EFS parameters in future studies.

In some previous ACh release studies (Baker et al. 1992; D'Agostino et al. 1990; Kilbinger et al. 1991), the airway smooth muscle strips were kept isometric for the purpose of simultaneous tension measurements. Since a potential prejunctional modulator often also has a direct postjunctional effect, the release of ACh from the control and treated tissue might have been studied under a different muscle tone. Active contractions or passive stretch of airway smooth muscle can release mediators such as PGE₂ (Grodzinska et al. 1975; Orehek et al. 1973; Türker and Zengil 1976) that can themselves prejunctionally modulate neurotransmission. Therefore, it seemed reasonable to determine if alterations of muscle preload influence ACh release.

In the present study, I used high-performance liquid chromatography (HPLC) plus electrochemical detection to measure ACh release from horse airway cholinergic nerves in order to determine the effect of alterations in stimulation parameters and muscle preload on ACh release. In this type of study, it is necessary to prevent ACh breakdown by treating the tissues with a cholinesterase inhibitor. The resultant accumulation of ACh may inhibit further ACh release due to the autoinhibitory effect of released ACh on prejunctional muscarinic autoreceptors. This autoinhibitory effect may interfere with the prejunctional regulatory effect of other modulators. For example, Deckers et al. (1989a) reported that the potentiating effect of the cyclooxygenase inhibitor indomethacin on ACh release from canine bronchial tissue can be demonstrated only in the presence of atropine but not in its absence. Because future studies may often require the administration of atropine in order to study the effect of other prejunctional modulators, it was necessary

to conduct the present study both in the presence and absence of atropine. In addition, administration of atropine allowed us to determine if muscarinic autoreceptors are present on cholinergic nerve terminals.

Horse airway smooth muscle was used for two reasons. First, the horse trachea provides a large amount of smooth muscle for many tissue baths. This removes the interanimal variability that occurs when it is necessary to use the trachea of one animal per bath in order to obtain detectable ACh release. More importantly, our laboratory has a long-term interest in studying heaves, a type of chronic obstructive pulmonary disease that affects horses and bears similarities to human asthma. Results of previous studies indicate that the airway obstruction is partly due to increased ACh release from the airway cholinergic nerve terminals (Broadstone et al. 1991). However, the evidence is indirect and it is unclear why ACh release may be elevated in heavy horses. It was therefore necessary to establish a technique to directly measure ACh release from horse airway cholinergic nerves.

Materials and methods

Experiments were approved by the All-University Committee on Animal Use and Care of Michigan State University. Five horses, 438.9 ± 10.9 kg, approximately 5.8 ± 0.8 years old, were used in this study. Other investigators also utilized tissues for a variety of investigations. Horses had no history of heaves, showed no clinical signs of respiratory tract disorder and, post-mortem, their lungs and airways were normal in gross appearance. Immediately after euthanasia by intravenous injection of an overdose of pentobarbital sodium, the thoracic cavity was opened. The trachea was quickly removed

and immersed in Krebs-Henseleit solution (composition in mM: NaCl 118.4, NaHCO₃ 25.0, dextrose 11.7, KCl 4.7, CaCl₂ · 2H₂O 2.6, MgSO₄ · 7H₂O 1.19, KH₂PO₄ 1.16) that had been bubbled with 95% O₂/5% CO₂. Tracheal smooth muscle strips with epithelium intact were prepared from the tracheal segment between the 21st and 30th cartilaginous rings above the carina. The muscle was cut with a template along the fiber direction, and four strips (each measuring 2 x 30 mm) were cut each time. These four strips were tied together at four places with silk and cut into two bundles of four muscle strips, 15 mm long, each with the silk tied at both of their ends. Twelve such strip bundles were prepared from the trachea of each animal. They were bubbled in Krebs-Henseleit solution with 95% O₂/5% CO₂ in a 30-ml tissue bath at 38°C for approximately 2 hours in order to remove the excessive foam formed during bubbling. The bundles were then suspended in twelve 2-ml tissue baths (Radnoti Glass Technology, Inc., Monrovia, CA). Each bath had a pair of parallel platinum wire electrodes built against the wall in the vertical direction. One end of the bundle was fixed to the bottom of the bath with a glass tissue holder, and the suture on the other end was passed over a pulley and attached to weights that maintained a passive tension. Tissues were equilibrated for a further hour in these baths. During equilibration, the bathing solution was changed at 10–15 min intervals.

After equilibration, tissues were incubated with Krebs-Henseleit solution containing 10⁻⁵ M neostigmine (a cholinesterase inhibitor to prevent the breakdown of released ACh) with or without 10⁻⁶ M atropine for 30 min. Electrical field stimulation (20 V, 4 Hz, 2 ms, except otherwise specified) was then applied to each tissue for 30 min. Tissues were stimulated 3–5 times with a 30-min resting period between

consecutive stimuli. Tissue bath solution was collected after each EFS or at the end of a 30-min resting period. Before the start and at the end of each EFS, the bath solution was replaced with Krebs-Henseleit solution along with appropriate concentrations of drugs. Each time the bath solution was changed, the tissues were rinsed three times with Krebs-Henseleit solution containing 10^{-5} M neostigmine. All bundles, except for the two bundles used for the muscle preload study, were studied under a preload of 8 g, since previous studies had determined that the optimal resting tension for each of the four strips in the bundle is 2 g (Yu et al. 1992a). Electrical impulses were produced by a stimulator (S88, Grass Instrument Co., Quincy, MA) and passed through a stimulus power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO). The electrical impulses consisted of square waves. When the applied voltage was 20 volts, the current going through each bath was 331.3 ± 4.3 mA at 38°C as measured with the stimulus power booster. The specific protocol for each strip bundle is shown in Table 2-1.

The wet weight of strip bundles determined at the end of the experiment after blotting with filter paper averaged 235.3 ± 5.4 mg ($n = 60$).

ACh analysis

High-performance liquid chromatography coupled with electrochemical detection was used to measure the amount of ACh in the tissue bath liquid. The HPLC system consisted of an Epson computer, a pump (Model 620, Perkin-Elmer, Norwalk, CT), an autosampler (ISS100C, Perkin-Elmer), an electrochemical detector (LC-4C, Bioanalytical System, Inc., West Lafayette, IN), an ACh and choline analytical column (Bioanalytical System Inc.), and an enzyme reaction column with immobilized AChE and choline

oxidase (Bioanalytical System, Inc.). The software program used to control the system was Perkin-Elmer's *LC analyst* (version 2.01). The principle of the technique was that: 1) ACh and choline were separated in the analytical column; 2) ACh was hydrolyzed to acetate and choline by AChE, and choline was oxidized to betaine and H_2O_2 by choline oxidase; and 3) H_2O_2 was then oxidized at 0.5 V applied potential. The current generated was proportional to the amount of H_2O_2 and therefore to the amount of ACh and choline. The lower limit of ACh determination was 1 pmol per injection. If 200 μl bath liquid was injected into the column each time, the lowest concentration of ACh that could be correctly determined by this technique would be 5×10^{-9} M. The detector response was linearly proportional to the amount of ACh over the range of 1 to 100 pmol. The mobile phase was 30 mM or 50 mM Na_2HPO_4 (pH = 8.5) and the flow rate was 0.5 or 1 ml/min. A typical chromatogram is shown in Fig. 2-1.

Collected samples were filtered through 0.2 μ nylon membrane Acrodiscs (Gelman Sciences, Ann Arbor, MI) and stored in sample vials at 4°C if they were to be analyzed within 12 hours or at -15°C if they were to be stored overnight. Two hundred μl was injected into the column for both samples and calibration standards. An external standard of 20 pmol ACh and choline was used for calibration. The standard was injected every 6 samples and the ACh amount in the samples was calculated based on bracketed calibration. Approximately 6 samples were loaded onto the autosampler each time and efforts were made to ensure that samples from the same bath remained in the autosampler for about the same length of time, because ACh content in the samples tended to decrease over time at room temperature. Analysis of samples began as soon as they were collected and finished within 24 hours.

Drugs

Neostigmine methylsulfate, atropine, and hexamethonium (Sigma Chemical Co., St. Louis, MO.) were diluted with water on the day of experiment. Tetrodotoxin (Sigma) stock solution (1 mg tetrodotoxin in 5 ml Krebs-Henseleit solution and 5 ml acetic acid) stored at 4°C was diluted with water on the day of experiment. Neostigmine and atropine were directly mixed into Krebs-Henseleit solution. Hexamethonium and tetrodotoxin solutions were pipetted into the tissue bath in 20 μ l volume (1% of the bath volume). The concentrations of all drugs were expressed as their final bath concentrations.

Statistical analysis

Single factor repeated design ANOVA followed by the Tukey HSD test was used to compare ACh release from the same tissues over different stimulation periods. An unpaired *t*-test was used to compare the release between tissues with and without atropine. All values are expressed as mean $\pm$ SE. $P < 0.05$ was considered statistically significant.

Results

Among all the samples ($n = 210$), the ACh content of only three samples was below measurable range. Of the three samples, two were collected when EFS was not applied and one was collected after a 5 volts-EFS period in the absence of atropine.

Sample stability was checked at room temperature (23°C), refrigerator temperature (4°C), and after freezing (-15°C). At room temperature, ACh concentration decreased

13.9 $\pm$ 1.9% (means 5.6 pmol/200 μ l versus 4.9 pmol/200 μ l, $p < 0.05$, paired t -test, $n = 6$) in eight hours. Refrigeration for 18 hours or freezing for 10 days did not result in a decrease in ACh amount, the means being 3.8 pmol/200 μ l versus 3.7 pmol/200 μ l for refrigeration ($n = 5$) and 6.9 pmol/200 μ l versus 6.8 pmol/200 μ l for freezing ($n = 8$).

In the absence of EFS, the spontaneous ACh release was small. The dramatic elevation of ACh release induced by EFS was abolished by tetrodotoxin (Fig. 2-2). The release of ACh was frequency- and voltage-dependent. The frequency response curve was sigmoid, with release reaching a plateau at 8 and 2 Hz in the absence and presence of atropine respectively (Fig. 2-3). Acetylcholine release increased with increasing voltage. In the absence of atropine, there was no difference in release at 15 and 20 volts, whereas, in the presence of atropine, a plateau in release was achieved at 10 volts (Fig. 2-4).

In the absence of atropine, increases in pulse duration caused a slight but significant increase in ACh release. In the presence of atropine, there was no clear relationship between pulse duration and ACh release (Fig. 2-5).

Alterations in preload had no influence on ACh release (Fig. 2-6). When the same tissue was stimulated five times, the amount of ACh released each time was fairly constant (Fig. 2-7). Although the release of ACh was significantly higher in the second than in some of the other stimulation periods, the magnitude of the difference was small in comparison to the total amount of released ACh.

Examination of the curves for frequency, voltage, pulse duration, preload-response, and time-control (Figs. 2-3 to 2-7) shows that atropine significantly and

dramatically augmented ACh release. In the time-control tissues stimulated at 4 Hz, 20 volts, 2 ms pulse duration, the release of ACh was approximately 10 and 45 pmol/g/min in the absence and presence of atropine, respectively (Fig. 2-7).

Fifteen min of EFS released the same amount of ACh as 30 min EFS when the ACh amount was expressed as pmol/g/min, suggesting that ACh release rate was constant during the 30-min period of stimulation. Ganglion blockade with hexamethonium did not influence the ACh release (Fig. 2-8).

Discussion

The present study demonstrated that EFS-induced ACh release from horse trachealis strips was frequency- and voltage-dependent. The neural origin of ACh was indicated by the observation that tetrodotoxin abolished the EFS-induced release.

When the length of stimulation period is fixed, as in the present study, nerves receive more pulses during each stimulation period as the frequency of stimulation is increased. At lower frequencies, the total release of ACh increased with frequency, while at higher frequencies, total ACh release reached a plateau. At each frequency, the ACh release was much greater in the presence than in the absence of atropine. The mechanisms of the plateau at higher frequencies may be different in the absence and presence of atropine. In the absence of atropine, the plateau was mainly due to the autoinhibitory effect of released ACh on prejunctional muscarinic autoreceptors. The potential ability of tissue to further release ACh was clearly indicated by the high output of ACh in tissues treated with atropine. For example, at a frequency of 8 Hz, ACh release was 17.1 ± 4.4 and 41.9 ± 10.6 pmol/g/min in the absence and presence of

atropine, respectively. There could be several possible mechanisms for the plateau of ACh release in atropine-treated tissues. First, the concentration of atropine applied may not have been high enough to block all the prejunctional autoreceptors. Second, the maximal ability of the tissue to synthesize or release ACh may have been reached. Third, other neurotransmitters may have been released during EFS. Horse trachea has parasympathetic, adrenergic, and NANC nervous systems (Broadstone et al. 1991). Norepinephrine and the neuropeptides released by the NANC system can all potentially modulate ACh release. Conclusions cannot be reached based on the results of the present study, which was not designed to investigate these mechanisms.

The shape of the voltage-response curve obtained in the present study bears similarity to that obtained in tension studies where EFS causes a voltage-dependent contractile response of airway smooth muscle (Broadstone et al. 1991). Maximal ACh-release and muscle tension both occur at 15–20 V. Blockade of autoinhibitory receptors increased the amount of ACh released at each voltage and the maximal release appeared at 10 rather than 15 V.

The increase in ACh release with increase in pulse duration observed when the tissues untreated with atropine were stimulated at 20 V is similar to the pulse-duration-dependent increase in NE release from sympathetic nerves stimulated supramaximally (Kalsner 1990b). Since the individual action potential is an all or none event, the increase in per pulse release has been attributed to the increased recruitment of terminal filaments of autonomic nerves.

Airway smooth muscle contractions induced by a variety of stimuli trigger the synthesis of prostaglandins, particularly PGE₂ (Grodzinska et al. 1975; Orehek et al.

1973; Türker and Zengil 1976). Synthesis is thought to be related to the mechanical consequence of the contraction (Orehek et al. 1973). Stretch of muscle can also elicit the release of PGE-like material from isolated cat trachea (Türker and Zengil 1976). It has been repeatedly demonstrated that PGEs, particularly PGE₂, have an inhibitory effect on airway cholinergic neurotransmission (Waters et al. 1984; Deckers et al. 1989a). Our results indicate that muscle preload alterations over the range of 2–20 g had no influence on ACh release. The results obtained in the presence of atropine reflect the effect solely of changes in passive tension, because postjunctional muscarinic receptors were blocked and the muscle did not contract during EFS. In the absence of atropine, the muscle contracted and developed active tension equal to the preloads. The fact that alterations of muscle preload over the 2–20 g range did not change ACh release indicates that either 1) the muscle does not release PGE₂ or other prejunctional modulators in response to different preloads, or 2) the cholinergic nerves are insensitive to these modulators. Therefore, if ACh release is studied under isometric conditions, the difference in tension between control and treated tissues is not likely to affect the outcome of results. Furthermore, if the release is studied under isotonic conditions as in the present study, the release would not be affected over a wide range of muscle preloads.

Starke (1987) defines presynaptic autoreceptors as receptors located on or close to the axon terminals of a neuron, through which the neuron's own transmitter can and, under appropriate conditions, does modify transmitter biosynthesis or release. The presence of prejunctional muscarinic autoreceptors on airway cholinergic nerves has been evidenced for several species (Barnes 1989c; Fryer and MacLagan 1984; Minette and Barnes 1988). Most evidence suggests that these muscarinic autoreceptors belong to the

M_2 subtype. In horse trachealis strips, we have examined the presence of M_2 receptors by comparing the effect of M_2 receptor antagonist gallamine on the contractile response to EFS and exogenous ACh. The contractile response to EFS is due to activation of the cholinergic nerves. Gallamine has no effect on the ACh dose-response curve. It also does not potentiate the response to EFS. The results suggest that there are no functional M_2 -receptors on the prejunctional site (Yu et al. 1992a). However, in the present study, ACh release was augmented by atropine in all the treatment groups. The augmentation of ACh release by atropine was not due to displacement of ACh molecules from muscarinic receptors of the tissue into the incubation solution (Chapter 3). Therefore, the results indicate the presence of muscarinic autoreceptors on the airway cholinergic nerve terminals. Since atropine is a non-specific muscarinic antagonist, it is impossible to judge the muscarinic autoreceptor subtype based on this finding. It is yet to be understood why our indirect and direct experiments have produced different results. One possibility is that, in the presence of cholinesterase inhibitor, the accumulated ACh exerted an enhanced negative feedback effect on the autoreceptors and therefore the function of these receptors is easier to demonstrate when a receptor antagonist is administered. Another possibility is that during EFS, the NANC nerves were activated, and their neurotransmitters postjunctionally modulated the contractile response to EFS. The enhanced contractile response to EFS due to blocking of autoreceptors might have been canceled by the direct inhibitory effect of the neuropeptides on the airway smooth muscle. Such an effect could only be reflected in indirect studies. The third possibility is that the prejunctional muscarinic autoreceptors on cholinergic nerves innervating horse trachea do not belong to the M_2 subtype. In rabbit airways, similar circumstances have

been encountered: the presence of prejunctional muscarinic autoreceptors on cholinergic nerves can be detected by direct measurements of ACh release (Loenders et al. 1992) but not by comparing the bronchoconstricting response to vagal stimulation and exogenous ACh (MacLagan and Faulkner 1989). These differences highlight the importance of direct measurement of ACh release.

The magnitude of augmentation of ACh release after atropine observed in the present study is much higher than that reported by D'Agostino et al. (1990), who used the tritiated choline method. The difference could be due to the 10 times higher concentration of atropine used in the present study. Other possibilities include failure to label all ACh storage pools by [^3H]choline, and an enhanced negative feedback effect in our study due to the use of a cholinesterase inhibitor, which resulted in accumulation of ACh.

The ACh release was the same when expressed as pmol/g/min whether the tissue was stimulated for 15 or 30 min, suggesting that the ACh release rate was constant and stable over the 30-min stimulation period. When the same tissue was stimulated for 5 consecutive times of 30 min each, the release of ACh was fairly consistent over different stimulation periods. Although the ACh release was higher in the second than in the other stimulation periods, the magnitude of difference was small relative to the total amount of released ACh.

The cholinergic nerves in the isolated trachealis preparation may comprise the preganglionic autonomic nerve terminals and the postganglionic parasympathetic neurons. In tension studies, it has been found that, during EFS, postganglionic neuron excitation is not dependent on activation of the preganglionic nerves (Chapter 4). In the present

study, ACh may be released from both the pre- and post-ganglionic neurons. However, it is impossible to determine the proportions from each site. In ferret, it has been found that there are only a small number of ganglia in the dorsal membrane of the trachea (Baker et al. 1986). If this holds true in horse trachea, our trachealis preparations may contain few ganglia and the ACh release from the presynaptic nerve terminals may contribute only a trivial proportion to the total release. The fact that the ganglionic blocker hexamethonium did not influence ACh release confirms that ACh release from the postganglionic nerve terminals is independent of activation of preganglionic neurons.

Several techniques have been used to measure ACh release from airway cholinergic nerves. Incubation with [^3H]choline followed by monitoring of tritium outflow from airway smooth muscle (Aas 1990; D'Agostino et al. 1990; Kilbinger et al. 1991) is very sensitive and does not require the addition of an AChE inhibitor to prevent ACh break down. However, all the neural compartments from which ACh is released are not equally labelled with tritium and the tritium stores are easily exhausted (Beani et al. 1984; Luz et al. 1983). The radioenzymatic method is complicated, time-consuming, and not as sensitive as HPLC plus electrochemical detection (Martin and Collier 1986). Radioimmunoassay is very sensitive, but the antibodies against ACh have cross reactions with some other choline esters (Kawashima et al. 1980; Tsuchiya et al. 1990). Use of HPLC plus electrochemical detection to measure ACh was originally developed by Potter et al. (1983) to measure ACh in rat brain tissues. It is highly specific and sensitive. Its high specificity can be attributed to several factors: 1) different chemical compounds have different retention times when they pass through the analytical column; 2) the enzymes in the reaction column have specificity; and 3) only the chemicals that can be oxidized

at less than or equal to the applied voltage can be detected. This technique has recently been used to measure ACh release from airway cholinergic nerves of guinea pigs, dogs, cattle, and rabbits (Baker et al. 1992; Baker and Brown 1991; Deckers et al. 1989a; Loenders et al. 1992a). Our data indicate that the HPLC technique provides a sensitive method to detect the effects of stimulus intensity and autoinhibitory feedback on ACh release from horse airway parasympathetic nerves.

Table 2-1. Detailed protocols

Control		Frequency		Voltage		Pulse Duration		Preload		Spontaneous & TTX	Stimu. Duration & HEX
Bath #											
1	2	3	4	5	6	7	8	9	10	11	12
Equilibration											
Incubation with neostigmine with (Y) or without (N) atropine for 30 min											
Sample #	Y	N	Y	N	Y	N	Y	N	Y	N	Y
1	20 V, 4 Hz, 2 ms		1 Hz	1 Hz	5 V		0.5 ms	2 g	No EFS		EFS (15 min)
2	20 V, 4 Hz, 2 ms		2 Hz	2 Hz	10 V		1 ms	10 g	EFS		EFS (30 min)
3	20 V, 4 Hz, 2 ms		4 Hz	4 Hz	15 V		2 ms	20 g	EFS with TTX		EFS (30 min) with HEX
4	20 V, 4 Hz, 2 ms		8 Hz	8 Hz	20 V		3 ms				
5	20 V, 4 Hz, 2 ms		0.5 Hz	16 Hz							

Note: 1) All samples were collected after electrical field stimulation (EFS) except the first sample of Bath 11, which was collected after 30 min incubation without EFS; 2) Except otherwise specified, voltage = 20 V, frequency = 4 Hz, pulse duration = 2 ms, EFS duration = 30 min; 3) Neostigmine and (in some baths) atropine were replenished each time the bath solution was changed; 4) In the five pairs of baths (first 10 baths), the bath receiving atropine and that not receiving it were alternated in each experiment; 5) A different frequency was applied to the baths with and without atropine, because in the presence of atropine, ACh release plateaued early, while in the absence of atropine, EFS at lower frequencies (< 1 Hz) did not always release a measurable amount of ACh; 6) The concentrations of tetrodotoxin (TTX) and hexamethonium (HEX) were 10^{-6} M.

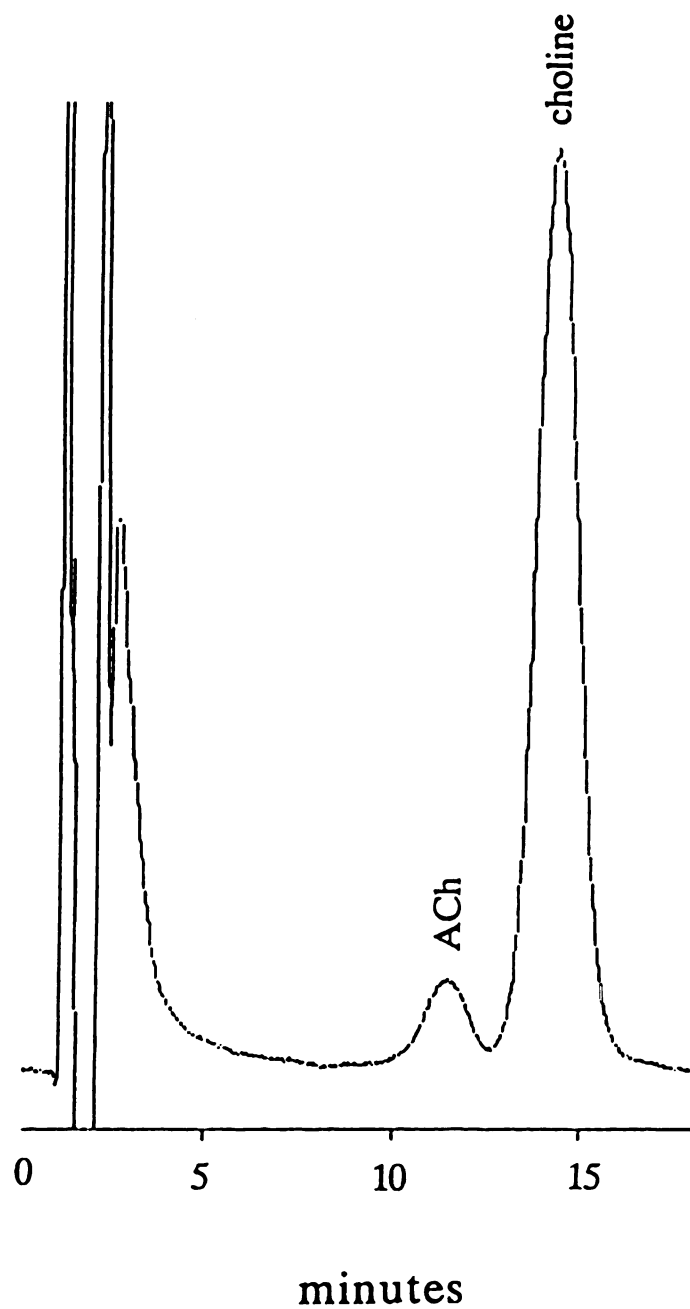


Fig. 2-1. A typical chromatogram showing the separation of acetylcholine (ACh) and choline in a sample. The mobile phase was 50 mM Na_2HPO_4 and the flow rate was 0.5 ml/min. Two hundred μl bath liquid was injected into the HPLC system. The ACh and choline peaks represent 8.6 and 92.4 pmol, respectively.

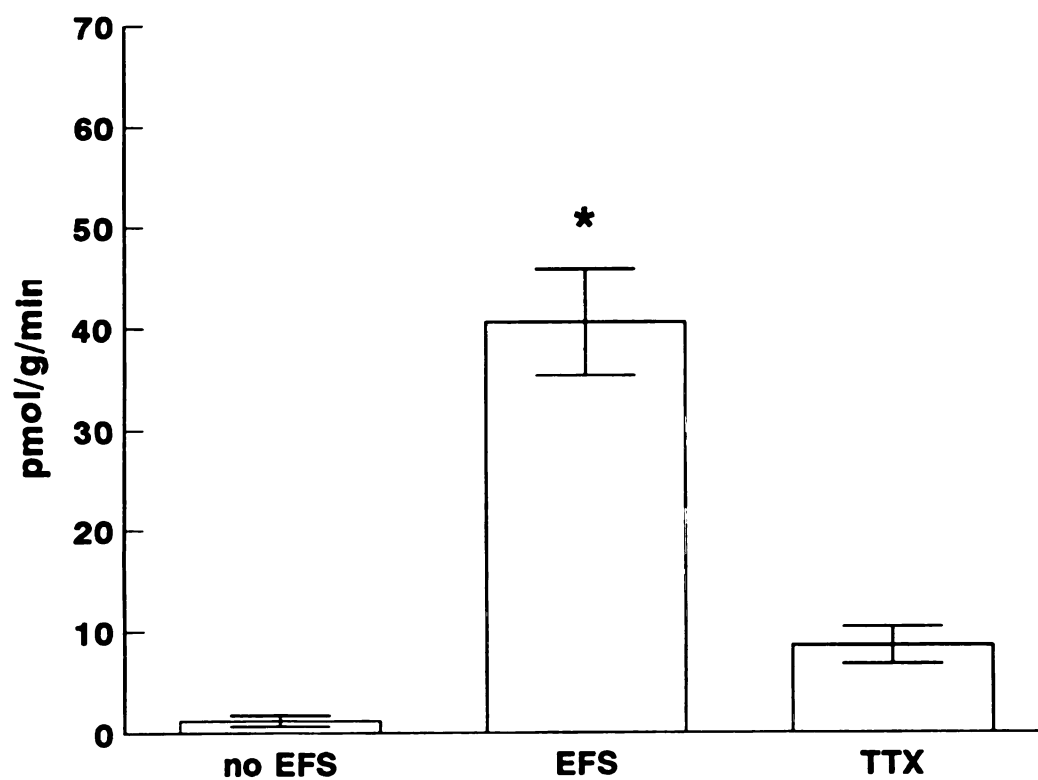


Fig. 2-2. Effect of electrical field stimulation (EFS, 20 V, 4 Hz, 2 ms) on acetylcholine release from horse airway cholinergic nerves in the presence of 10^{-6} M atropine and the effect of 10^{-6} M tetrodotoxin (TTX) on the EFS-induced release. * significantly different from the other two treatment groups. There was no significant difference between the "no EFS" and "TTX" groups.



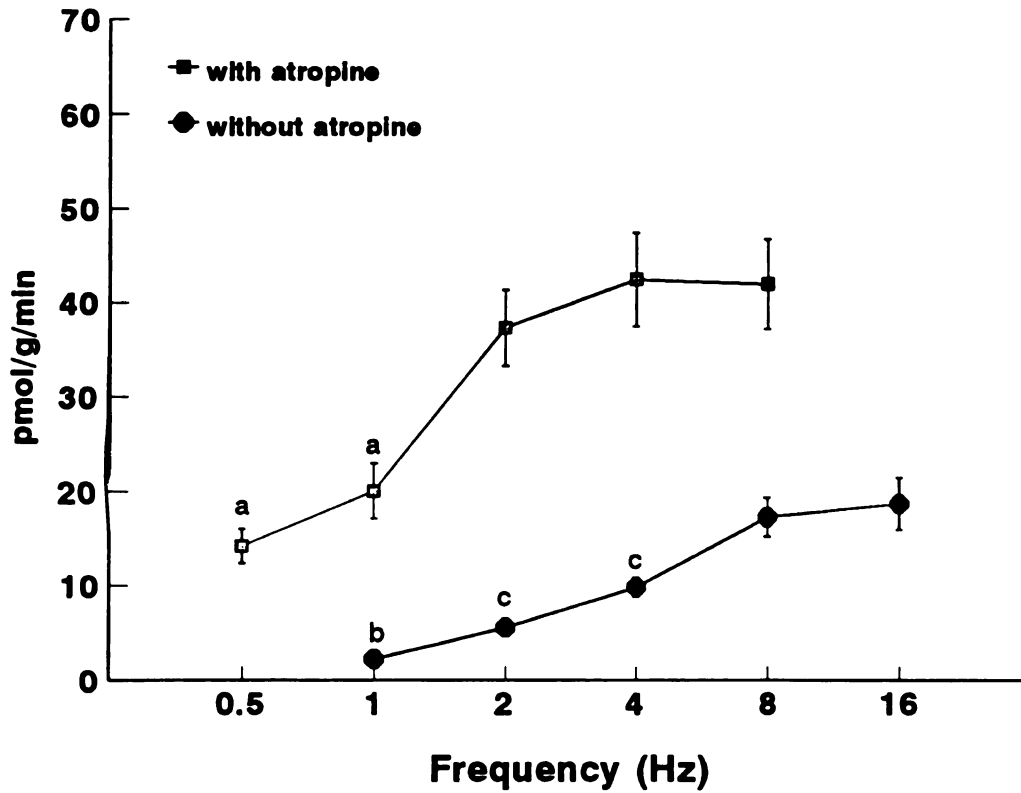


Fig. 2-3. Effect of frequency on electrical field stimulation-induced acetylcholine release from horse airway cholinergic nerves in the presence and absence of 10^{-6} M atropine. Significantly different from 2, 4, 8 Hz (a), 4, 8, 16 Hz (b), or 8, 16 Hz (c). Values in the presence and absence of atropine were significantly different at all frequencies.

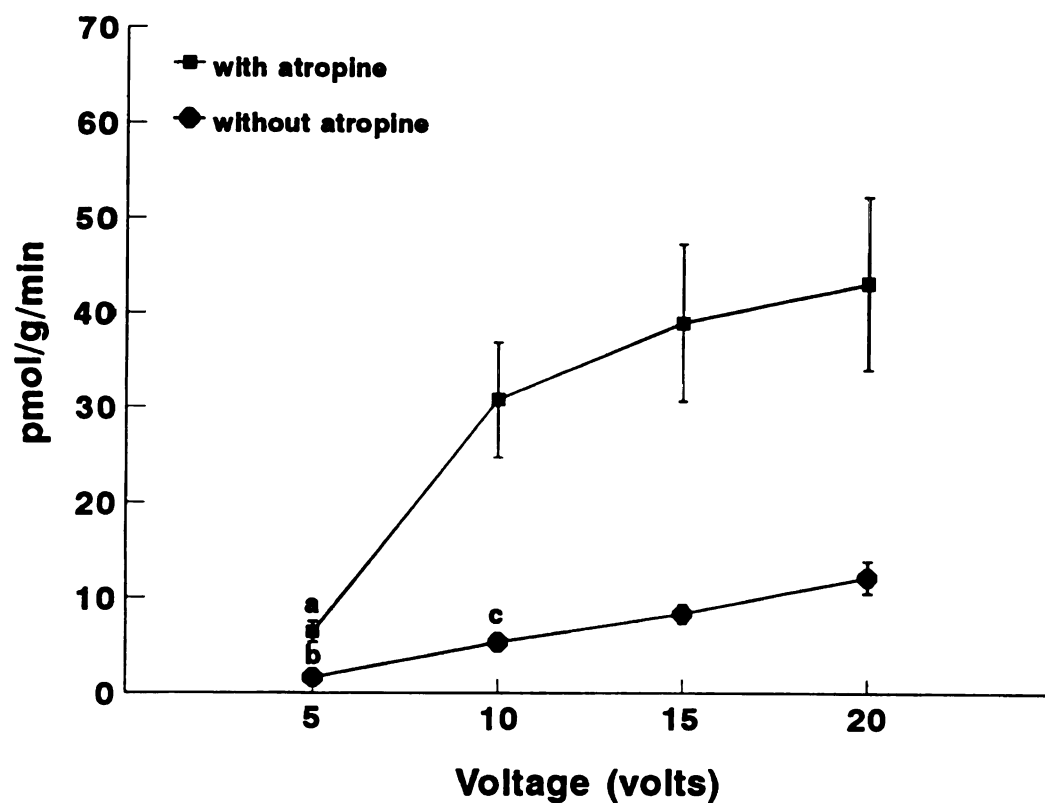


Fig. 2-4. Effect of voltage on electrical field stimulation-induced acetylcholine release from horse airway cholinergic nerves in the presence and absence of 10^{-6} M atropine. Significantly different from 10, 15, 20 V (a), 15, 20 V (b), or 20 V (c). Values in the presence and absence of atropine were significantly different at all voltages.

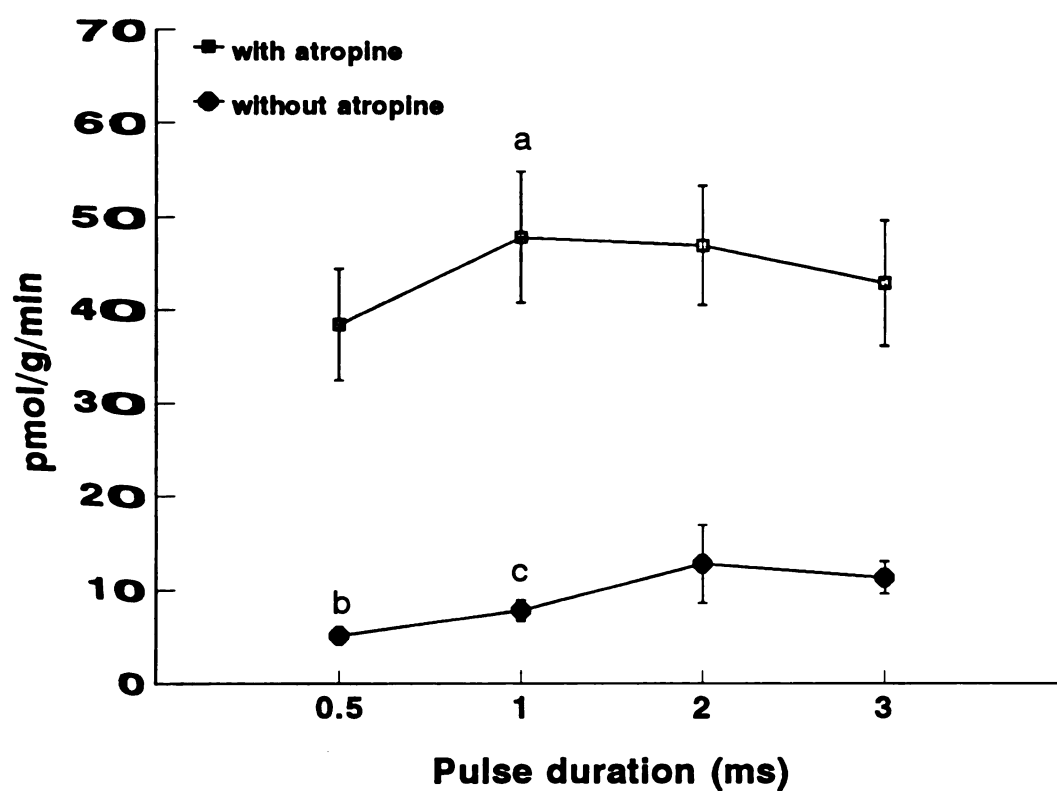


Fig. 2-5. Effect of pulse duration on electrical field stimulation-induced acetylcholine release from horse airway cholinergic nerves in the presence and absence of 10^{-6} M atropine. Significantly different from 0.5 ms (a), 2, 3 ms (b), or 3 ms (c). Values in the presence and absence of atropine were significantly different at all pulse durations.

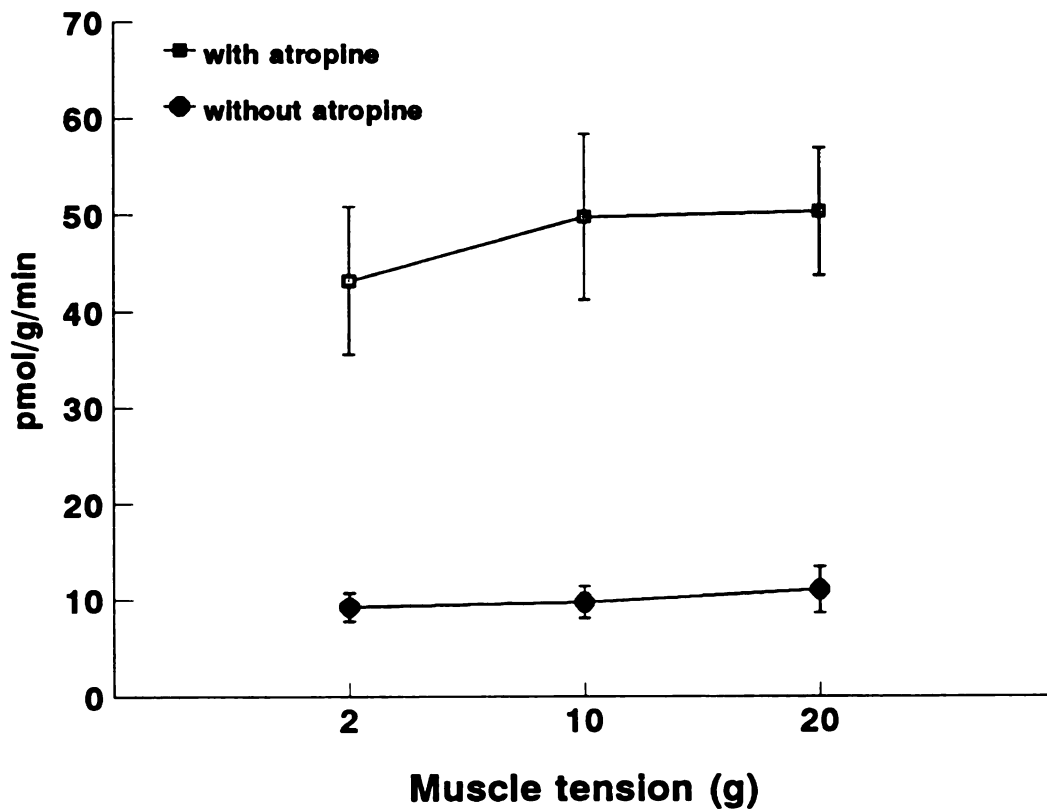


Fig. 2-6. Effect of preload on electrical field stimulation-induced acetylcholine (ACh) release from horse airway cholinergic nerves in the presence and absence of 10^{-6} M atropine. Preload alterations had no influence on ACh release. Values in the presence and absence of atropine were significantly different at all preloads.

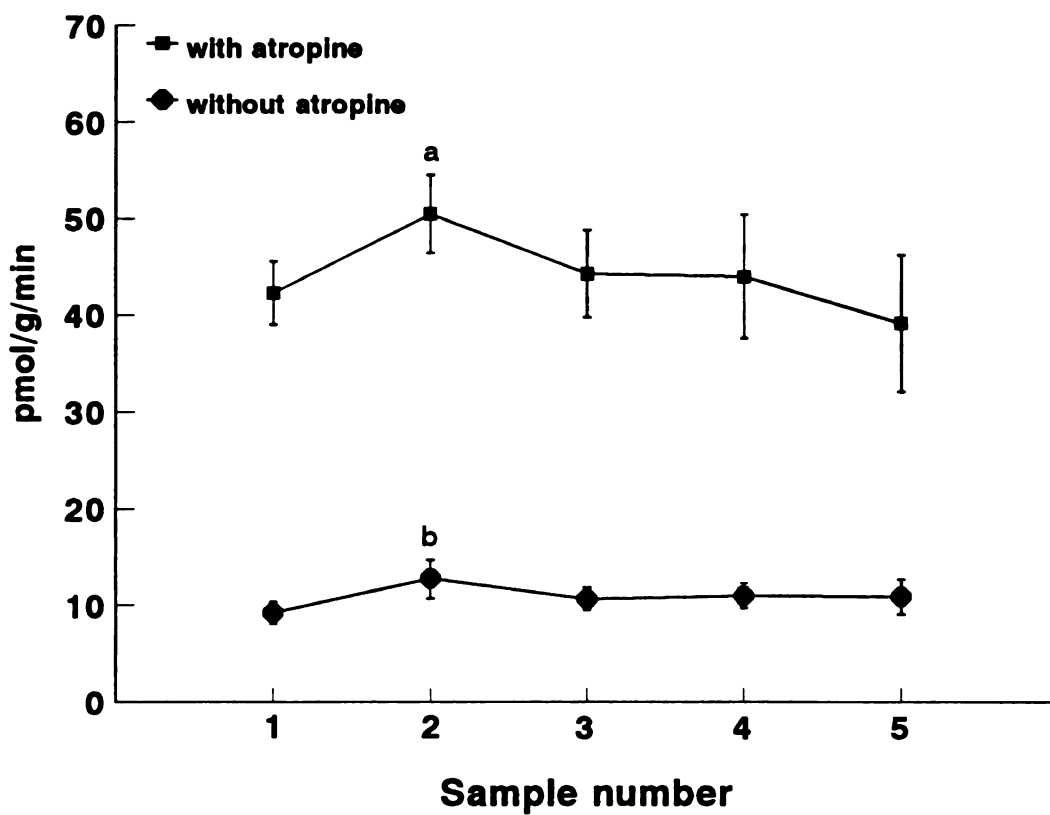


Fig. 2-7. Electrical field stimulation-induced acetylcholine release from horse airway cholinergic nerves over 5 stimulation periods in the presence and absence of 10^{-6} M atropine. Significantly different from S1, S4, S5 (a), or S1 (b). Values in the presence and absence of atropine were significantly different at all stimulation periods.

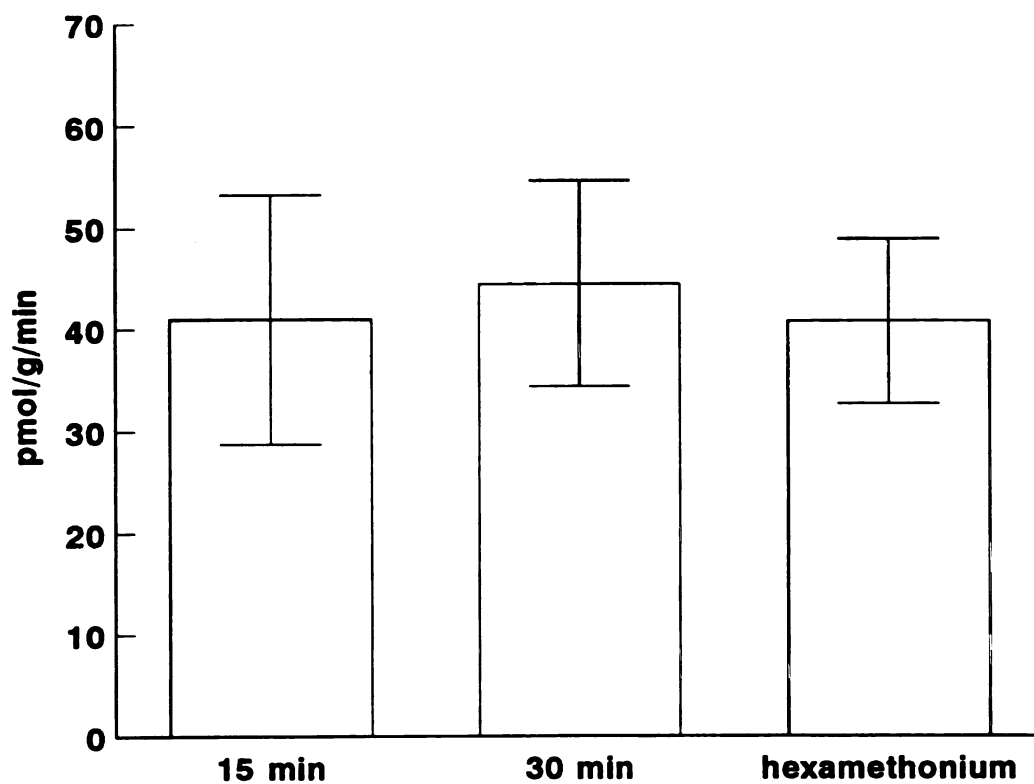


Fig. 2-8. Effects of stimulation duration alterations and hexamethonium on acetylcholine release from horse airway cholinergic nerves in the presence of 10^{-6} M atropine. If expressed as pmol/g/min, ACh release was the same whether the tissue was stimulated for 15 or 30 min. Hexamethonium had no influence on ACh release.



CHAPTER 3

MUSCARINIC AUTORECEPTORS

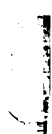
ON HORSE AIRWAY CHOLINERGIC NERVES

Introduction

The release of ACh from airway cholinergic nerves, the major bronchoconstrictor neural pathway in all mammals, is regulated by prejunctional muscarinic autoreceptors. Muscarinic autoreceptors are probably dysfunctional in asthmatic patients (Minette et al. 1989) and in guinea pigs infected with parainfluenza virus (Fryer et al. 1990) or challenged by ovalbumin (Fryer and Wills-Karp 1991). This dysfunction may lead to increased ACh release and airway obstruction.

Although five subtypes of muscarinic receptors have been cloned, only three, M_1 , M_2 , and M_3 , can usually be identified by the use of selective muscarinic antagonists. Most investigators conclude that the muscarinic autoreceptors on airway cholinergic nerves belong to the M_2 or a " M_2 -like" subtype (Aas and MacLagan 1990; Brichant et al. 1990; Kilbinger et al. 1991; Minette and Barnes 1988). However, some have suggested that they belong to the M_1 (Janssen and Daniel 1990b), M_3 (Deckers et al. 1989b), or M_2/M_4 (Loenders et al. 1992b) subtype.

Information about prejunctional muscarinic autoreceptors on horse airway cholinergic nerves is limited. Direct measurements of ACh release from airway



cholinergic nerves demonstrate that the nonselective muscarinic receptor antagonist atropine dramatically augments ACh release (Chapter 2), indicating that inhibitory muscarinic receptors are present. However, the selective M_2 receptor antagonist gallamine does not augment the response of horse trachealis to EFS, suggesting that there are no functional inhibitory M_2 receptors (Yu et al. 1992a). Therefore, the subtype of autoreceptors remains to be determined.

Measurement of ACh release from airway cholinergic nerves is the most direct and reliable method to study prejunctional modulation. The present study was conducted to characterize the prejunctional muscarinic autoreceptors by studying the effects of several competitive muscarinic antagonists on ACh release.

Materials and methods

Tissue preparation and equilibration

Trachealis tissues of 10 horses (body weight 405.8 ± 35.6 kg) were used for this study. Horses had no clinical signs of respiratory disease for several weeks before they were euthanized by injection of an overdose of pentobarbital sodium. Trachealis strip bundles with epithelium were prepared from the 16th to 25th cartilaginous rings above the carina using the method previously described (Chapter 2). Each bundle was suspended in a 2-ml tissue bath that contained Krebs-Henseleit solution (composition in mM: 118.4 NaCl, 25.0 NaHCO_3 , 11.7 dextrose, 4.7 KCl, 2.6 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.19 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.16 KH_2PO_4) maintained at 38°C and bubbled with 95% O_2 / 5% CO_2 . The tension applied to each bundle was well within the range of 2–20 grams, which had been determined to have no influence on ACh release from horse airway

cholinergic nerves (Chapter 2). Tissues were equilibrated in the baths for 2 to 3 hours, during which time the bath solution was replaced at 10–20 min intervals.

Each 2-ml tissue bath has a pair of built-in platinum wire electrodes. Electrical impulses were produced by a stimulator and passed through a stimulus power booster as described in Chapter 2.

Protocol

After equilibration, the tissues were incubated with 10^{-5} M neostigmine, a cholinesterase inhibitor, and 10^{-5} M guanethidine for 60 min. Four trachealis strip bundles from each animal were used to study the effects of atropine (non-selective, 10^{-8} – 10^{-4} M), pirenzepine (M_1 -selective, 10^{-7} – 10^{-3} M), AF-DX 116 (M_2 -selective, 10^{-7} – 10^{-3} M), and hexahydrosiladifenidol (HHSiD, M_3 -selective, 10^{-8} – 10^{-4} M) on ACh release, respectively. Electrical field stimulation (20 V, 4 Hz, 2 ms) was applied to the tissues for six 20-min periods with a 30-min interval between consecutive periods. During period 1, the tissues were stimulated in the absence of muscarinic antagonists. During subsequent periods, each bundle was stimulated in the presence of logarithmically increasing concentrations of a muscarinic antagonist. The tissue was preincubated with each concentration of an antagonist for 25–30 min. Bath liquid was collected for ACh analysis 1–2 min after each EFS period. The tissue bath solution was replaced before the start and at the end of each EFS period.

One bundle from each animal was stimulated for 6 periods in the absence of any muscarinic antagonist and served as time control. Two bundles were used to examine the effect of the vehicles of AF-DX 116 and HHSiD. To determine if ACh release could

be sustained at a high level following the administration of muscarinic antagonists, one bundle was stimulated for 6 periods in the presence of 10^{-5} M atropine.

Two bundles were used to determine if the increase in ACh concentration in the tissue bath following muscarinic antagonists was partly due to displacement of ACh molecules from muscarinic receptors in the tissues into the bath solution. These two bundles were stimulated for two periods. Bath liquid samples were collected 15 min after each EFS period. During period 1, no muscarinic antagonist was administered. During period 2, one bundle was stimulated in the presence of 10^{-5} M atropine after incubation with it for 20–25 min, whereas the other bundle was stimulated in the absence of atropine but incubated with 10^{-5} M atropine for 15 min after EFS prior to collecting the sample.

At the end of experiment, tissues were blotted and weighed. The wet weight averaged 0.197 ± 0.006 g.

In order to compare the effect of HHSiD on tracheal smooth muscle contraction with that on ACh release, two trachealis strips (10 x 2 mm) with epithelium were suspended in tissue baths and equilibrated using the method described previously (Yu et al. 1992a). The isometric force generated by the muscle strip was recorded on a polygraph. After equilibration, 10^{-5} M ACh was added to the baths to precontract the strips. When the effect of ACh had reached a plateau, HHSiD was added to one bath at concentrations beginning from 10^{-8} M in log increments until the tension had returned to baseline. The other strip was treated with HHSiD solvent and served as time control.

ACh analysis

ACh content was determined by HPLC coupled with electrochemical detection (Chapter 2).

Drugs

Neostigmine methylsulfate, guanethidine monosulfate, atropine sulfate, and pirenzepine dihydrochloride (Sigma Chemical, St. Louis, MO) were dissolved and diluted with either water or Krebs-Henseleit solution on the day of experiment. Frozen stock solutions of HHSiD hydrochloride (Research Biochemicals Inc., Natick, MA) and AF-DX 116 (courtesy Boehringer Ingelheim, Ridgefield, CT) were diluted with water before use. The concentrations of all drugs are expressed as their final bath concentrations.

Statistical analysis

ACh release was expressed as pmol/g/min. One way analysis of variance (ANOVA) for repeated measures followed by Newman-Keuls test was used to compare the difference in ACh release rate before and after the treatment with the muscarinic antagonist, and to compare the maximal effects and the negative logarithm of EC_{50} (the molar concentration of an antagonist required to reach 50% of its maximal augmenting effect) of different muscarinic antagonists. All values are expressed as mean $\pm$ SE. $P < 0.05$ was considered statistically significant.

Results

All samples contained a measurable amount of ACh. All the muscarinic antagonists augmented ACh release concentration-dependently (Fig. 3-1). Atropine was the most potent antagonist in augmenting ACh release. A significant increase appeared at 10^{-8} M with atropine, 10^{-6} M with HHSiD, and 10^{-5} M with pirenzepine and AF-DX 116. However, the magnitude of augmentation by 10^{-5} M pirenzepine and AF-DX 116 was larger than that by 10^{-8} M atropine. Therefore, 3×10^{-6} M might be the approximate concentration required for pirenzepine and AF-DX 116 to produce an effect comparable to that of 10^{-8} M atropine. The release rate decreased at the highest applied concentrations of all muscarinic antagonists except for pirenzepine. The maximal effect (pmol/g/min, $n = 5$) of atropine (78.3 ± 8.8) was significantly greater than that of pirenzepine (50.3 ± 7.1) and AF-DX 116 (50.4 ± 7.0). The $-\log EC_{50}$ ($n = 5$) of atropine (7.4 ± 0.3) was significantly greater than that of pirenzepine (5.5 ± 0.2) and AF-DX 116 (5.7 ± 0.2). The maximal effect and EC_{50} of HHSiD could not be determined because ACh release did not reach a plateau following HHSiD administration.

In the absence of muscarinic antagonists, ACh release was constant over the six stimulation periods ($n = 5$). The vehicles of AF-DX 116 and HHSiD had no influence on ACh release ($n = 4$). The augmenting effect of 10^{-5} M atropine was consistent over the six stimulation periods ($n = 4$) (Fig. 3-2).

Incubation of tissues with atropine during EFS augmented ACh release ($n = 3$). However, incubation of tissues with atropine after EFS but before sample collection had no effect on the measured amount of ACh ($n = 4$) (Fig. 3-3).

The tension increase following 10^{-5} M ACh was stable over the experimental period. The selective M_3 muscarinic receptor antagonist HHSiD concentration-dependently reduced the tension of the ACh-precontracted tissues. HHSiD reduced the tension to about 20% of the precontracted level at 10^{-6} M and relaxed the muscle completely at 10^{-5} M (Fig. 3-4).

Discussion

In the present study, all four muscarinic antagonists augmented ACh release. Augmentation was not simply a result of displacement of ACh molecules from the receptors of the tissue into the bath liquid because incubation with atropine after EFS but prior to sample collection had no influence on the measured amount of ACh. During the 6th EFS period that coincided with the highest concentration of each antagonist, the augmenting effect of some antagonists decreased. This was a result of nonspecific antagonist effects rather than of depletion of ACh, because the high ACh release rate in response to 10^{-5} M atropine was maintained over six EFS periods.

The increase in ACh release in response to atropine confirms the presence but does not identify the subtype of prejunctional muscarinic autoreceptors on horse airway cholinergic nerves. Although all three selective muscarinic antagonists, including Pirenzepine (M_1 selective), AF-DX 116 (M_2 selective), and HHSiD (M_3 selective), augmented ACh release, none had the same potency or maximal effect as atropine. Even though the muscarinic autoreceptors may comprise a single subtype, the augmentation by all antagonists was not unexpected because they are receptor-selective rather than receptor-specific.

M₃ receptors are responsible for horse airway smooth muscle contraction in response to ACh (Yu et al. 1992a). Therefore, to determine if the prejunctional muscarinic autoreceptors are also M₃, I compared the effects of HHSiD on airway smooth muscle contraction and ACh release. If the prejunctional autoreceptors are also M₃, HHSiD should have a similar potency on both muscle contraction and ACh release. The observation that HHSiD (1 μ M) inhibited approximately 80% of the 10 μ M ACh-induced contraction but augmented ACh release only slightly indicates that the prejunctional muscarinic autoreceptors are not M₃. Using a similar approach, Loenders et al. (1992b) reached the same conclusion about rabbit tracheal parasympathetic nerves. By contrast, Deckers et al. (1989b) concluded that the prejunctional muscarinic autoreceptors are M₃ because the M₃-selective muscarinic antagonist 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) augmented ACh release from canine bronchial segments. I have made a similar observation in horse trachealis strips (unpublished data). Because 4-DAMP is a selective rather than specific M₃ antagonist, its augmenting effect may be explained by actions on other receptor subtypes.

M₁ receptors are generally regarded as excitatory rather than inhibitory receptors on airway cholinergic neural pathways (Barnes 1989b,c). Pirenzepine has a higher affinity for M₁ receptors than other subtypes. Its K_d for M₁ receptors is approximately 12.6 nM based on the pA₂ value (Mei et al. 1989). In the present study, ~3 μ M Pirenzepine was required to augment ACh release significantly. This concentration is about 240 times its K_d, suggesting that the prejunctional autoreceptors are unlikely to be of the M₁ subtype.

The prejunctional muscarinic autoreceptors appear to be of the M_2 subtype in several species. However, in the present study, the concentration-response curve obtained with the M_2 -selective antagonist AF-DX 116 not only looked dramatically different from that of atropine but also superimposed on the concentration-response curves of the M_1 and M_3 antagonists. Although the augmenting effect of AF-DX 116 was not significant until 10 μ M, 3 μ M might be able to produce an effect comparable to that of 10 nM atropine. Even the latter concentration of AF-DX 116 is about 21 times of its K_d (141 nM calculated from pA_2 in Mei et al. 1989) for M_2 receptors, suggesting that prejunctional muscarinic autoreceptors are not M_2 . However, this conclusion is tempered by the observation that the concentration of atropine (10 nM) required to augment ACh release was also about 8 fold greater than its K_d for M_2 receptors (1.26 nM calculated from the pA_2 in Mei et al. 1989). The present study therefore does not allow elimination of the possibility that the prejunctional autoreceptor is M_2 . However, in a previous study the M_2 antagonist gallamine did not augment the response of equine trachealis to EFS, suggesting an absence of functional M_2 receptors (Yu et al. 1992a). It may be necessary to investigate if the autoreceptors on horse airway cholinergic nerves belong to other muscarinic receptor subtypes such as the M_4 or a novel uncharacterized subtype. M_4 receptors share the same transduction mechanism as M_2 receptors. Due to the lack of muscarinic antagonists with satisfactory selectivity for M_4 receptors, examination of the possibility that the autoreceptor is M_4 must await the development of new drugs.

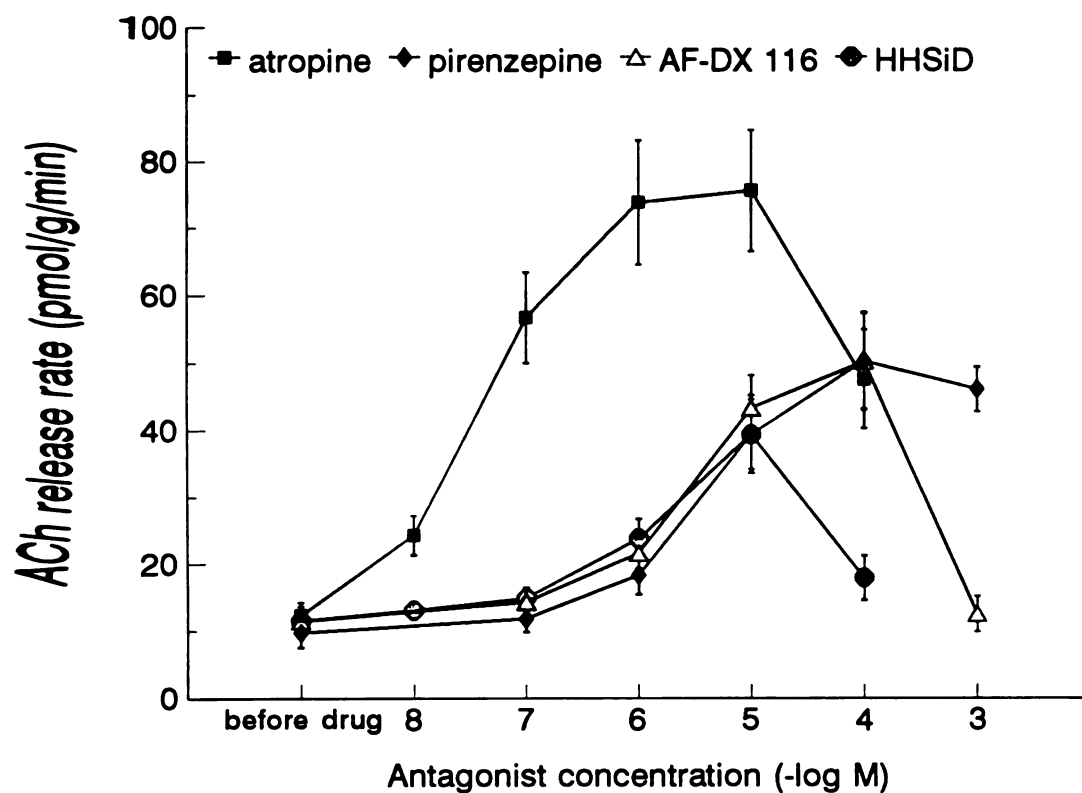


Fig. 3-1. Effects of muscarinic antagonists on acetylcholine (ACh) release from horse airway cholinergic nerves. All antagonists augmented ACh release. Significant effects appeared at 10 nM atropine, 1 μ M hexahydrosiladifenidol (HHSiD), and 10 μ M pirenzepine and AF-DX 116. The potency and maximal effect of atropine were much greater than those of the other antagonists.

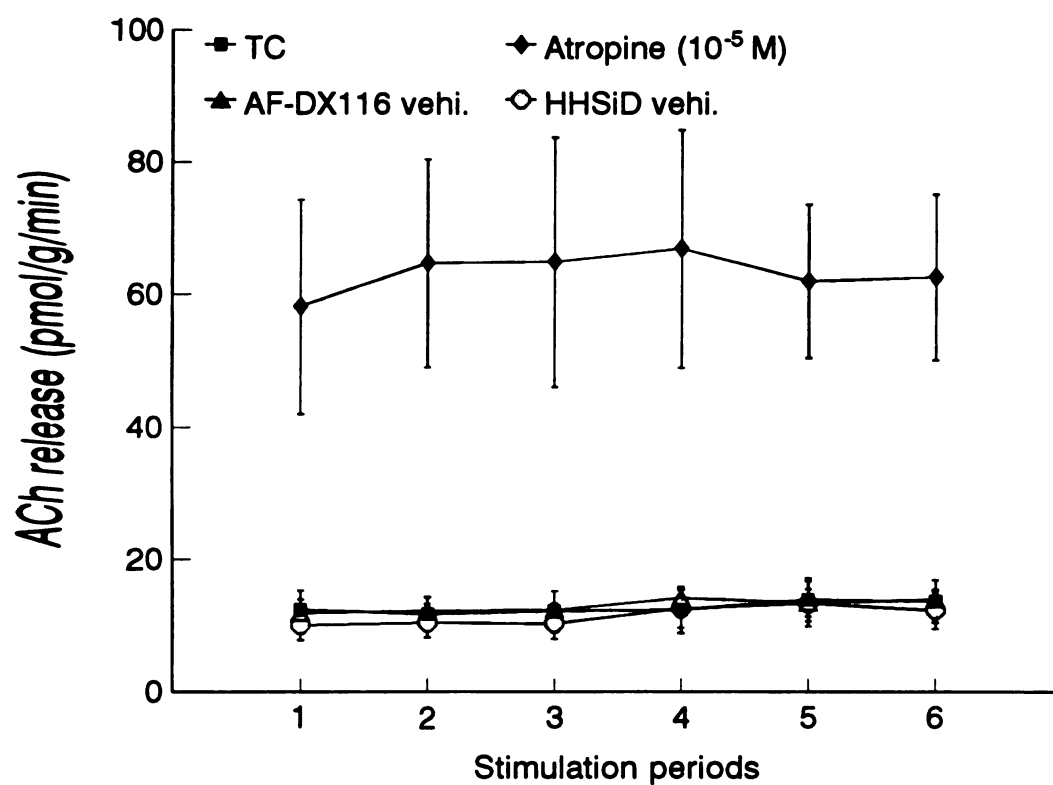


Fig. 3-2. ACh release in response to EFS in the time control tissue and in tissues treated with AF-DX 116 vehicle, HHSiD vehicle, and 10⁻⁵ M atropine. Atropine augmented ACh release. AF-DX 116 and HHSiD vehicles had no influence on ACh release. ACh release was constant over the six stimulation periods.

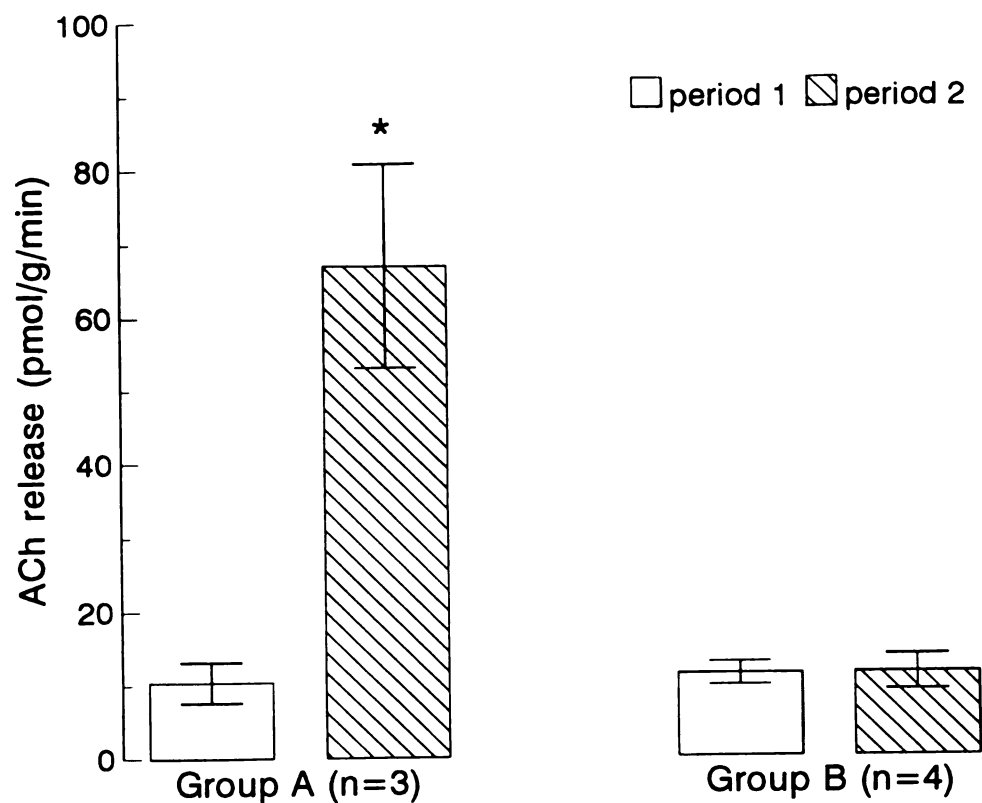


Fig. 3-3. ACh release in response to 10^{-5} M atropine administered before (Group A) and after EFS (Group B). Two periods of EFS were applied to each group. Tissue bath samples were collected 15 min after each period of EFS. During period 1, no muscarinic antagonist was administered. For period 2, atropine was administered before EFS in group A and after EFS but prior to sample collection in group B. Atropine did not augment ACh release in Group B, suggesting that the augmentation was not simply a result of displacement of ACh molecules from the receptors of the tissue into the bath liquid.

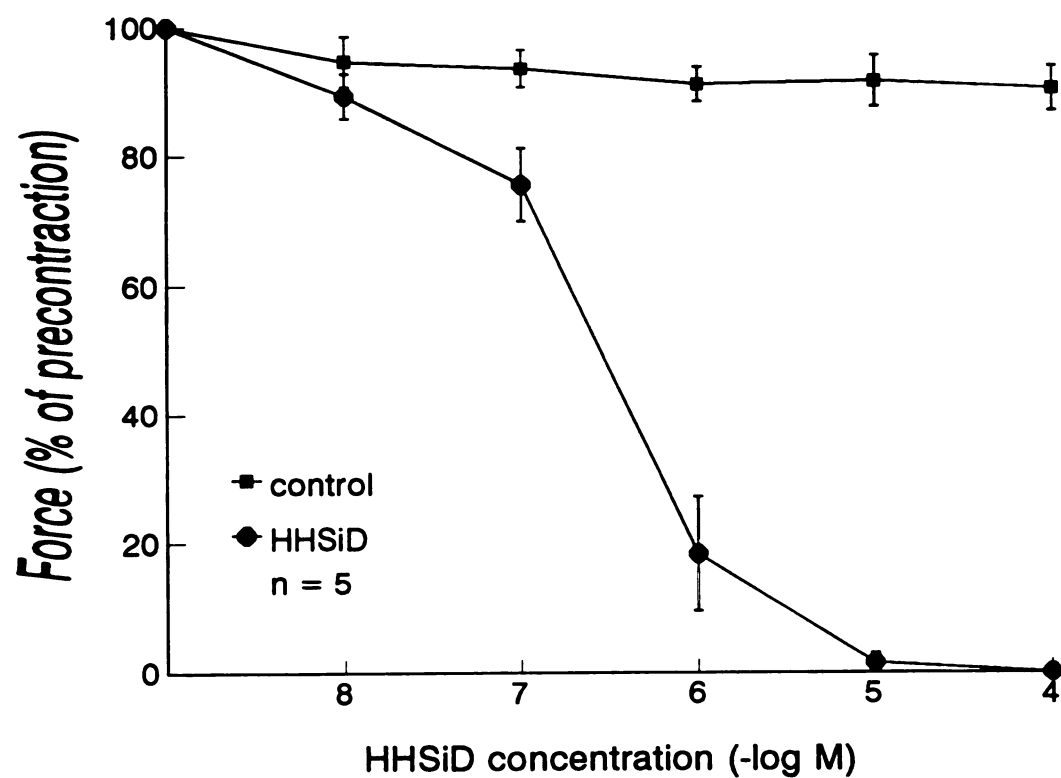


Fig. 3-4. Effect of the M_3 -selective antagonist hexahydrosiladifenidol (HHSiD) on the tension of horse airway smooth muscle precontracted by $10 \mu\text{M}$ ACh. HHSiD concentration-dependently relaxed the muscle, $1 \mu\text{M}$ and $10 \mu\text{M}$ eliminated about 80% and 100% of the precontraction, respectively.

12-1-19

CHAPTER 4

EXOGENOUS BUT NOT ENDOGENOUS PGE₂ MODULATES PONY TRACHEAL SMOOTH MUSCLE CONTRACTIONS

Introduction

Some horses and ponies that are housed in barns and fed hay develop a type of chronic obstructive pulmonary disease colloquially called "heaves." Like asthma in humans, heaves is characterized by periods of airway obstruction and hyperresponsiveness followed by periods of disease remission (Derksen et al. 1985a). The airway obstruction in affected animals occurs mainly through stimulation of muscarinic receptors because R_L decreases and C_{dyn} increases following atropine administration (Broadstone et al. 1988).

In vitro experiments utilizing isolated tracheal smooth muscle strips have shown that, compared to controls, muscle from heavy horses is slightly hyporesponsive to exogenous ACh, but hyperresponsive to EFS (Broadstone et al. 1991). One possible explanation for these observations is a defective regulation of cholinergic neurotransmission in affected animals, such that nerve stimulation results in increased ACh release at the neuromuscular junction.

The parasympathetic cholinergic innervation of the airways is modulated by a number of factors, including prostaglandins (Ito 1991). An inhibitory effect of PGE₂ on

parasympathetic nerves in canine airways is well documented (Waters et al. 1984; Nakanishi et al. 1976; Ito and Tajima 1981a; Ito et al. 1990; Daniel et al. 1987; Shore et al. 1987; Deckers et al. 1989a). Epithelium is an important source of prostanoids in the airways and, in the normal horse, PGE₂ is the major prostanoid released by tracheal epithelium (Gray et al. 1992b). Furthermore, airway mucosal tissues of heavy horses produced less PGE₂ in response to stimulation by certain agonists than those of control horses (Gray et al. 1992a). Therefore, it is possible that a lack of PGE₂ in the heavy horses may result in increased release of ACh from parasympathetic nerves. This would result in bronchospasm and the clinical signs of airway obstruction typical of heaves.

Before determining if a lack of PGE₂ is an important mechanism in the pathogenesis of heaves, it is first necessary to establish the role of PGE₂ in the modulation of airway parasympathetic nerves of normal animals. The purpose of this study was 1) to determine if exogenous PGE₂ can modulate the response of tracheal smooth muscle to EFS and ACh; and 2) to investigate, by means of cyclooxygenase inhibition, if endogenous PGE₂ modulates airway smooth muscle contraction.

Materials and methods

One female and 10 male ponies, weighing between 104 and 209 kg, were studied. They had no history of respiratory disease, showed no clinical signs of respiratory tract disorder and, post-mortem, their lungs and airways were normal in gross appearance. Immediately after euthanasia by intravenous injection of an overdose of pentobarbital sodium, the thoracic cavity was opened. The trachea was quickly removed and immersed in Krebs-Henseleit solution (composition in Mm: NaCl 118.4, NaHCO₃ 25.0, Dextrose

1 1.7, KCl 4.7, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.6, MgSO_4 1.19, KH_2PO_4 1.16), which had been bubbled with 95% O_2 and 5% CO_2 . It was then transported a short distance to the laboratory where it was gassed with 95% O_2 and 5% CO_2 continuously.

Tracheal smooth muscle strips with epithelium intact were prepared from the first 20 tracheal rings above the carina. The muscle was cut with a template along the fiber direction. Each strip measured 3.5 x 10 mm. The strips were suspended by 3-0 surgical silk in 10-ml tissue baths containing Krebs-Henseleit solution bubbled with 95% O_2 -5% CO_2 and maintained at 38°C. One end of the tissue was tied to the hook at the lower end of an electrode holder that was placed in the muscle bath. The other end was tied to a force transducer (Grass FTO3) mounted on micromanipulators so that the tissue length could be changed. A continuous recording of isometric force generation was obtained by a polygraph (Grass Model 7D, 7E, 79D) connected to the force transducer. Tissues were suspended between platinum electrodes for electrical stimulation. 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.

Tissues were equilibrated for approximately 90 min with an initial passive tension of 2 g applied and maintained. In preliminary experiments, it was determined that 2 grams is the optimal resting tension for the trachealis strips. During the equilibration, tissues were electrically stimulated for 2-3 min at 8-10 min intervals (15 v, 0.5 ms pulse duration, 16 Hz) until the baseline was stable and the magnitude of response to the same stimulus was consistent. The bath solution was changed every 15 min. At the end of equilibration, the Krebs-Henseleit solution was replaced by 127 mM KCl-substituted

1

Krebs-Henseleit solution, and the contractile response to this solution was recorded. Tissues were then rinsed with Krebs-Henseleit solution several times until the muscle tension returned to baseline.

Protocol 1: Effect of exogenous PGE₂ on contractions induced by EFS and ACh

Seven tracheal muscle strips from each pony were suspended between rectangular electrodes placed parallel to the muscle. Meclofenamate (10^{-6} M) was added to each tissue bath. After a 40-min incubation period, guanethidine (10^{-5} M) was added to the bath. Sixty min after adding meclofenamate, PGE₂ was added to six tissue baths and ethyl alcohol (vehicle) equivalent to the volume used to dissolve 10^{-6} PGE₂ was added to a seventh bath to give a final concentration of 0.1% of ethyl alcohol. One concentration of PGE₂ was added to each bath (10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} M). Twenty min after adding PGE₂ or its vehicle, contractile responses to increasing frequencies (0.5, 1, 2, 4, 8, 16, 32 Hz) of EFS (15 v, 0.5 ms pulse duration) were obtained. Each stimulus was applied until a plateau in tension was achieved, approximately 2–3 min. The interval between consecutive stimuli was 6 min.

Following EFS, ACh was added to the bath cumulatively at concentrations of 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} M. When tension had plateaued following addition of a concentration of ACh, the next concentration was added. The tissues were then removed from the bath, blotted with filter paper, and weighed (wet weights).

An experiment was conducted to determine if the PGE₂ concentration in the muscle bath remained stable after addition of exogenous PGE₂. One muscle strip was incubated with 10^{-6} M meclofenamate for one hour and exogenous PGE₂ was added to

the muscle bath. PGE₂ samples were taken immediately and after 110 min, the approximate time needed to conduct the experiments with EFS and ACh administration.

Protocol 2: Effects of cyclooxygenase inhibitors on airway smooth muscle contraction

Two sets of eight tracheal strips were prepared from each pony. One set was used to study the effect of cyclooxygenase blockade on the response to EFS, another set to study the effect on the ACh dose-response curve. Tissues were suspended between ring-shaped electrodes. Three concentrations of aspirin (10^{-3} , 10^{-4} , 10^{-5} M) and meclofenamate (10^{-4} , 10^{-6} , 10^{-8} M) and their solvents (methanol and distilled water, respectively) were added separately to eight pairs of organ baths. The amount of solvent administered to the control baths was equal to that used to dissolve the highest concentration of inhibitors. Forty min after adding the inhibitors, guanethidine (10^{-5} M) was added to each organ bath and 20 min later, 0.5 ml of bathing solution was taken from each bath for measurement of PGE₂ concentration and replaced with the same volume of Krebs-Henseleit. As soon as the PGE₂ samples had been taken, ACh was added cumulatively (10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} M) to one set of strips and the tissues of the other set were stimulated electrically as described under protocol 1.

Upon completion of the ACh administration or EFS, another 0.5 ml of bathing solution was taken for measurement of PGE₂. The tissues were then removed from the bath, blotted, and weighed as in protocol 1.

Measurement of PGE₂

Tissue bath samples were stored at -80°C and PGE₂ was measured by radioimmunoassay in batches after all samples had been collected. The PGE₂ antiserum was obtained from Advanced Magnetics (Cambridge, MA), tritiated PGE₂ from Dupont-New England Nuclear (Boston, MA), and authentic PGE₂ standard from Cayman Chemical Co. (Ann Arbor, MI). The crossreactivity of the antiserum at 50% B/B₀ was PGE₂ 100%, PGE₁ 50%, PGA₂ 6%, PGF_{2α} 1.3%, and all other eicosanoids 1% or less. The lower limit of PGE₂ detection was 3.05 pg/100 μl. When concentrations were below the limit of detection, the value of 3.05 pg/100 μl was used for statistical purposes. Tissue production of PGE₂ (ng/g wet wt) was calculated from bath concentration (pg/100 μl), bath volume, and tissue wet weight.

Drugs

The following drugs were used: acetylcholine chloride, aspirin (Sigma), sodium monohydrate meclofenamate (courtesy of Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company), guanethidine monosulfate (courtesy of CIBA Pharmaceutical Co.), PGE₂ (Cayman Chemical Co.). All drugs were dissolved in distilled water except PGE₂ and aspirin. Aspirin was dissolved in methanol to make a solution of 10⁻¹ M, which was subsequently diluted with distilled water. The PGE₂ was dissolved in ethyl alcohol to 10⁻³ M and stored at -20°C. The stock solution of PGE₂ was diluted with distilled water before use in each experiment. Drugs were added in a volume equal to 1% of the total volume of solution in the muscle bath. Concentration of drugs was expressed as the final bath concentration.

1

Statistical analysis

Responses to EFS and ACh were calculated as a percentage of the maximum contraction produced by 127 mM KCl. Data were analyzed by between-within/split-plot ANOVA, single factor randomized design, and paired t-test as appropriate. $P < 0.05$ was considered statistically significant. All values are expressed as mean $\pm$ SE. n = number of animals.

Results

Trachealis muscle contracted in a frequency-dependent manner in response to EFS. All contractions were abolished by atropine (10^{-6} M) and by tetrodotoxin (10^{-6} M) but not affected by hexamethonium (10^{-5} M), indicating that they resulted from postganglionic cholinergic nerve stimulation and activation of muscarinic receptors. ACh produced a dose-dependent trachealis contraction that was abolished by atropine.

Protocol 1: Effect of exogenous PGE₂ on contractions induced by EFS and ACh

Muscle contraction in response to EFS was inhibited in a dose-dependent manner by PGE₂. Concentrations of PGE₂ 10^{-8} M and above caused significant inhibition. This inhibitory effect was stronger at low than at higher frequencies. When PGE₂ concentration was equal to or less than 10^{-9} M, no inhibitory effect was observed (Fig. 4-1a). Prostaglandin E₂ also inhibited ACh-induced contractions, but the minimum concentration of PGE₂ needed to significantly inhibit ACh-induced contractions was 10^{-7} M, higher than that needed to inhibit EFS-induced contractions (Fig. 4-1b). The concentration of PGE₂ in the muscle bath remained stable during the 110-min incubation period, 197.0 ng/100

μl immediately after exogenous PGE_2 addition and 201.2 ng/100 μl at the end of incubation.

Protocol 2: Effects of cyclooxygenase inhibitors on tracheal smooth muscle contraction

After the 60-min incubation period, concentrations of PGE_2 in the organ bath averaged $9.8 \pm 1.7 \times 10^{-10}$ M (mean $\pm$ se) in the absence of cyclooxygenase inhibitors. Neither meclofenamate nor aspirin had any significant effect on the frequency-response curves or the ACh dose-response curves (Fig. 4-2, 4-3).

Meclofenamate (10^{-6} and 10^{-4} M) inhibited PGE_2 production both before and after EFS and ACh-induced contractions. At 10^{-8} M, the inhibitory effect of meclofenamate was variable (Fig. 4-4a, 4-5a). Aspirin (10^{-4} and 10^{-3} M) also inhibited PGE_2 production but 10^{-5} M did not (Fig. 4-4b, 4-5b). The increase in PGE_2 levels that followed EFS was suppressed by both 10^{-6} and 10^{-4} M meclofenamate but only by 10^{-3} M aspirin (Fig 4-4a, 4-4b). The tendency for PGE_2 to increase following ACh was suppressed by 10^{-6} and 10^{-4} M meclofenamate and by 10^{-4} and 10^{-3} M aspirin (Fig. 4-5a, 4-5b).

Discussion

The purpose of the present study was to determine the role of PGE_2 in the regulation of equine airway smooth muscle. Two approaches were used: the administration of exogenous PGE_2 and inhibition of endogenous PGE_2 synthesis by cyclooxygenase inhibitors.

Our results clearly show that exogenous PGE_2 inhibits tracheal smooth muscle contractions induced by both EFS and ACh. The action of PGE_2 on ACh-induced

contractions must be direct on smooth muscle, but the effect on EFS-induced contractions could be directly on smooth muscle, indirectly through nerves, or both. The minimum concentration of PGE_2 needed to inhibit contractions induced by EFS was 10^{-8} M. This was less than the 10^{-7} or 10^{-6} M needed to inhibit contractions induced by ACh. These observations suggest that the effect of PGE_2 on EFS-induced contraction was exerted in part directly on muscle and in part through nerves. The equine trachea receives parasympathetic, sympathetic, and NANC innervation (Broadstone et al. 1991). In the present experiments, the function of sympathetic nerves was blocked by the administration of guanethidine. We were unable to block the NANC system, because the neurotransmitter had not been identified at the time of this study and there is therefore no blocker. It is most likely that the contractions we observed during EFS were due to activation of postganglionic parasympathetic nerves, because contractions were eliminated by both atropine (10^{-6}) and tetrodotoxin (10^{-6}) and were unaffected by hexamethonium. The inhibitory effect of exogenous PGE_2 on nerves must therefore have been exerted on parasympathetic postganglionic nerves.

The inhibitory effect of PGE_2 on parasympathetic nerves of dog airways is well documented. As we observed in the pony, the parasympathetic nerves of dog airways have a higher sensitivity to the inhibitory effect of PGE_2 than does airway smooth muscle. The actual concentrations of PGE_2 needed to inhibit the nerves and muscle vary widely in different studies. Ito et al. reported that when PGE_2 concentration is between 10^{-11} and 10^{-9} M, it only inhibits EJPs. In doses over 10^{-8} M, PGE_2 also reduces muscle tone and dose-dependently suppresses the amplitude of twitch contractions (Ito and Tajima 1981a; Ito et al. 1990). Daniel et al. (1987) observed that 10^{-9} and 10^{-8} M PGE_2

inhibits the contractile response to EFS as well as EJPs remarkably, but the inhibitory effect on ACh-induced contractions is significantly less. Waters et al. (1984) found that 3×10^{-8} M PGE_2 inhibits the contractile response to EFS significantly but shows little effect on ACh-induced contractions. Similar results have also been obtained by Nakanishi et al. (1976) but with much higher concentrations of PGE_2 (2.8×10^{-7} and 2.8×10^{-6} M). In equine trachealis electrically stimulated using parameters that caused muscle contraction by both a direct and a neurally mediated mechanism, Gill and Kroeger (1990) observed that exogenous PGE_2 at 10^{-8} M inhibited the neural component and at 10^{-7} M inhibited both the neural and myogenic components. The inhibitory PGE_2 concentrations reported by Gill and Kroeger are identical to those of the present study.

From our results with exogenous PGE_2 , it is apparent that endogenous PGE_2 could affect the frequency-response curve only at concentrations equal to or greater than 10^{-8} M. The concentration of PGE_2 in the muscle bath after a one-hour incubation period averaged 9.8×10^{-10} M and after EFS was 1.9×10^{-9} M in the absence of cyclooxygenase inhibitors. These concentrations were well below those that inhibited the response to EFS. Because PGE_2 is lipid soluble and can easily pass through cell membranes, it is unlikely that the PGE_2 concentration in the muscle was much higher than that in the organ bath. Therefore, even if production of all the endogenous PGE_2 was inhibited, a significant leftward shift of either the frequency-response curves or ACh dose-response curves would not be expected. Moreover, the muscle contractions in response to EFS should have been markedly inhibited if the PGE_2 concentration inside the muscle strip had exceeded 10^{-8} M. Based on the measured levels of PGE_2 in the muscle bath and the observed inhibitory effect of exogenous PGE_2 , it seems unlikely that endogenous PGE_2

has inhibitory effects in pony trachealis muscle. To further test this possibility, we examined the effect of cyclooxygenase blockade on the muscle's response to EFS and ACh.

Indomethacin is the most frequently used cyclooxygenase inhibitor for this type of experiment. We had planned to use indomethacin, but it frequently induced spontaneous contractions of tracheal smooth muscle, an effect that appeared to increase at higher concentrations. Because of the spontaneous contractions, it was difficult to measure the contractile responses to EFS and ACh accurately. Therefore, two other cyclooxygenase inhibitors instead of indomethacin were used in this study.

Both meclofenamate and aspirin blocked PGE_2 synthesis. At the highest concentrations of these drugs used, PGE_2 concentration after a one-hour incubation period was decreased from 9.8×10^{-10} M to 3.3×10^{-10} M with 10^{-3} M aspirin, to 3.0×10^{-10} M with 10^{-6} M meclofenamate, and to 1.7×10^{-10} M with 10^{-4} M meclofenamate. The value of PGE_2 after 10^{-4} M meclofenamate is even an overestimate of the actual PGE_2 concentration, because several PGE_2 samples were below the measurable range yet were reported as the lowest detectable value. These same high concentrations of aspirin and meclofenamate also blocked the increase in PGE_2 following EFS.

Despite the decrease in PGE_2 concentration in our organ baths, cyclooxygenase inhibitors did not affect the contractile response of tracheal smooth muscle to either EFS or ACh. These results are analogous to those of Daniel et al. (1987) obtained in canine tracheal strips dissected free of epithelium. In their experiments, indomethacin had little effect on the amplitude of EJPs elicited by electrical stimulation. By contrast, others, in both in vitro (Waters et al. 1984; Shore et al. 1987) and in vivo (Bethel and McClure

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1990) studies, reported that cyclooxygenase blockade enhances the canine trachealis response to electrical stimulation. In the only other reported study of the effect of cyclooxygenase blockade on equine trachealis, Gill and Kroeger used stimulus parameters that were very different from those of the present study in that they had direct effects on muscle as well as activating nerves. These investigators observed that indomethacin increased the contractile response to EFS, especially when the neurogenic component of contraction was blocked with atropine. These observations led them to conclude that endogenous prostanoids exert modulatory effects in horse trachealis (Gill and Kroeger 1990). Because the stimulus parameters were so different and because we studied ponies rather than horses, it is difficult to compare our results with those of Gill and Kroeger. However, the failure of both meclofenamate and aspirin to shift the EFS and ACh response curves suggests that, under the conditions of the present experiments, endogenous prostanoids do not exert a tonic inhibition on either parasympathetic nerves or airway smooth muscle.

Our conclusion that endogenous prostanoids do not exert an important modulatory effect on pony airway smooth muscle is supported by in vivo observations. Several days' administration of the cyclooxygenase inhibitor flunixin meglumine to normal ponies did not induce airway obstruction (Gray et al. 1989). This is unlike the observation of Ito and Tajima (1981b), who reported that dogs treated for 5 days with indomethacin began wheezing and coughing. The lack of an important role for PGE₂ in normal ponies makes it unlikely that an absence of PGE₂ is an important factor in the airway hyperresponsiveness and airway obstruction characteristic of heaves.

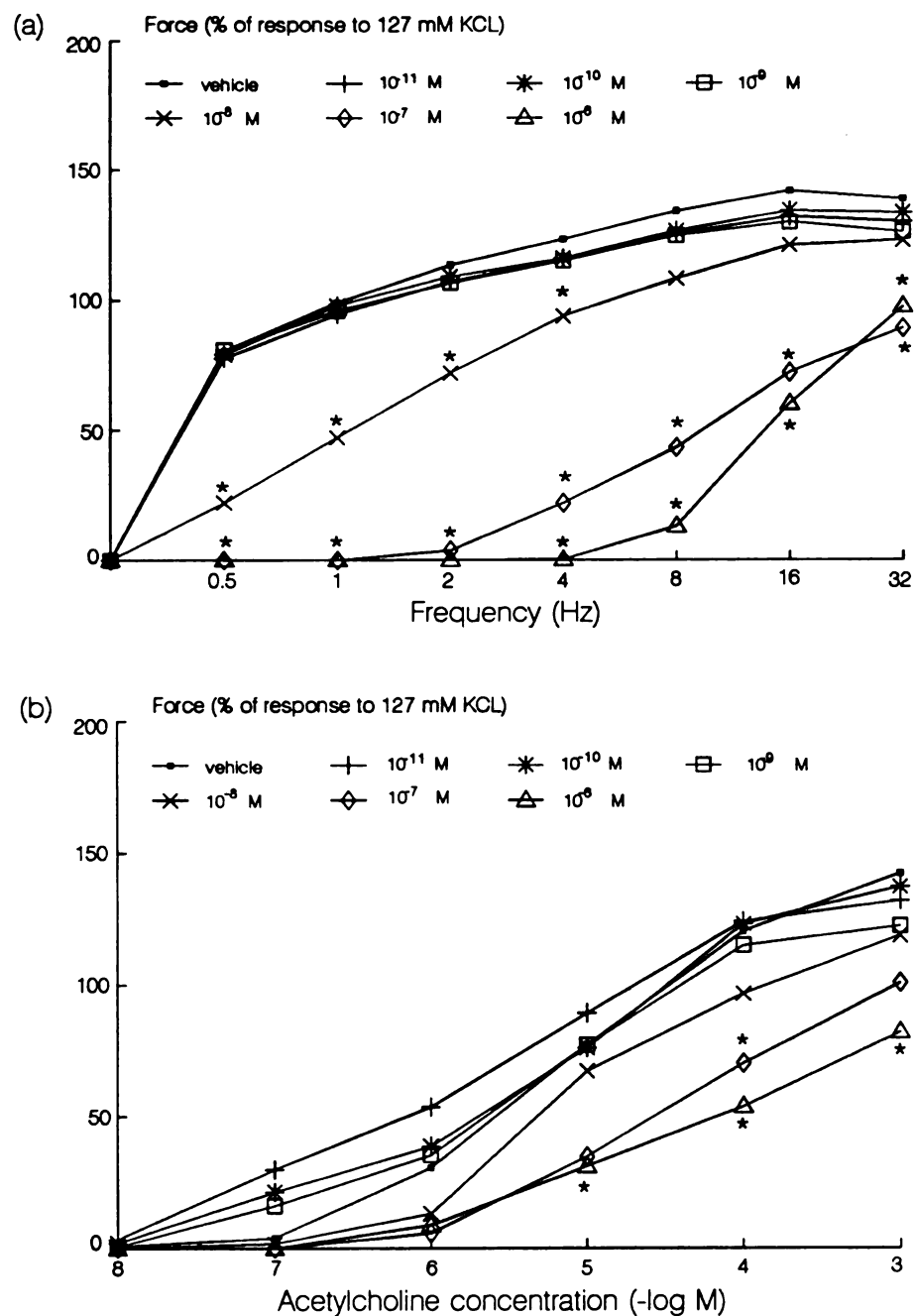


Fig. 4-1. Effect of exogenous PGE₂ on trachealis contractions induced by EFS (Graph a) and ACh (Graph b). $n = 5$. Single factor randomized design ANOVA was used to compare force at each frequency of stimulation and at each concentration of ACh. * = significantly different from the corresponding value in the vehicle-treated group.

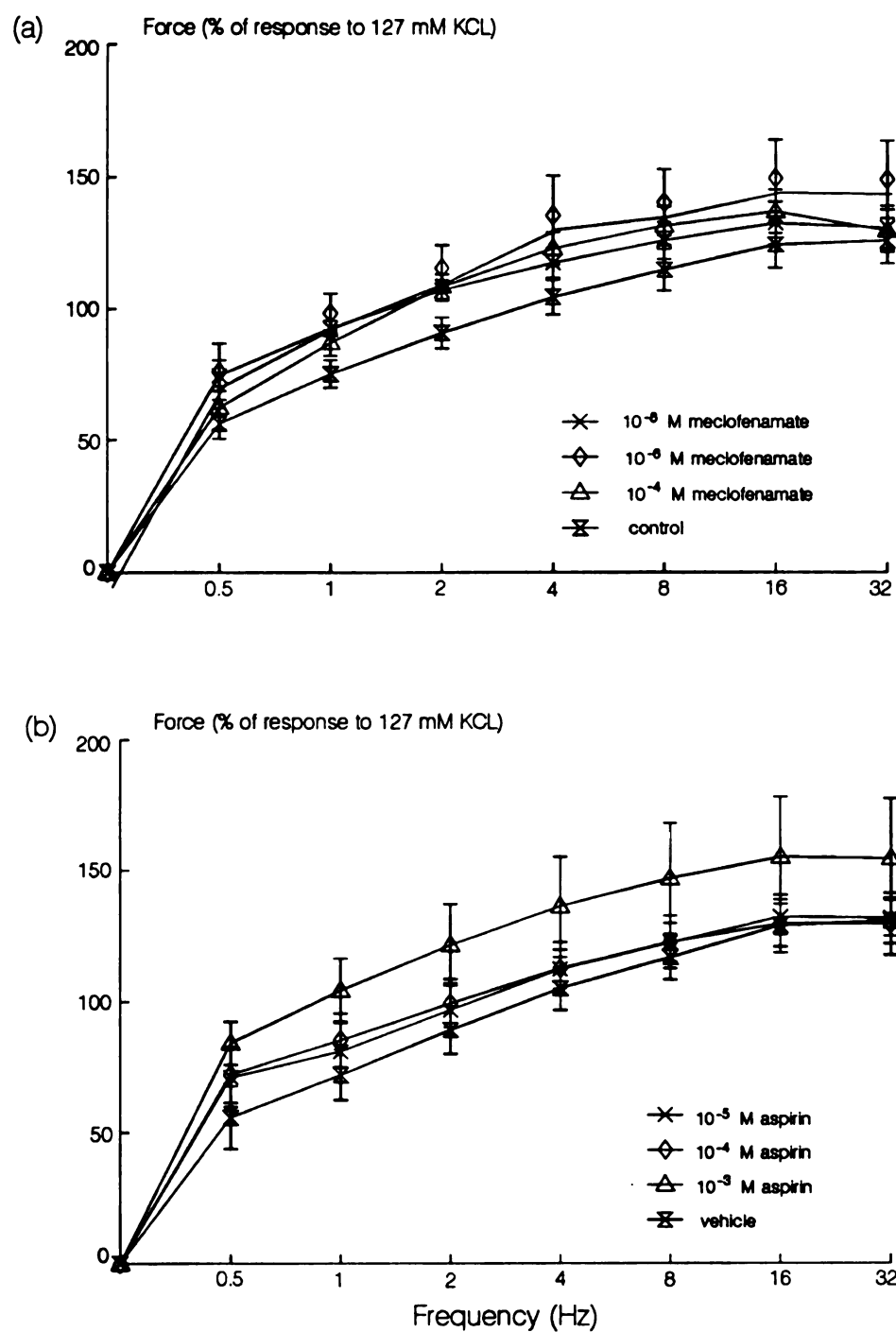


Fig. 4-2. Effect of meclofenamate and aspirin on contractions induced by EFS in trachealis. Single factor randomized design ANOVA was used to compare force at each frequency of stimulation. No statistically significant differences were observed. $n = 8$ in aspirin vehicle, 10^{-5} and 10^{-4} M aspirin-treated groups. $n = 7$ in the rest.

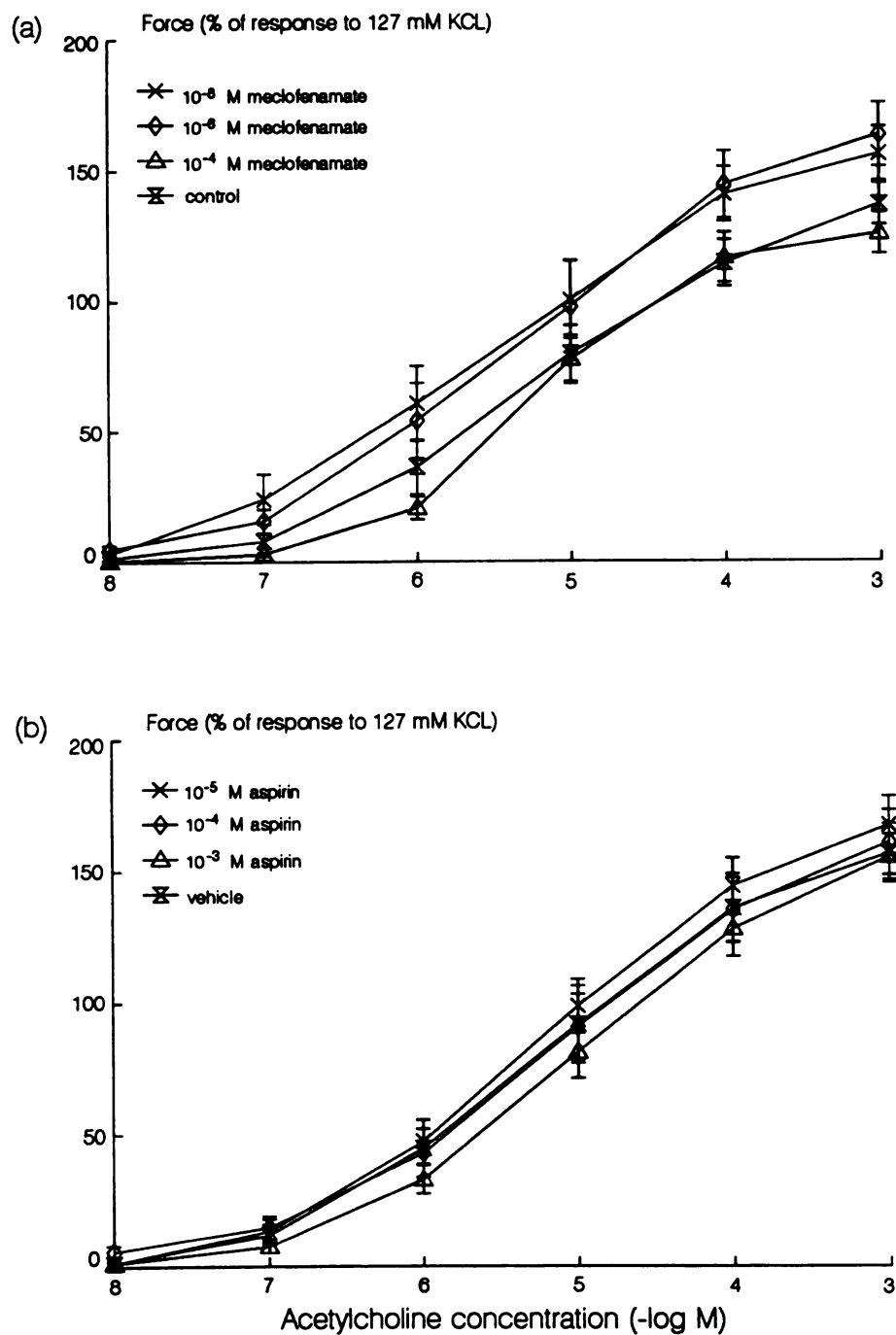


Fig. 4-3. Effect of meclofenamate and aspirin on contractions induced by ACh (ACh) in trachealis. Single factor randomized design was used to compare force at each concentration of ACh. No statistically significant differences were found. $n = 6$ in 10^{-8} and 10^{-4} M meclofenamate-treated groups. $n = 7$ in 10^{-6} M meclofenamate and 10^{-3} aspirin-treated groups. $n = 8$ in the rest.

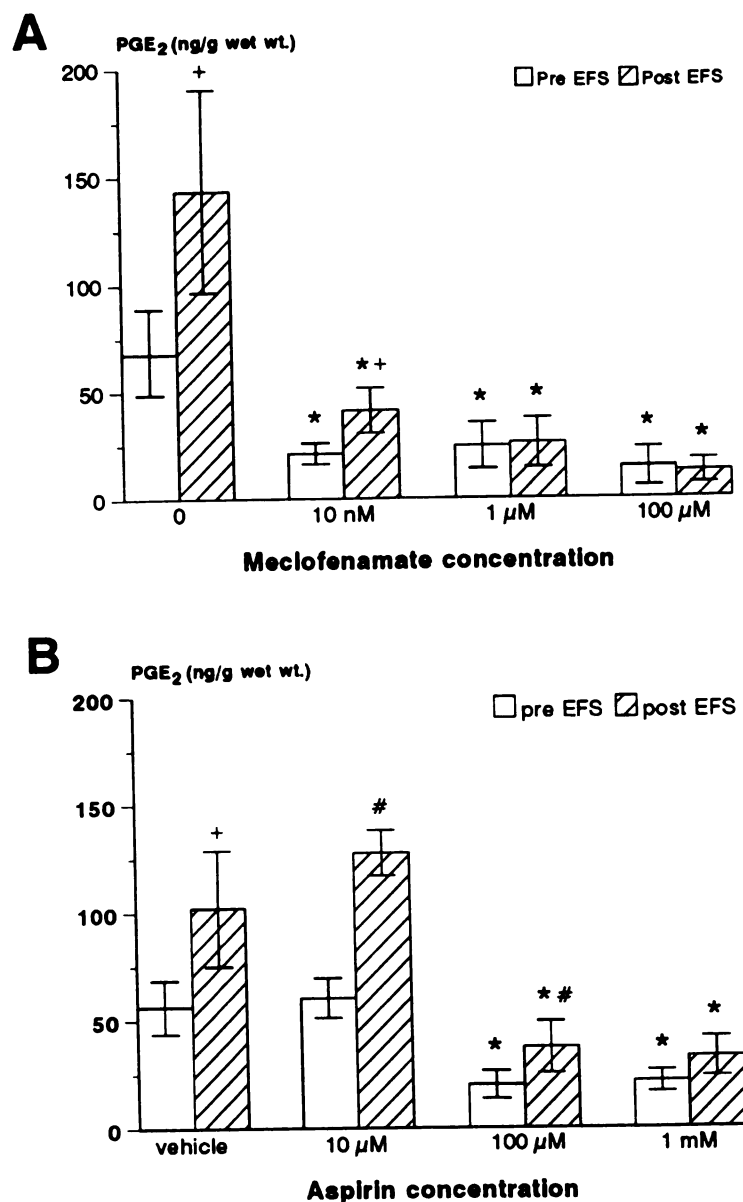


Fig. 4-4. Effect of meclofenamate and aspirin on PGE₂ production by tracheal strips subjected to EFS. Single factor randomized design ANOVA was used to compare PGE₂ levels at different concentrations of the two drugs and paired t-test was used to compare values before and after EFS. * = significantly different from the corresponding control value. # = significant increase in PGE₂ production after EFS. + = p values of 0.051, 0.052, and 0.055 for comparison of pre- and post-EFS PGE₂ production in the meclofenamate vehicle, 10⁻⁸ M meclofenamate and aspirin vehicle-treated tissues, respectively. n = 5 in all groups.

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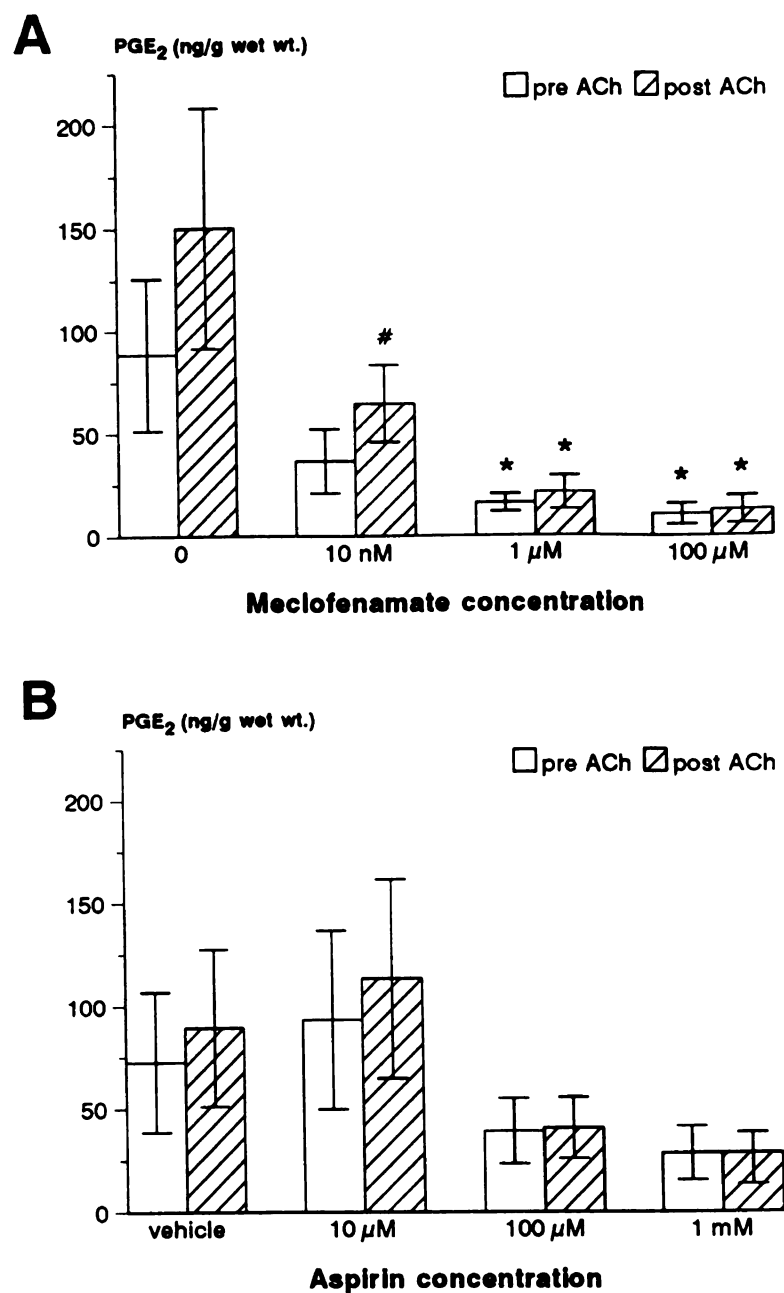


Fig. 4-5. Effect of meclofenamate and aspirin on PGE₂ production by tracheal tissues subjected ACh. Single factor randomized design ANOVA was used to compare PGE₂ production at different concentrations of the two inhibitors and paired t-test to compare values before and after ACh. * = significantly different from the corresponding control value. # = significant increase in PGE₂ production after EFS. n = 4 in 10⁻⁸ M meclofenamate-treated group. n = 5 in the rest.

CHAPTER 5

PGE₂ INHIBITS ACETYLCHOLINE RELEASE FROM CHOLINERGIC

NERVES IN CANINE BUT NOT EQUINE AIRWAYS

Introduction

Heaves is a type of chronic obstructive pulmonary disease in horses and ponies. Affected animals develop airway obstruction when stabled and fed hay and enter remission at pasture. Atropine markedly reduces the increased airway resistance of horses with heaves, indicating the involvement of muscarinic receptors in airway obstruction (Broadstone et al. 1988). A comparison of the response of airway smooth muscle to ACh and EFS suggests that increased ACh release from airway cholinergic nerves is at least in part responsible for the increased response to muscarinic blockade in horses with heaves (Broadstone et al. 1991). The elevated release of ACh from cholinergic nerves could be due to alterations in prejunctional regulation.

Prostanoids have an important modulatory effect on airway cholinergic neurotransmission. An inhibitory effect of PGE₂ on airway cholinergic neurotransmission is well documented in dogs. Low concentrations of exogenous PGE₂ inhibit the contractile response of airway smooth muscle to EFS but have little or no effect on that to exogenous ACh. Furthermore, inhibition of endogenous prostanoid production by cyclooxygenase inhibitors potentiates the response to EFS with little or no effect on the

response to ACh (Nakanishi et al. 1976; Waters et al. 1984). PGE₂ also inhibits the amplitude of EFS-induced EJPs (Inoue et al. 1984; Ito and Tajima 1981a). Direct measurements of ACh release have shown that PGE₂ inhibits ACh release from airway cholinergic nerves and that cyclooxygenase inhibition has the opposite effect (Deckers et al. 1989a; Shore et al. 1987).

The airway subepithelial tissues of heavy horses have a reduced ability to release PGE₂ when compared with those of control horses (Gray et al. 1992a). Based on the above evidence from dogs, I hypothesized that PGE₂ can inhibit ACh release from the cholinergic nerve terminals of control horses and that this effect of PGE₂ is lacking or attenuated in heavy horses. In order to test this hypothesis, it was first necessary to determine if PGE₂ has a modulatory effect on airway cholinergic neurotransmission in control horses.

In the study that I described in Chapter 4, I concluded that exogenous PGE₂ has an inhibitory effect on cholinergic neurotransmission in pony trachea because it has a greater inhibitory effect on the contractile response of trachealis to EFS than to ACh, and that endogenous prostanoids have no modulatory effect on cholinergic neurotransmission in pony trachea because inhibition of cyclooxygenase has no influence on the contractile response of trachealis to EFS. However, this second conclusion is not in agreement with results obtained in horse airways. Yu et al. (in press a) observed that cyclooxygenase inhibition augmented the contractile response of horse tracheal and, in particular, bronchial smooth muscle preparations to EFS to a much greater extent than the response to exogenous ACh, suggesting that endogenous prostanoids inhibit cholinergic neurotransmission in the horse airways. In order to clarify the role of PGE₂ on equine

airway cholinergic neurotransmission, it became essential to directly measure ACh release from airway cholinergic nerves.

In pilot studies conducted on several horses, I was unable to detect an effect of either PGE₂ or cyclooxygenase inhibition on ACh release from airway cholinergic nerves even though such an effect had been demonstrated in dogs. The purpose of the present experiment was therefore to compare the effect of exogenous PGE₂ and endogenous prostanoids on ACh release from horse and dog tracheal and bronchial cholinergic nerves.

Materials and methods

Preparation of tissues

Seven dogs (three female, four male, body weight 18.8 ± 1.2 kg) and five horses (two female, three male, body weight 421.4 ± 41.5 kg) were used for this study. The horses had no history of heaves. Immediately after euthanasia by intravenous injection of an overdose of pentobarbital sodium, the thoracic cavity was opened and the trachea and lungs were removed. The lungs from all the animals were normal in gross appearance. In dogs, the trachea and both lungs were immersed in Krebs-Henseleit solution (composition in mM: NaCl 118.4, NaHCO₃ 25.0, dextrose 11.7, KCl 4.7, CaCl₂·2H₂O 2.6, MgSO₄·7H₂O 1.19, KH₂PO₄ 1.16) that had been bubbled with 95% O₂/5% CO₂. In horses, the trachea between the twenty-first and thirtieth cartilaginous rings above the carina and a piece of lung tissue from the middle part of one lung were immersed in Krebs-Henseleit solution. The tissues were transferred to the laboratory

within 10–15 min after dissection, where they were continually gassed with 95% O₂/5% CO₂.

Tracheal and bronchial smooth muscle preparations with epithelium were made from the airways of both species. Typically, six tracheal and six bronchial preparations were prepared from each animal. In dogs, the trachea was cut into segments containing two or three cartilage rings. The loose connective tissue on the serosal side was dissected away. The dorsal tracheal membrane adjacent to the tips of the cartilage was tied with silk suture. The tissue, which included mucosa, serosa and all tissues between, was then cut free from the cartilage. Bronchial rings (3–5 mm in outside diameter, 15 mm in length) were prepared from random locations of both lungs by dissecting away the surrounding lung parenchyma.

In horses, trachealis strip bundles were prepared using the method described previously (Chapter 2). In addition, bronchial rings of size similar to those of the dogs were prepared.

Protocols

The tissue preparations were then suspended in twelve 2-ml tissue baths (Radnoti Glass Technology, Inc., Monrovia, CA). Each bath had a pair of parallel platinum wire electrodes built against the wall in the vertical direction. For tracheal strips or bundles, one end of the preparation was fixed to the tip of a glass tissue holder. The suture on the other end was attached to a steel bar through a piece of elastic. The tension applied to the tissue was well within the range of 2–20 grams, which has been determined to have no influence on ACh release from airway cholinergic nerves (Chapter 2). The

bronchial tubes were gently guided on to the shorter arm of an "L"-shaped glass tissue holder. To prevent the bronchial tube from floating above the surface of the bath solution, a piece of silk suture was passed through a hole in the bronchial wall and tied to the hook at the end of the tissue holder. Tissue baths contained Krebs-Henseleit solution, which was maintained at 38°C and bubbled with 95% O₂/5% CO₂.

Tissues were equilibrated in the baths for approximately two hours, during which time the bath solution was replaced at 10–15 min intervals. This extended equilibration period was necessary in order to reduce the amount of foam formed during bubbling. In a 2-ml bath, foam formation results in overflow of bath solution so that ACh release cannot be accurately determined. After equilibration, tissues were used to study the effect of either endogenous prostanoids or exogenous PGE₂ on ACh release. The general protocols are described below and the specific experimental procedures for each bath are listed in Tables 5-1 and 5-2.

To determine the effect of endogenous prostanoids on airway cholinergic neurotransmission, tissues were incubated with atropine (3×10^{-7} M) and neostigmine (1.5×10^{-6} M) for 30 min before four periods of 12-min EFS were applied. Period 1 was applied in the absence of cyclooxygenase inhibitors, whereas periods 2-3 were in the presence of a cyclooxygenase inhibitor or its vehicle. A 60-min period was allowed between period 1 and period 2 for incubation with a cyclooxygenase inhibitor or vehicle. Intervals of 30 min were allowed between subsequent stimulation periods. Indomethacin (3×10^{-6} M) was used as the cyclooxygenase inhibitor for dog tissues. For horses, it was necessary to compare the results with those obtained from dogs as well as to closely mimic the conditions of an earlier indirect study (Yu et al. in press a) in which

meclofenamate was used as a cyclooxygenase inhibitor. For these reasons, both meclofenamate (10^{-6} M) and indomethacin were used, and EFS with a set of different parameters was applied to the meclofenamate-treated tissues.

Tissues used to determine the effect of PGE_2 were preincubated with atropine (3×10^{-7} M), neostigmine (1.5×10^{-6} M), and indomethacin (3×10^{-6} M) for 120 min before four 12-min periods of EFS were applied at 30-min intervals. The ACh release during period 1 served as control. After period 1, in addition to the three preincubation drugs, either PGE_2 or its vehicle was added to each tissue bath. Because Shore et al. (1987) had reported a time-dependency of the effect of PGE_2 on ACh release in dog airways, only one concentration of PGE_2 was added to each bath containing dog tissues, and ACh release was measured following the three subsequent EFS periods. However, analysis of our dog data revealed no such time-dependency, and therefore PGE_2 was added to horse tissues in a cumulative way. The tissues were incubated with each concentration of PGE_2 or its vehicle for 20–25 min before they were electrically stimulated in the presence of this concentration of PGE_2 or its vehicle. The concentration of PGE_2 vehicle used in the dog experiment was equivalent to that used to dissolve 10^{-7} M PGE_2 . In the horse experiment, vehicle concentration matched that used to dissolve each concentration of PGE_2 .

At the end of each EFS period, 1.5 ml of the bath solution was collected from each bath for ACh analysis. The tissue bath was flushed with Krebs-Henseleit solution containing atropine (3×10^{-7} M) and neostigmine (1.5×10^{-6} M) with or without indomethacin (3×10^{-6} M) for 2–3 times before and after each EFS period. Each time the bath solution was replaced, various drugs were replenished at appropriate concentrations.

Electrical impulses were produced by a stimulator (S88, Grass Instrument Co., Quincy, MA) and passed through a stimulus power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO). The electrical impulses consisted of square waves. The parameters of EFS were 15 V, 5 Hz, and 2 ms for all tissues except those used to study the effect of meclofenamate. In the latter case, the parameters were 15 V, 1 Hz, and 0.5 ms, which had been used in the previous indirect study (Yu et al. in press a).

The wet weight of each tissue preparation was determined at the end of the experiment after blotting with filter paper. It averaged in mg 153.7 ± 5.2 ($n = 36$), 151.8 ± 5.3 ($n = 36$), 210.7 ± 5.6 ($n = 30$), and 193.5 ± 16.8 ($n = 30$) for dog tracheal, dog bronchial, horse tracheal, and horse bronchial preparations, respectively.

ACh analysis

Bath liquid samples were first filtered through 0.2μ nylon membranes (Acrodiscs 13, Gelman Sciences, Ann Arbor, MI) and then analyzed by HPLC coupled with electrochemical detection. The method was the same as that I described in Chapter 2 except for the following modifications: 1) A precolumn (Bioanalytical System, Inc., West Lafayette, IN) containing choline oxidase and catalase was added before the analytical column to remove choline in the samples. This enabled me to shorten the length of each run without concern about the separation of ACh and choline. 2) The mobile phase was 50 mM Na_2HPO_4 (pH = 8.5) and the flow rate was 1 ml/min. 3) A calibration standard of 20 pmol ACh was injected every 4 samples and the ACh amount in the samples was calculated based on the bracketed calibrations. 4) A new autosampler (ISS200, Perkin-Elmer, Norwalk, CT) replaced the old model.

Drugs

Neostigmine methylsulfate, atropine sulfate, and indomethacin (Sigma Chemical Co., St. Louis, MO.) solutions were prepared on the day of experiment. Neostigmine and atropine were dissolved in water to concentrations of 10^{-2} and 10^{-3} M, respectively. Indomethacin was dissolved in solutions of equimolar sodium carbonate (Malinckrodt, Inc., Paris, KY) to a concentration of 10^{-2} M. These solutions of drugs were mixed into a bulk volume of Krebs-Henseleit solution to reach their final concentrations. PGE₂ (Cayman Chemical Company, Ann Arbor, MI) was first dissolved with methanol (1 mg/ml) and subsequently diluted to 10^{-5} M with distilled water. The 10^{-5} M PGE₂ was stored as a stock solution at -15°C and diluted with water before use. Meclofenamate sodium monohydrate (courtesy of Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI) was dissolved in and diluted with water on the day of experiment. The PGE₂ and meclofenamate solutions were pipetted into the tissue bath in 20 μl volume (1 % of the bath volume). The concentrations of all drugs were expressed as their final bath concentrations.

Statistical analysis

To determine the effects of cyclooxygenase inhibition and exogenous PGE₂ on ACh release, the ACh release in each bath during the first EFS period was regarded as 100%, and the release during subsequent periods was expressed as a percentage of this value. Depending on the number of treatment groups, either an unpaired *t*-test or one-way ANOVA for randomized measures followed by Tukey's HSD test was used to determine the effects of PGE₂ or cyclooxygenase inhibition. To compare data between

the two species, ACh release was expressed as pmol/g/min and analyzed by unpaired *t*-test. All values were expressed as mean $\pm$ SE. *P* < 0.05 was considered statistically significant.

Results

All tissue preparations released a measurable amount of ACh during each EFS period. Typical chromatograms are shown in Figure 5-1. There was a significant difference in ACh release rate (pmol/g/min) between the two species. In tracheal preparations, the ACh release rate in horse was about twice of that in dog. In bronchial preparations, the ACh release rate in dog was approximately three times that in the horse (Fig. 5-2).

Dog airways

In the absence of cyclooxygenase inhibition, ACh release was fairly constant over the four stimulation periods. Cyclooxygenase inhibition by indomethacin significantly augmented ACh release in both the tracheal (Fig. 5-3A) and bronchial (Fig. 5-3B) preparations. The effect was greater in the trachea than in the bronchi. Although ACh release tended to increase gradually following indomethacin administration in the trachea, there was no significant difference among periods 2 to 4 (Fig. 5-3A).

PGE₂ concentration-dependently inhibited ACh release from dog airway cholinergic nerves and the effect was greater in the trachea (Fig. 5-4A) than in the bronchi (Fig. 5-4B). There was no difference in ACh release values among the three EFS periods subsequent to administration of a dose of PGE₂.

Horse airways

In both trachea and bronchi, the release of ACh was constant over the four stimulation periods in the absence of cyclooxygenase inhibition. In contrast to the results obtained in dog tissues, neither indomethacin (Fig. 5-5) nor meclofenamate (Fig. 5-6) significantly influenced ACh release from either the tracheal or bronchial preparations of horses.

In the trachea, 10^{-9} and 10^{-8} M PGE₂ did not affect ACh release. However, 10^{-7} M PGE₂ significantly augmented ACh release (Fig. 5-7A). PGE₂ had no effect on ACh release from horse bronchial preparations (Fig. 5-7B).

Discussion

The major findings of the present study were that exogenous PGE₂ inhibited ACh release from canine airway cholinergic nerves and inhibition of endogenous prostanoid production by a cyclooxygenase inhibitor had the opposite effect. These results could not be reproduced in horses.

An inhibitory effect of exogenous PGE₂ and endogenous prostanoids on ACh release from canine airway tissues has also been reported by Deckers et al. (1989a) and Shore et al. (1987). Like me, they also observed that cyclooxygenase inhibition by indomethacin augmented ACh release. With respect to the effect of exogenous PGE₂, Shore et al. measured ACh release induced by three repeated 15-min EFS periods in indomethacin-pretreated tissue and observed that 10^{-8} M PGE₂ prevented the gradual increase in ACh release on consecutive stimulation periods. This is similar to my observation except that Shore et al. observed a greater time-dependency in the effect of

PGE₂. Because the effect of PGE₂ was not observed during the first period of EFS but developed slowly over time, Shore et al. reasoned that PGE₂ may affect the synthesis or mobilization of ACh rather than the release process itself. In the present study, PGE₂ (10⁻⁹ - 10⁻⁷ M) inhibited ACh release in a concentration-dependent manner after 20-25 min preincubation and the effect of PGE₂ did not change over time. Therefore, my results favor an effect of PGE₂ on the release rather than the synthesis of ACh.

The mechanism of action of PGE₂ on ACh release from canine airway cholinergic nerves is not fully elucidated. In sympathetic nerves, the mechanism whereby PGE₂ inhibits norepinephrine release is believed to be inhibition of Ca²⁺ influx (Malik and Sehic 1990), probably via the EP3 receptor (Racké et al. 1992). PGE₂ could be working through a similar mechanism in canine airway cholinergic nerves.

My results with dog tissues indicate that both indomethacin and PGE₂ had a greater effect on ACh release in the trachea than in the bronchi. These observations suggest that the higher response of tracheal tissue to PGE₂ and indomethacin is more likely a result of higher sensitivity to PGE₂ rather than an increased PGE₂ concentration in the tracheal tissue.

In the present study, I did not observe either an inhibitory effect of exogenous PGE₂ or an augmenting effect of cyclooxygenase inhibitors on ACh release from horse airway cholinergic nerves. Although the results obtained with PGE₂ and cyclooxygenase inhibitors are in mutual agreement, they are contradictory to the conclusion of a previous study (Yu et al. in press a), which, based on comparisons of the contractile response of horse airway smooth muscle preparations to EFS and exogenous ACh, suggests that endogenous prostanoids inhibit ACh release from horse airway cholinergic nerves.

Failure to observe an inhibitory effect of exogenous PGE_2 and endogenous prostanoids on ACh release from horse airway cholinergic nerves is unlikely to be due to a lack of sensitivity of my ACh measuring technique. The sensitivity of my technique is well exemplified by the fact that: 1) under the same experimental conditions, I was able to confirm the inhibitory effect of prostanoids on ACh release from dog airway cholinergic nerves; and 2) using the same technique, I have detected a clear concentration-dependent inhibition of ACh release from horse airway cholinergic nerves by α_2 -adrenoceptor agonists (Chapter 6).

For technical reasons, direct measurement of ACh release from airway cholinergic nerves became possible only recently. Previously, most of our knowledge about prejunctional modulation of airway cholinergic neurotransmission was inferred by comparing the effect of a potential neuromodulator on the contractile response of airway smooth muscle to EFS and exogenous muscarinic agonist such as ACh. A greater effect on the response to EFS than ACh suggests prejunctional modulation. To use this approach, it is necessary to assume that exogenous and neurally released ACh behave similarly. However, this is not completely true. Endogenous ACh is released in small amounts. Its effect is quickly terminated due to its breakdown to choline and acetate by cholinesterase, which is located almost exclusively in or around the nerve terminals (Baker et al. 1986). The density of innervation of airway smooth muscle is generally sparse. Although smooth muscle cells near the varicosities of cholinergic nerve terminals are believed to be directly activated by endogenous ACh, it is questionable if ACh molecules can diffuse to the cells further away before its degradation. These latter cells are probably activated by electrical coupling through gap junctions or other structures

(Gabella 1987; Kannan and Daniel 1980). By contrast, exogenous ACh is usually administered in larger amounts so that the ACh molecules distribute throughout the tissue preparation and induce smooth muscle contractions by acting on numerous muscarinic receptors of each individual cell. Because the amount of exogenous ACh is usually in great excess to the cholinesterase activity of the tissue, a long-lasting plateau in tension is often observed following addition of each concentration of ACh. Because of these differences in the actions of endogenous and exogenous ACh, factors that directly affect airway smooth muscle cells influence the response of the tissue to endogenous ACh more easily than to exogenous ACh. Among all the factors that have been raised as prejunctional modulators of airway cholinergic nerves, few lack a direct postjunctional effect. In addition, all kinds of nerves in the tissue preparation are activated during EFS, release of other neurotransmitters may influence the response to EFS but not to exogenous ACh. Furthermore, if a factor has a modulatory effect on gap junctions of airway smooth muscle cells, it may influence the contractile response to EFS but not to exogenous ACh. A remarkable modulatory effect of PGE_2 on gap junctions in canine airway smooth muscle has already been reported (Agrawal and Daniel 1986). For the above reasons, it may be not always reliable to infer conclusions about prejunctional modulation simply by comparing the contractile responses of airway smooth muscle to EFS and exogenous ACh.

I was not the first to observe a species difference in the response of airway cholinergic nerves to PGE_2 . Based on results of an indirect study, Black et al. (1989) concluded that PGE_2 inhibits cholinergic neurotransmission in rabbit but not human bronchus. They explained that the difference in the response to PGE_2 between species

results from a difference in the dependency of ACh release on voltage dependent Ca^{2+} channels, based on the observation that the Ca^{2+} channel agonist BAY K8644 augmented the contractile response to EFS in the rabbit but not human bronchus. However, it may be questionable to assume a prejunctional effect of BAY K8644 in the rabbit bronchus when a postjunctional effect was observed in the same preparation.

ACh release from airway cholinergic nerves is subject to feedback inhibition through muscarinic autoreceptors. The nonspecific muscarinic antagonist atropine can remove this autoinhibitory effect (Chapters 2, 3). It has been reported that the augmenting effect of indomethacin on ACh release from canine bronchial tissue can be demonstrated in the presence but not in the absence of atropine (Deckers et al. 1989a). Therefore, all tissue preparations were treated with atropine in the present study. In order to demonstrate the neuromodulatory effect of a factor, blockade of muscarinic autoreceptors may be necessary only in such direct studies in which ACh accumulates due to prolonged EFS and inhibition of ACh breakdown by a cholinesterase inhibitor. The fact that the inhibitory effect of PGE_2 on ACh release can be observed in the presence of atropine indicates that PGE_2 does not exert its inhibitory effect through muscarinic receptors.

In conclusion, the present study demonstrated that exogenous PGE_2 and endogenous prostanoids inhibit ACh release from canine but not equine airway cholinergic nerves. The lack of an important role for PGE_2 in the regulation of ACh release from airway cholinergic nerves of the control horses makes it unlikely that a decrease of PGE_2 production is an important factor in the cholinergically mediated airway obstruction characteristic of heaves.

Table 5-1. Detailed protocols for dog tissues

Table 5-1. Detailed protocols for dog tissues

Bath #	Cyclooxygenase inhibition				Exogenous PGE ₂							
	1	2	3	4	5	6	7	8	9	10	11	12
Trachea (T) or Bronchi (B)	T	B	T	B	T	B	T	B	T	B	T	B
Equilibration												
Incubation	Atropine (3×10^{-7} M) and neostigmine (1.5×10^{-6} M), and indomethacin (3×10^{-6} M) for 120 min											
EFS parameters	15 V, 5 Hz, 2 ms											
S P	1	Control										
	2	indomethacin (3×10^{-6} M)	indomethacin vehicle	PGE ₂ vehicle	PGE ₂ (10^{-9} M)	PGE ₂ (10^{-8} M)	PGE ₂ (10^{-7} M)					
	3	as above	as above	as above	as above	as above	as above	as above	as above	as above	as above	as above
	4	as above	as above	as above	as above	as above	as above	as above	as above	as above	as above	as above

EFS = electrical field stimulation; SP = stimulation period

Table 5-2. Detailed protocols for horse tissues

Exogenous PGE ₂	

Table 5-2. Detailed protocols for horse tissues

		Cyclooxygenase inhibition							Exogenous PGE ₂						
Bath #		1	2	3	4	5	6	7	8	9	10	11	12		
Trachea (T) or Bronchi (B)		T	B	T	B	T	B	T	B	T	B	T	B		
Equilibration															
Incubation		Atropine (3 x 10 ⁻⁷ M) and neostigmine (1.5 x 10 ⁻⁶ M) for 30 min							Atropine (3 x 10 ⁻⁷ M), neostigmine (1.5 x 10 ⁻⁶ M), and indomethacin (3 x 10 ⁻⁶ M) for 120 min						
EFS parameters		15 V, 1 Hz, 0.5 ms				15 V, 5 Hz, 2 ms									
S P		Control													
		1													
		2	meclofenamate (10 ⁻⁶ M)	control			indomethacin (3 x 10 ⁻⁶ M)		indomethacin vehicle		PGE ₂ (10 ⁻⁹ M)		PGE ₂ vehicle		
		3	as above	as above			as above		as above		PGE ₂ (10 ⁻⁸ M)		PGE ₂ vehicle		
4		as above	as above			as above		as above		PGE ₂ (10 ⁻⁷ M)		PGE ₂ vehicle			

EFS = electrical field stimulation; SP = stimulation period

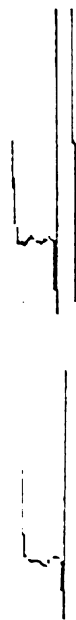


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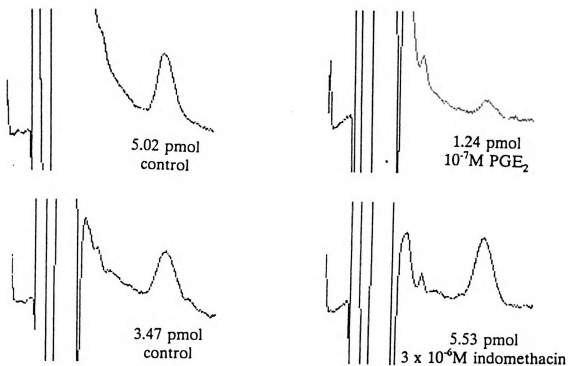


Fig. 5-1. Typical chromatograms showing the effects of PGE₂ and indomethacin on acetylcholine (ACh) release from cholinergic nerves innervating dog trachea. Each pair of chromatograms represents the ACh amount of two samples from the same tissue bath. Because the two samples of each pair were analyzed a few hours apart and the detector sensitivity tended to decrease over time, the proportionality between ACh amount and the peak height was not constant.

ACh release (pmol/g/min)

150

125

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75

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25

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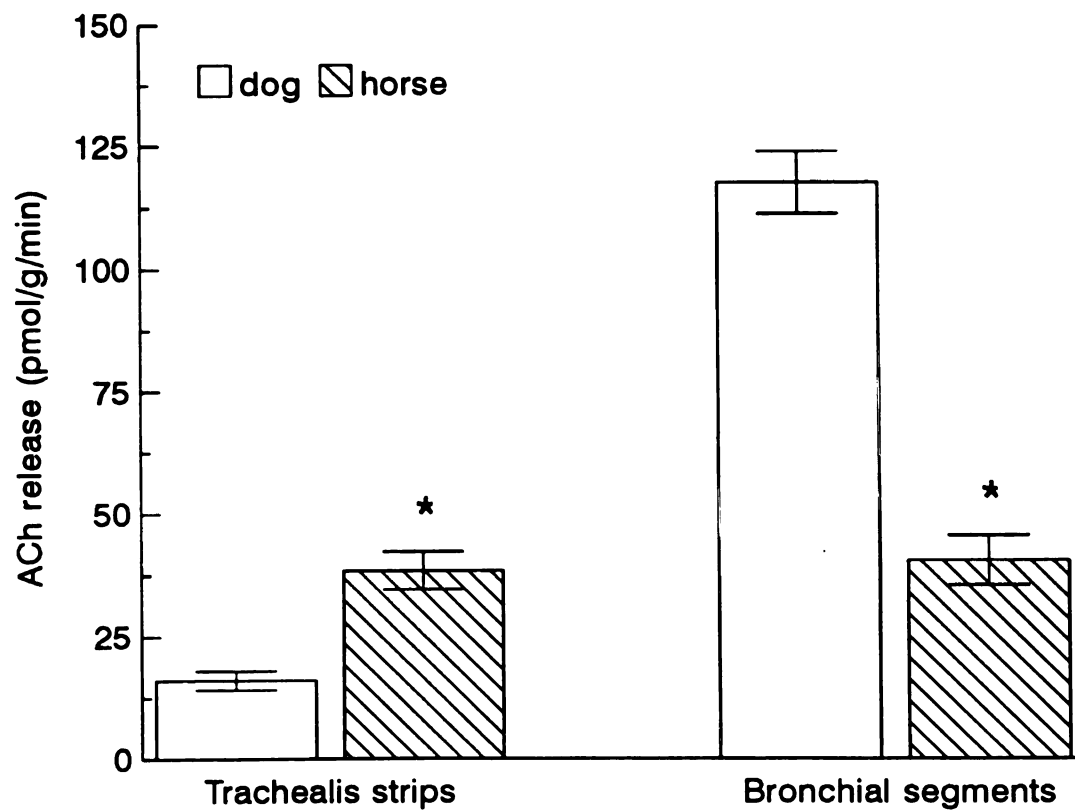


Fig. 5-2. Acetylcholine (ACh) release (pmol/g/min) from cholinergic nerves innervating dog and horse airways. The values represent the ACh release rate during period 1 in tissues used to study the effect of indomethacin and its vehicle. n equals 12 and 10 for dog and horse tissues, respectively. * $P < 0.05$ compared with the dog value.

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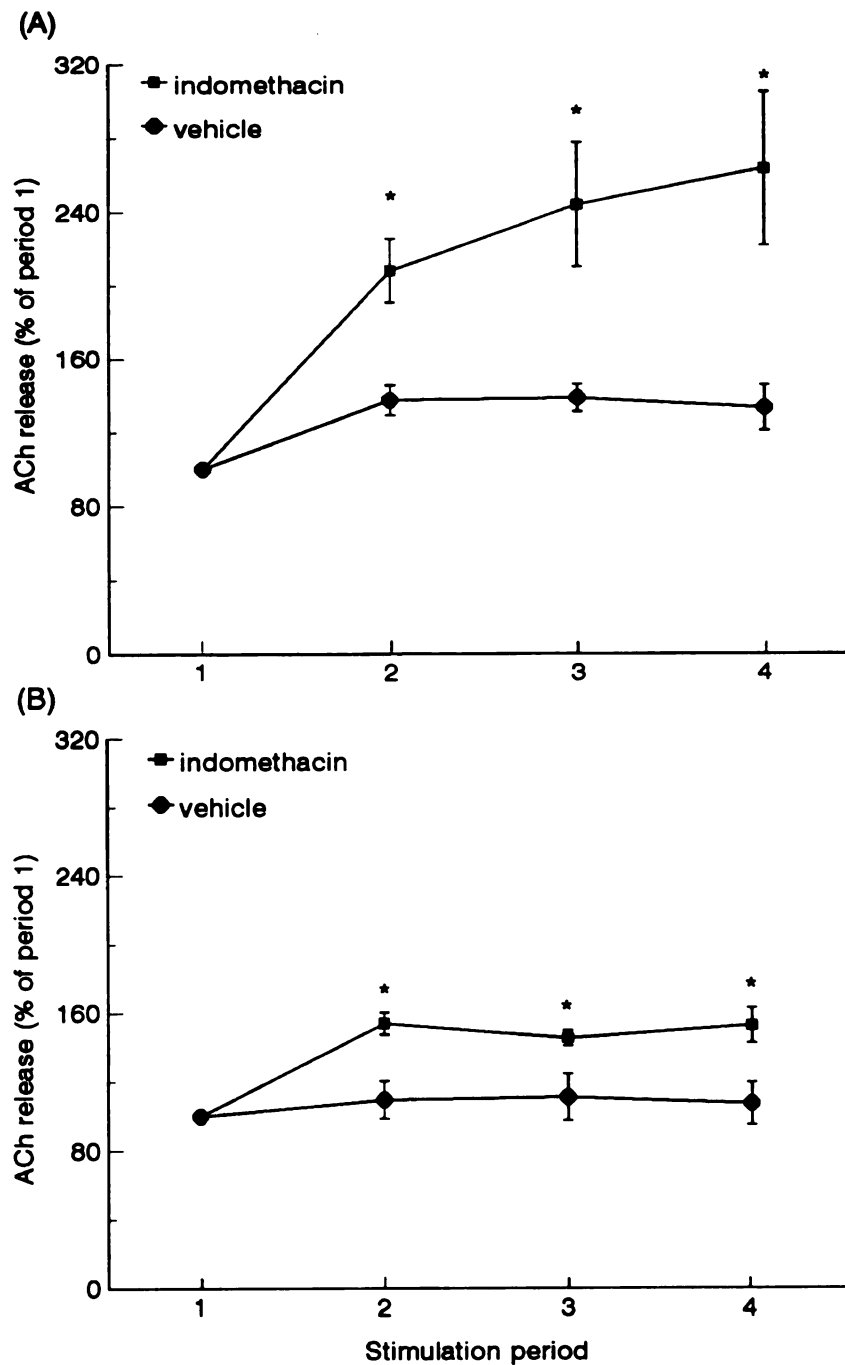


Fig. 5-3. Effect of indomethacin on acetylcholine (ACh) release from cholinergic nerves innervating dog trachea (A) and bronchi (B). Four periods of electrical field stimulation were applied to two groups ($n = 6$ in each group) of tissue. No indomethacin was administered during period 1. During periods 2 to 4, one group was treated with indomethacin (3×10^{-6} M), the other with its vehicle. Indomethacin augmented ACh release significantly. Although ACh release tended to increase progressively following indomethacin in the trachea, there was no significant difference among periods 2 to 4. * $P < 0.05$ compared with the vehicle group.

ACh release (% of period 1)

(B)

160

120

80

40

0

ACh release (% of period 1)

Fig. 5-4. Effects of indomethacin on ACh release in innervating dogs. Indomethacin (10 mg/kg) was applied to the dogs. In period 1, one group was given indomethacin, and the magnitude of ACh release was compared with the control group.

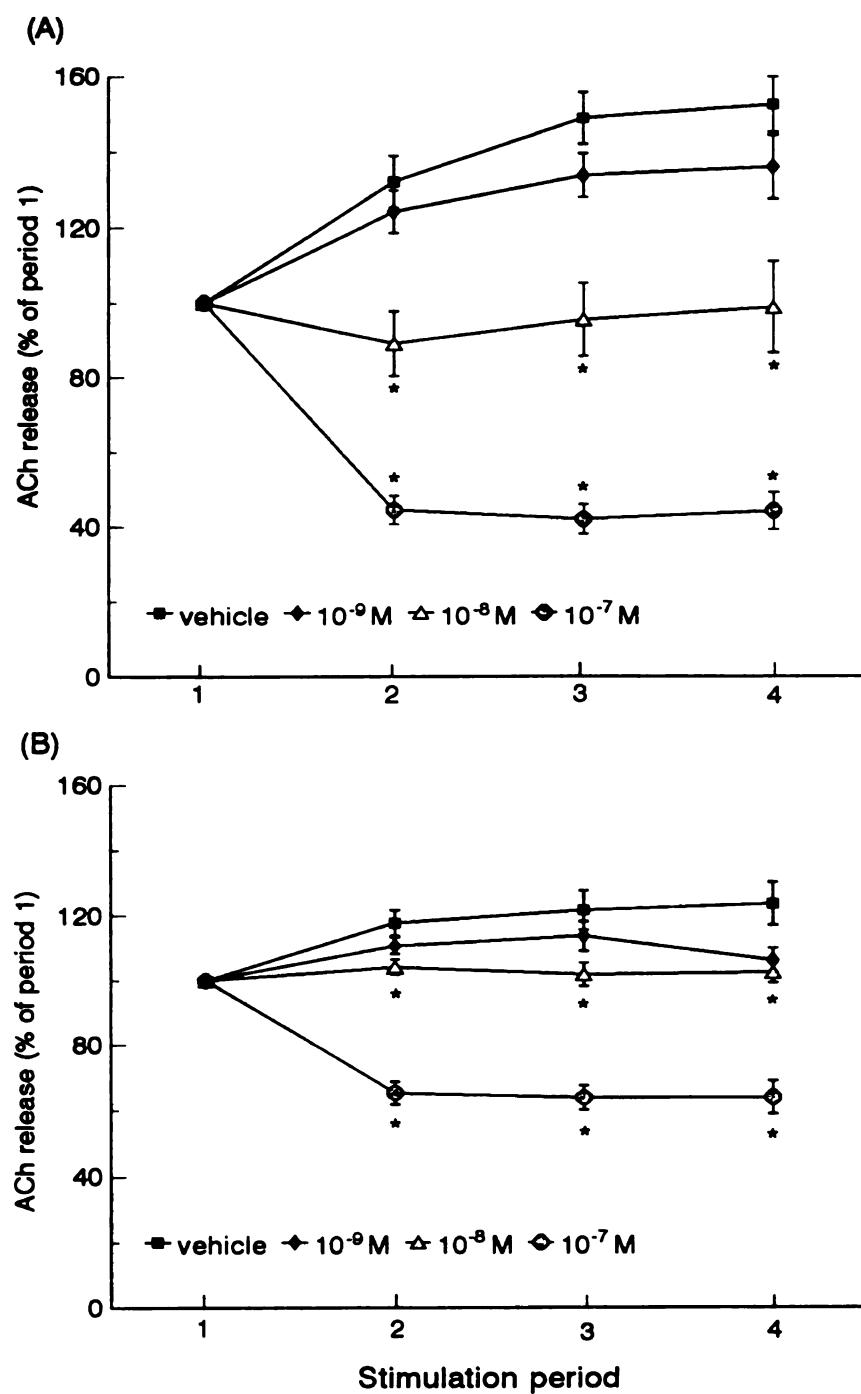


Fig. 5-4. Effect of PGE_2 on acetylcholine (ACh) release from cholinergic nerves innervating dog trachea (A) and bronchi (B). Four periods of electrical field stimulation were applied to four groups ($n = 6$ in each group) of tissue which had been pretreated with indomethacin. No PGE_2 was administered during period 1. During periods 2 to 4, one group was treated with PGE_2 vehicle, whereas the other three were treated with different concentrations of PGE_2 . PGE_2 inhibited ACh release concentration-dependently and the magnitude of inhibition did not change over time. * $P < 0.05$ compared with the control group.

(A)

ACh release (% of period 1)

(B)

ACh release (% of period 1)

Fig. 5-5
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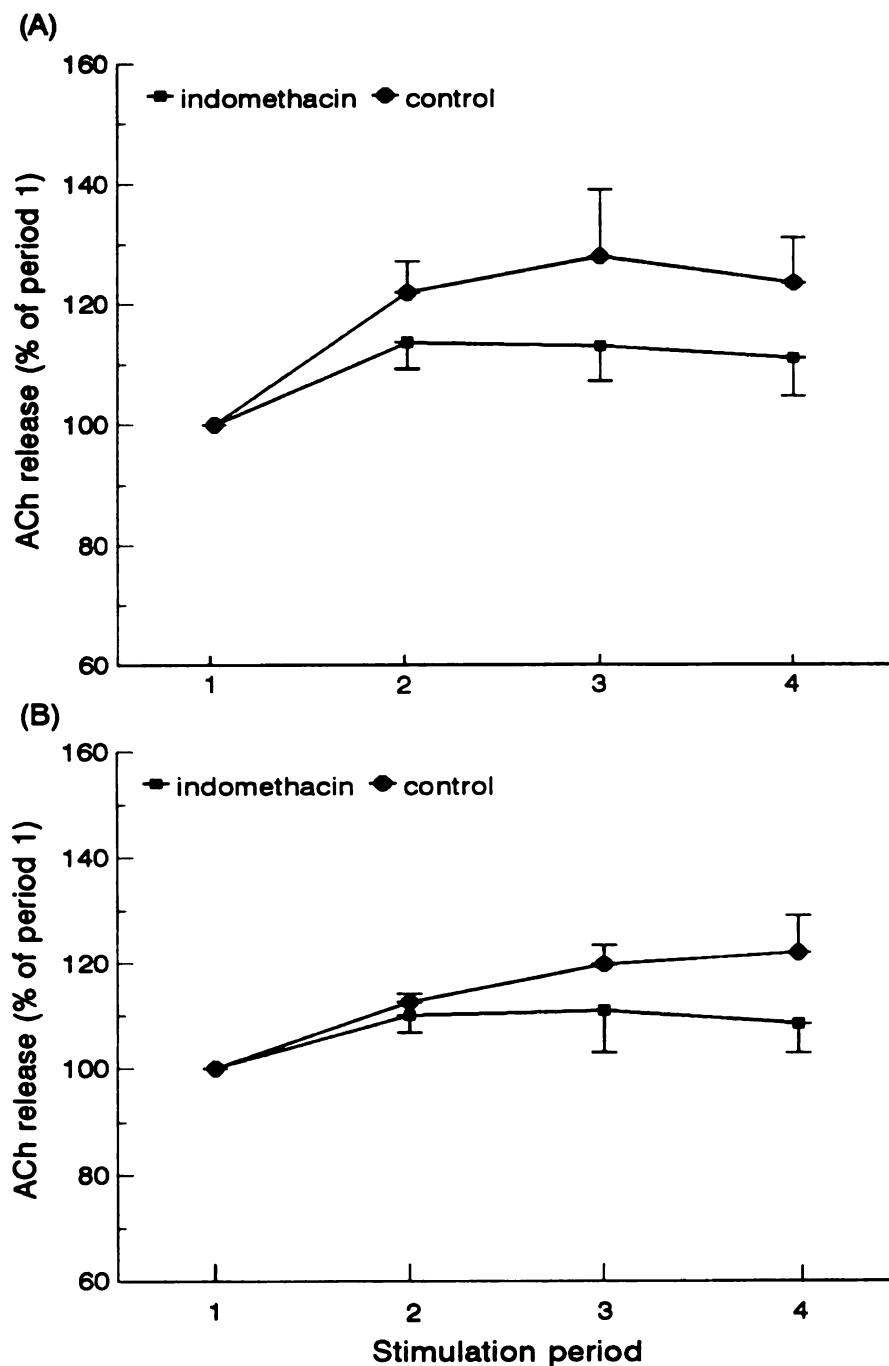


Fig. 5-5. Effect of indomethacin on acetylcholine (ACh) release from cholinergic nerves innervating horse trachea (A) and bronchi (B). Four periods of electrical field stimulation were applied to two groups ($n = 5$ in each group) of tissue. No indomethacin was administered during period 1. During periods 2 to 4, one group was treated with indomethacin (3×10^{-6} M) whereas the other with its vehicle. Indomethacin had no effect on ACh release.

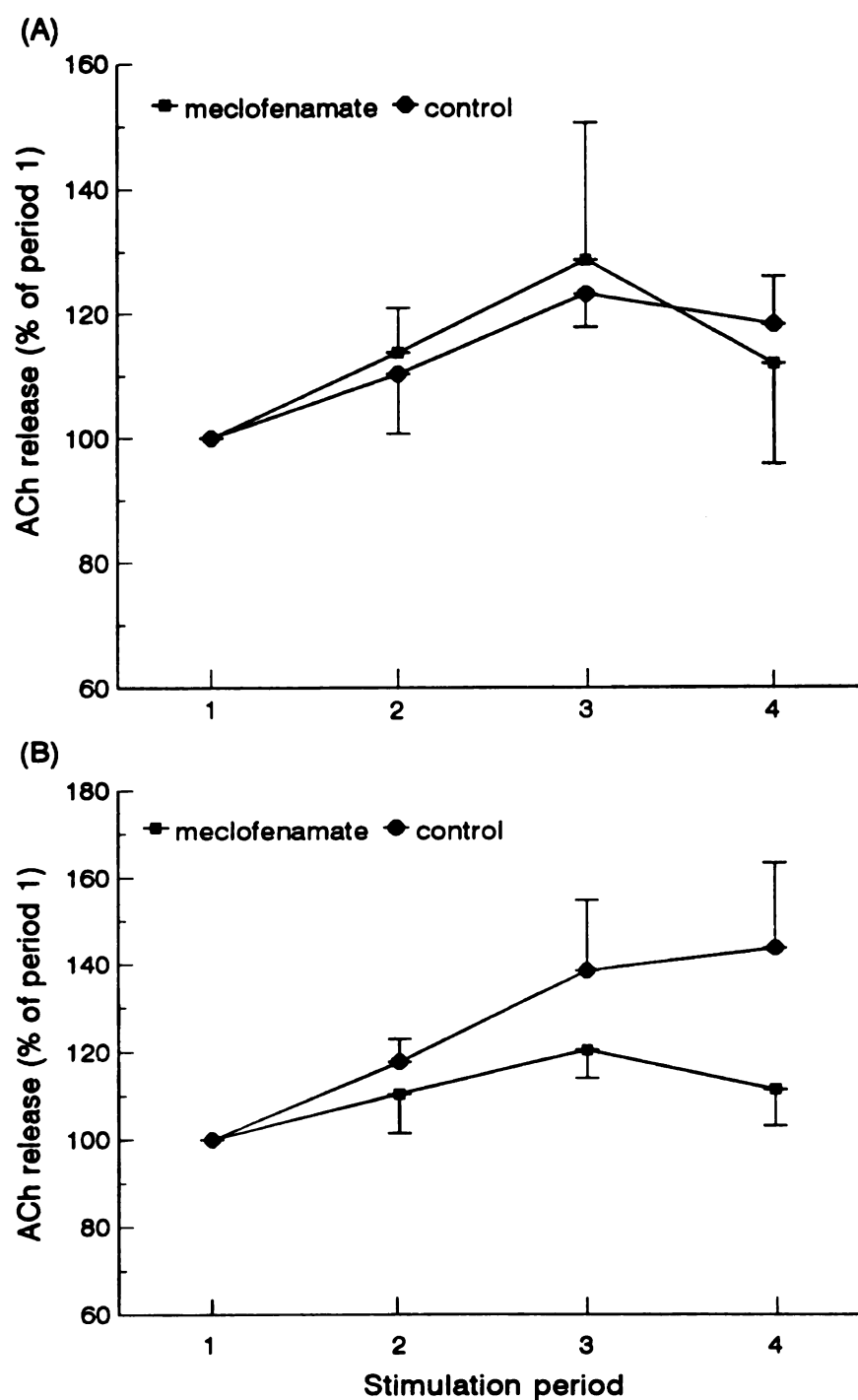


Fig. 5-6. Effect of meclofenamate on acetylcholine (ACh) release from cholinergic nerves innervating horse trachea (A) and bronchi (B). Four periods of electrical field stimulation were applied to two groups ($n = 5$ in each group) of tissue. No meclofenamate was administered during period 1. During periods 2 to 4, one group was treated with meclofenamate (10^{-6} M) whereas the other served as control. Meclofenamate had no effect on ACh release.

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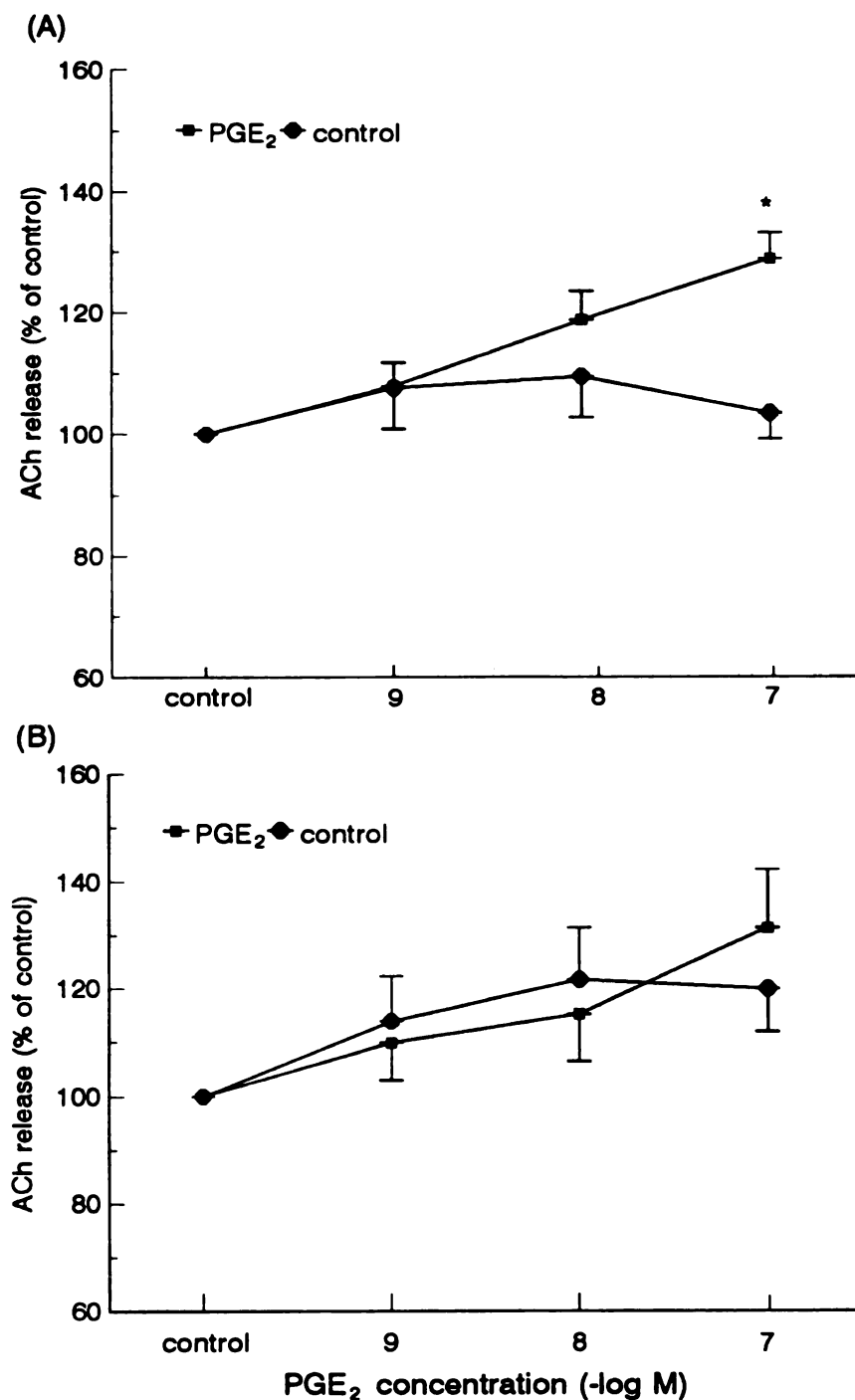


Fig. 5-7. Effect of PGE₂ on acetylcholine (ACh) release from cholinergic nerves innervating horse trachea (A) and bronchi (B). Four periods of electrical field stimulation were applied to two groups ($n = 5$ in each group) of tissue that had been pretreated with indomethacin. No PGE₂ was given during period 1. Periods 2 to 4 were in the presence of increasing concentrations of either PGE₂ or its vehicle. PGE₂ was without effect on ACh release except that 10^{-7} M PGE₂ augmented ACh release significantly in the trachea. * $P < 0.05$ compared with the vehicle group.

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CHAPTER 6

PREJUNCTIONAL α_2 -ADRENOCEPTORS INHIBIT ACETYLCHOLINE RELEASE FROM CHOLINERGIC NERVES IN EQUINE AIRWAYS*

Introduction

The parasympathetic nervous system is the predominant excitatory neural pathway in mammalian airways (Barnes 1986). Its activity can be modulated by a number of factors through actions on prejunctional receptors. Inhibitory α_2 -adrenoceptors have been suggested to be present on airway cholinergic nerves of guinea pigs (Grundström et al. 1981a; Thompson et al. 1990) and humans (Grundström and Andersson 1985). It is yet unclear if prejunctional α_2 -receptors are present on cholinergic nerves supplying equine airway smooth muscle. The interest in equine airways was stimulated by the observation that there is a predominant muscarinic component to airway obstruction in the equine airway disease known as heaves (Broadstone et al. 1988).

The most common approach for studying prejunctional modulation is to compare the effect of a potential neuromodulator on the contractile response of airway smooth muscle to EFS and to an exogenous muscarinic agonist such as ACh. A greater effect on the response to EFS than to ACh suggests that this factor has a prejunctional

* This study was conducted in conjunction with Mingfu Yu, who performed the experiments involving muscle tension measurements.

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modulatory effect. In this indirect approach, ACh release is inferred solely from the contractile response of smooth muscle. Conclusions about prejunctional modulation may not be reliable, because inhibitory neurotransmitters released concurrently with ACh or factors that inhibit the spread of excitation between gap junctions would also cause modulation of the response to EFS but not to ACh. Although direct measurement of ACh release can provide dependable evidence about prejunctional modulation, previous studies on prejunctional α_2 -receptors of airway cholinergic nerves have been mostly indirect (Grundström et al. 1981a; Grundström and Andersson 1985; Thompson et al. 1990). In a study in which ACh release from canine airway cholinergic nerves was directly measured, NE did not influence ACh release (Martin and Collier 1986).

In the present study, I investigated the presence and function of prejunctional α_2 -adrenoceptors on cholinergic nerves innervating horse airway smooth muscle by comparing the responses to EFS and ACh and by measurement of ACh release. I concluded that α_2 -adrenoceptors are present on equine airway cholinergic nerves and that activation of these receptors inhibits cholinergic neurotransmission.

Materials and methods

Sixteen horses (body weight: 381.0 ± 25.8 kg, $\bar{X} \pm \text{SE}$) were used in this study which was approved by the All-University Committee on Animal Use and Care of Michigan State University. Horses had no clinical signs of respiratory disease for several weeks before they were euthanized by injection of an overdose of pentobarbital sodium through the jugular vein. Post-mortem, their lungs were normal in gross appearance. A variety of non-pulmonary tissues was utilized by several other investigators. A

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segment of trachea between the 6th and 15th cartilaginous rings above the carina and a part of the caudal lung were removed quickly, immersed in Krebs-Henseleit solution (composition in mM: NaCl 118.4, NaHCO₃ 25.0, dextrose 11.7, KCl 4.7, CaCl₂ · 2H₂O 2.6, MgSO₄ · 7H₂O 1.19, KH₂PO₄ 1.16), and gassed continuously with 95% O₂ - 5% CO₂.

The trachea was opened longitudinally through the cartilages in its anterior aspect and was pegged flat. Trachealis strips (2 × 30 mm) were cut with a template along the muscle fiber direction. Two bronchial rings (OD = 3–5 mm, length = 15 mm) were dissected free from the surrounding tissues from each of 5 animals. The epithelium was left intact in all the preparations. Trachealis strips were used for both muscle tension studies and ACh release measurement, while bronchial rings were used only in ACh release measurement.

Muscle tension study

Trachealis strips (2 x 10 mm) were tied with 3-0 silk suture and suspended in 15-ml tissue baths containing Krebs-Henseleit solution bubbled with 95% O₂ - 5% CO₂ and maintained at 38°C. One end of each strip was tied to a hook at the lower end of an electrode holder that was placed in the muscle bath. The other end was attached to a force transducer (Grass FT03, Grass Instrument Company, Quincy, MA) mounted on a micromanipulator so that the tissue length could be adjusted. The isometric force generated by the muscle was recorded on a Grass polygraph (model 7E or 7D). Tissues were suspended between platinum electrodes for EFS. Electrical pulses were produced

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by a stimulator (Grass S88) and passed through a stimulus power booster (Stimu-Splitter II, Med Lab Instrument, Loveland, CO).

Tracheal strips were equilibrated for approximately 100 min with a predetermined optimal passive tension of 2 grams applied and maintained. During the equilibration, EFS (20 V, 16 Hz, 0.5 ms) was applied to tissues for durations of 2–3 min at 8- to 10-min intervals until the baseline was stable and the magnitude of the response to this stimulus was consistent. The bath solution was changed every 15 min. At the end of the equilibration, the contractile response to 127 mM KCl-substituted Krebs-Henseleit solution was determined. This response was used to normalize the strength of subsequent muscle contractions. Tissues were then rinsed with Krebs-Henseleit solution until the muscle tension returned to baseline.

Protocol 1: Effect of α_2 -receptor agonists clonidine and UK 14,304 on EFS-induced contraction—Control frequency-response curves (0.1, 0.5, 2, 8, 32 Hz) were constructed by applying increasing frequency of supramaximal EFS (20 V, 0.5 ms) to the tissues. Each stimulus lasted approximately 2–3 min until the tension reached a plateau. The interval between consecutive stimuli was 6 min. Afterwards, three concentrations of clonidine (10^{-7} , 10^{-6} , and 10^{-5} M) and three concentrations of UK 14,304 (10^{-8} , 10^{-7} and 10^{-6} M) were added to six muscle baths. Each bath received only one drug at one concentration. One strip from each animal did not receive drug treatment and served as time control. After a 30-min incubation period for clonidine or a 10-min incubation period for UK 14,304, the second frequency-response curve was generated.

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Protocol 2: Effect of clonidine (10^5 M) and UK 14,304 (10^6 M) on the contractile response to exogenous ACh—This protocol was similar to protocol 1 except that ACh concentration-response curves were constructed instead of frequency-response curves. Acetylcholine was added to the muscle baths cumulatively at concentrations from 10^{-8} to 10^{-3} M in log increments. When the response to one concentration of ACh had reached a plateau, the next concentration of ACh was added. After the control ACh concentration-response curves had been obtained, tissues were washed with warmed Krebs-Henseleit solution until the tension returned to baseline.

Protocol 3: Effects of α_1 - or α_2 -receptor antagonists on the actions of clonidine or UK 14,304—When the frequency-response curves before and after clonidine (10^{-6} M) had been obtained (as described in protocol 1), an α_2 -receptor antagonist yohimbine (10^{-7} M or 10^{-6} M) was added to the tissue baths. After a 30-min incubation period, the third frequency-response curves were generated.

To confirm that the effects of clonidine and UK 14,304 were mediated through α_2 -receptors, the highly selective α_2 -antagonist idazoxan and the α_1 -antagonist prazosin were used. Tissues were first incubated with idazoxan for 20 min and then with idazoxan plus either clonidine (10^{-6} M) or UK 14,304 (10^{-7} M) for another 10 min. EFS frequency-response curves were constructed before and after the drug treatment. The effect of prazosin on the clonidine response was studied with a similar protocol.

Protocol 4: Effect of yohimbine on EFS-induced smooth muscle contraction—In order to determine if prejunctional α_2 -receptors are normally activated by endogenous NE released from adrenergic nerves, EFS frequency-response curves were obtained before and after incubation of the tissues with yohimbine (10^{-7} and 10^{-6} M). To determine if any

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effect of yohimbine was due to inhibition of activation of prejunctional α_2 -receptors or nonspecific effect, the effect of yohimbine was also studied in the presence of guanethidine (10^{-5} M). This concentration of guanethidine abolishes the EFS-induced contraction of equine digital artery.

Measurement of ACh release induced by EFS

Four trachealis strips (each measuring 2 x 15 mm) were tied into a bundle with 3-0 silk suture. Four such trachealis bundles (weight: 221.3 ± 13.6 mg, $\bar{X} \pm \text{SE}$) and 2 bronchial rings (231.1 ± 19.9 mg, $\bar{X} \pm \text{SE}$) were prepared from each animal. Each preparation was suspended in a 2-ml tissue bath (Radnoti Glass Technology Inc., Monrovia, CA) containing Krebs-Henseleit solution bubbled with 95% O_2 - 5% CO_2 and maintained at 38°C. A pair of parallel platinum wire electrodes was built against the wall of the muscle bath in the vertical direction. One end of the tissues was tied to a glass tissue holder that was placed in the muscle bath; the other end was attached to a steel bar above the bath by an elastic thread. As indicated in Chapter 2, ACh release is unaffected by muscle preload alterations.

Tissues were equilibrated for approximately 2 hours, during which time the bathing solution was changed every 10–15 min. After equilibration, tissues were incubated with 10^{-6} M neostigmine (a cholinesterase inhibitor to prevent the breakdown of released ACh), 10^{-7} M atropine (to block the muscarinic autoreceptors on the cholinergic nerves), and 10^{-5} M guanethidine for 30 min. Two bundles of trachealis were incubated with 10^{-7} M and 10^{-6} M yohimbine, respectively, in addition to neostigmine, atropine, and guanethidine. Electrical field stimulation (20 V, 0.5 ms, 0.5 Hz for

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trachea; 20 V, 1 ms, 2 Hz for bronchi) was applied to all the tissues for four 15-min periods. After the first EFS period, increasing concentrations of clonidine (10^{-7} , 10^{-6} , and 10^{-5} M) were administered to four tissue baths, including the two trachealis bundles with yohimbine and one without, and one bronchial segment. Electrical stimulation was applied after a 30-min incubation with each concentration of clonidine. In each experiment, one bundle of trachealis and one bronchial segment did not receive clonidine and served as time control. Tissue bath solution was collected upon the completion of each EFS for the measurement of ACh. At the end of the experiment, tissues were blotted dry and weighed. The effect of UK 14,304 alone or plus idazoxan on ACh release was studied in a similar way in the trachealis.

High-performance liquid chromatography coupled with electrochemical detection was used to measure the amount of ACh in the tissue bath liquid. The technique was the same as described in Chapter 2 except for some modifications, which were addressed in Chapter 5.

Drugs

The following drugs were used in the present study: ACh chloride, atropine sulfate, clonidine hydrochloride, neostigmine methylsulfate, prazosin hydrochloride, yohimbine hydrochloride (all from Sigma Chemical Co., St Louis, MO) and UK 14,304 (Research Biochemical International, Natick, MA). All the drugs except UK 14,304 were dissolved in deionized water and diluted with Krebs-Henseleit solution on the day of the experiment. UK 14,304 was dissolved in ethanol (10 mg/ml) and then diluted with water to 10^{-2} M. This solution was stored at 4°C as aliquots and diluted with

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Krebs-Henseleit solution on the day of the experiment. The concentration of ethanol in 10^{-6} M UK 14,304 is 0.0029%, which had no influence on EFS-induced smooth muscle contraction as determined in a previous study. Drug solution was pipetted into the tissue baths at 1% of the bath volume. The concentrations of the drugs were expressed as their final bath molar concentrations.

Statistical analysis

The contractile responses of the muscle strips to EFS and ACh were expressed as a percentage of the tension produced by 127 mM KCl. The ACh amount in the samples was expressed as a percentage of that measured in the first sample of each bath and also as pmol/g/min. A paired *t*-test was used to compare the contractile responses to each frequency of EFS or each concentration of ACh before and after the drug administration. To determine if the effect of clonidine was frequency- and concentration-dependent, the effect of clonidine on EFS-induced contraction was also calculated as % inhibition and analyzed by two-way analysis of ANOVA. The disinhibition of the effect of clonidine by yohimbine, and the ACh measurement data in trachea were analyzed by the use of single factor repeated and randomized design ANOVA, respectively. Tukey's post-Hoc test was used to identify significantly different means. The effect of clonidine on ACh release in bronchial rings was tested by unpaired *t*-test.

All values were expressed as $\bar{X} \pm \text{SE}$. $P < 0.05$ was considered statistically significant. *n* equals the number of animals.

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Results

Muscle tension study

Clonidine (10^{-7} – 10^{-5} M) had no effect on passive tension but inhibited the EFS-induced smooth muscle contraction in a concentration- and frequency-dependent manner. The inhibition was absent at 32 Hz but increased in magnitude as frequency decreased (Fig. 6-1). Inhibition induced by clonidine (10^{-6} M) was attenuated by the α_2 -receptor antagonists yohimbine (10^{-7} and 10^{-6} M, Fig. 6-2) and idazoxan (10^{-6} M, Fig. 6-3), but not by the α_1 -receptor antagonist prazosin (10^{-6} M, Fig. 6-3). UK 14,304 inhibited the EFS-induced smooth muscle contraction at 10^{-7} and 10^{-6} M (Fig. 6-4). The inhibition produced by 10^{-7} M UK 14,304 was prevented by pretreating the tissues with 10^{-7} M idazoxan (Fig. 6-4). Neither clonidine (10^{-5} M) nor UK 14,304 (10^{-6} M) affected the contractile response to exogenous ACh (data not shown). Yohimbine at 10^{-7} M had no influence on the response to EFS (data not shown), but yohimbine at 10^{-6} M significantly increased the response to 0.1 Hz (Fig. 6-5). Although a statistically significant effect of yohimbine was also observed at 32 Hz, the effect was trivial and probably biologically irrelevant. The effect of 10^{-6} M yohimbine was unaffected by pretreating the tissues with 10^{-5} M guanethidine.

ACh measurement

During the first stimulation period, the ACh release rate of trachealis strips was 7.23 ± 2.10 pmol/g/min. Incubation with yohimbine or idazoxan alone had no influence on ACh release. In trachealis strips, UK 14,304 (10^{-8} – 10^{-6} M) inhibited ACh release concentration-dependently and this inhibition was attenuated by pretreating the tissues

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with idazoxan (Fig. 6-6A). Similarly, clonidine (10^{-7} to 10^{-5} M) decreased ACh release concentration-dependently and this inhibition was antagonized by pretreating the tissues with yohimbine (10^{-7} and 10^{-6} M, Fig. 6B and C). Clonidine (10^{-7} – 10^{-5} M) also significantly inhibited ACh release in bronchi (Fig. 6-7). All of the above-mentioned results were obtained at a single stimulation frequency (0.5 Hz for trachea, 2 Hz for bronchi) and voltage (20 V). The clonidine-induced inhibition on ACh release was also studied at several frequencies (0.5, 2, 8, 32 Hz) and voltages (10, 15, 20, 24 V) in trachealis. Clonidine significantly inhibited ACh release at 0.5 and 2 Hz but not at 8 and 32 Hz (20 V, 0.5 ms, Fig. 8A). The inhibition observed at 0.5 Hz was present at all voltages (Fig. 6-8B).

Discussion

The role of α_2 -adrenoceptors on equine airway cholinergic neurotransmission was examined in the present study. In the indirect experiment, α_2 -receptor agonists clonidine and UK 14,304 inhibited the contractile response of trachealis to EFS but did not affect the response to ACh. The lack of an effect of α_2 -agonists on ACh-induced contractions suggests either that there are no α_2 -receptors on smooth muscle cells or that their activation does not interfere with the muscarinic response of smooth muscle. Therefore, the inhibition of EFS-induced contraction by clonidine or UK 14,304 could be explained by 1) a decreased ACh release from cholinergic nerves, 2) an increased release of neurotransmitters from inhibitory nerves; or 3) other possible effects of clonidine or UK 14,304, such as modulation of the spread of excitation through gap junctions. It is difficult to determine which possibility is true simply through the measurement of muscle

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tension. However, direct measurements indicated that clonidine and UK 14,304 inhibited ACh release significantly. Thus the inhibition by clonidine or UK 14,304 of EFS-induced contractions must be a result of decreased ACh release from cholinergic nerves.

Clonidine is a selective α_2 -receptor agonist (Doxey et al. 1985) and yohimbine is an antagonist specific for α_2 -receptors at concentrations equal to or below 10^{-7} M (Kenakin 1987). In the present study, yohimbine concentration-dependently attenuated the clonidine-induced inhibition of both the contractions and the ACh release in response to EFS, and this effect of yohimbine was apparent at 10^{-7} M concentration. The antagonism by yohimbine of the action of clonidine is most likely a result of competition at the receptor level (genuine antagonism) rather than functional antagonism, because yohimbine *per se* had little or no effect on either EFS-induced contractions or ACh release. Furthermore, prazosin, a selective α_1 -antagonist, did not prevent clonidine's effect. These observations suggest that the inhibitory effect of clonidine on cholinergic neurotransmission and its reversal by yohimbine were through their actions on α_2 -receptors. This conclusion was confirmed by the observation that the highly selective α_2 -receptor agonist, UK 14,304, concentration-dependently inhibited both the contractile response and ACh release induced by EFS. This inhibition was antagonized by idazoxan, a highly specific α_2 -antagonist (Doxey et al. 1985). Based on the above observations, we conclude that α_2 -receptors are present on equine airway cholinergic nerves and the activation of these receptors inhibits cholinergic neurotransmission.

Isolated trachealis strips may contain both the preganglionic nerve terminals and the postganglionic neurons of the parasympathetic nerves. Because ganglionic blockade with hexamethonium has no effect on either muscle contractions (Chapter 4) or ACh

release in response to EFS (Chapter 2), the prejunctional α_2 -receptors that inhibited ACh release must be located on postganglionic neurons. However, the possibility cannot be excluded that α_2 -adrenoceptors may also exist on parasympathetic ganglia (Baker et al. 1983).

Based on the dominance of the parasympathetic nerves in control of airway tone and the huge depressant effect of clonidine and UK 14,304 on cholinergic neurotransmission, it seems likely that prejunctional α_2 -adrenoceptors constitute a potent regulatory mechanism of smooth muscle tone in equine airways. The physiological stimulants of these receptors could be NE released by sympathetic nerves and/or circulating catecholamines. The observation that sympathetic and parasympathetic nerves are in close association in guinea pig trachea (Jones et al. 1980) furnishes the anatomical possibility for interaction between these two nervous systems. Electrical field stimulation of isolated trachealis strips activates all the nerves in the preparation. If NE released from the sympathetic nerves in response to EFS can activate α_2 -receptors on cholinergic nerves, blockade of the α_2 -receptors with yohimbine should enhance ACh release and the contractile response to EFS. In the present study, EFS-induced contraction was augmented by yohimbine (10^{-6} M) but only at a frequency of 0.1 Hz. It is unlikely that this augmentation was a result of blockade of α_2 -receptors, because depletion of the sympathetic neurotransmitter with guanethidine did not abolish the augmentation. These results suggest that, under the present experimental conditions, endogenous NE does not inhibit ACh release from equine airway cholinergic nerves via prejunctional α_2 -receptors. This conclusion is not unexpected considering the sparse sympathetic innervation in most species and the rapid removal by neuronal and extraneuronal uptake or enzymatic

breakdown of NE (Barnes 1986). Thompson et al. (1990) reached a similar conclusion in experiments with guinea pig tracheal tissues.

Circulating catecholamines may also stimulate α_2 -receptors on cholinergic nerves. In the guinea pig airways, epinephrine and NE, in the presence of β -blockers, inhibit airway smooth muscle contraction induced by nerve stimulation but not by exogenous ACh (Grundström et al. 1981a) suggesting prejunctional modulation by catecholamines. However, it is not known if circulating catecholamines have such an effect at their physiological concentrations. The concentration of epinephrine and NE in equine plasma are 9×10^{-10} M and 7×10^{-10} M respectively at rest and increase to 1.53×10^{-7} M and 1.48×10^{-7} M respectively after exercise (Snow et al. 1992). Whether these concentrations of catecholamines, especially after exercise, can modify equine airway cholinergic activity has not been determined in vitro. Failure of intravenous yohimbine to alter pulmonary function in ponies implies that the α_2 -inhibitory receptors are not activated under physiological conditions in the resting pony (Broadstone et al. 1992).

The existence of α_2 -receptors on cholinergic nerves may have some clinical importance in the treatment of airway obstruction. Xylazine, an α_2 -receptor agonist and potent tranquilizer that inhibits the EFS-induced contractions in equine peripheral airways (LeBlanc et al. 1993), causes bronchodilation in heavy ponies and this effect is reduced by yohimbine (Broadstone 1992). Although α_2 -receptors also occur on the airway smooth muscle cells of some species (Barnes et al. 1982; Takaganagi et al. 1990), their activation causes smooth muscle contraction. Therefore, if xylazine-induced

bronchodilation is mediated at the level of the airway rather than centrally, the most likely mechanism is its action on prejunctional α_2 -receptors.

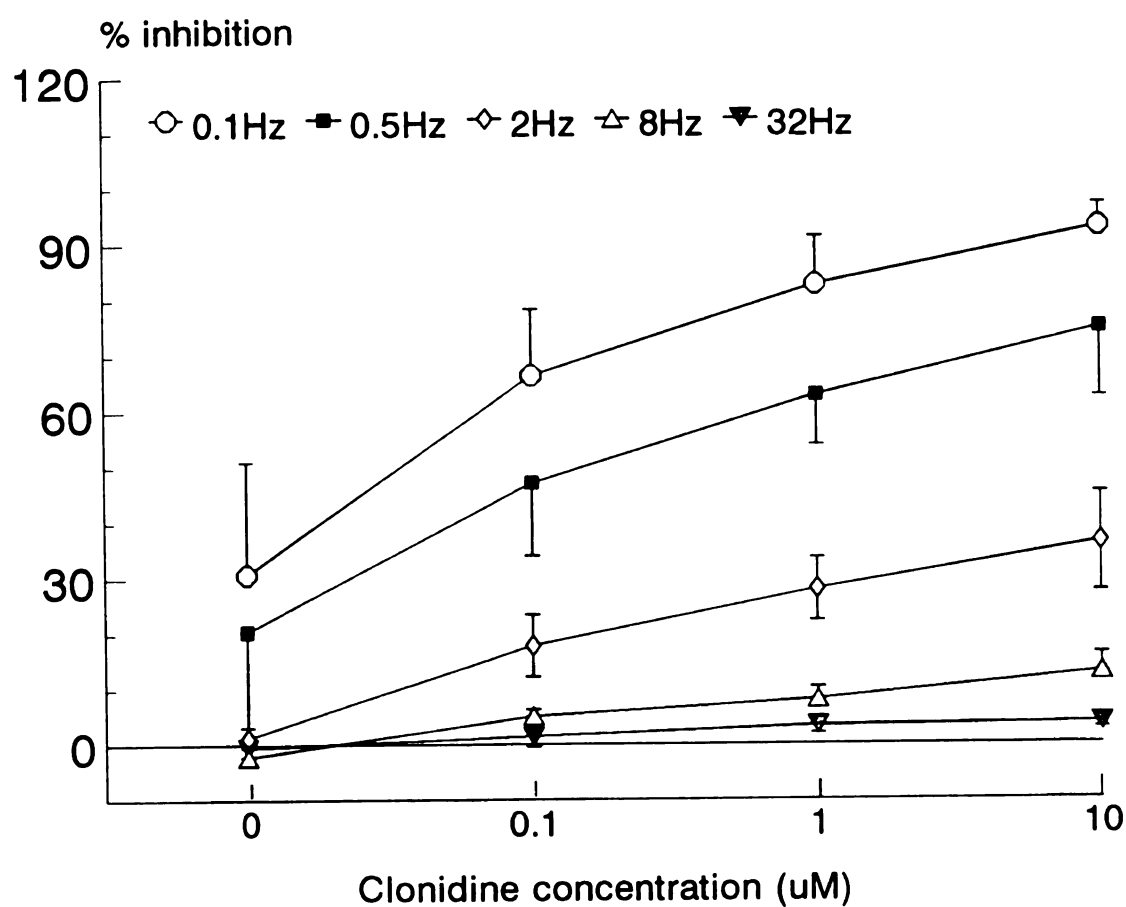


Fig. 6-1. Inhibitory effect of clonidine on the contractile response of equine trachealis to electrical field stimulation (EFS, 20 V, 0.5 ms). Percent inhibition was calculated from the amplitude of muscle contraction as $[(pre-drug - post-drug)/pre-drug] \times 100$. The inhibitory effect of clonidine was concentration- ($P = 0.0012$) and frequency-dependent ($P = 0.0000$).

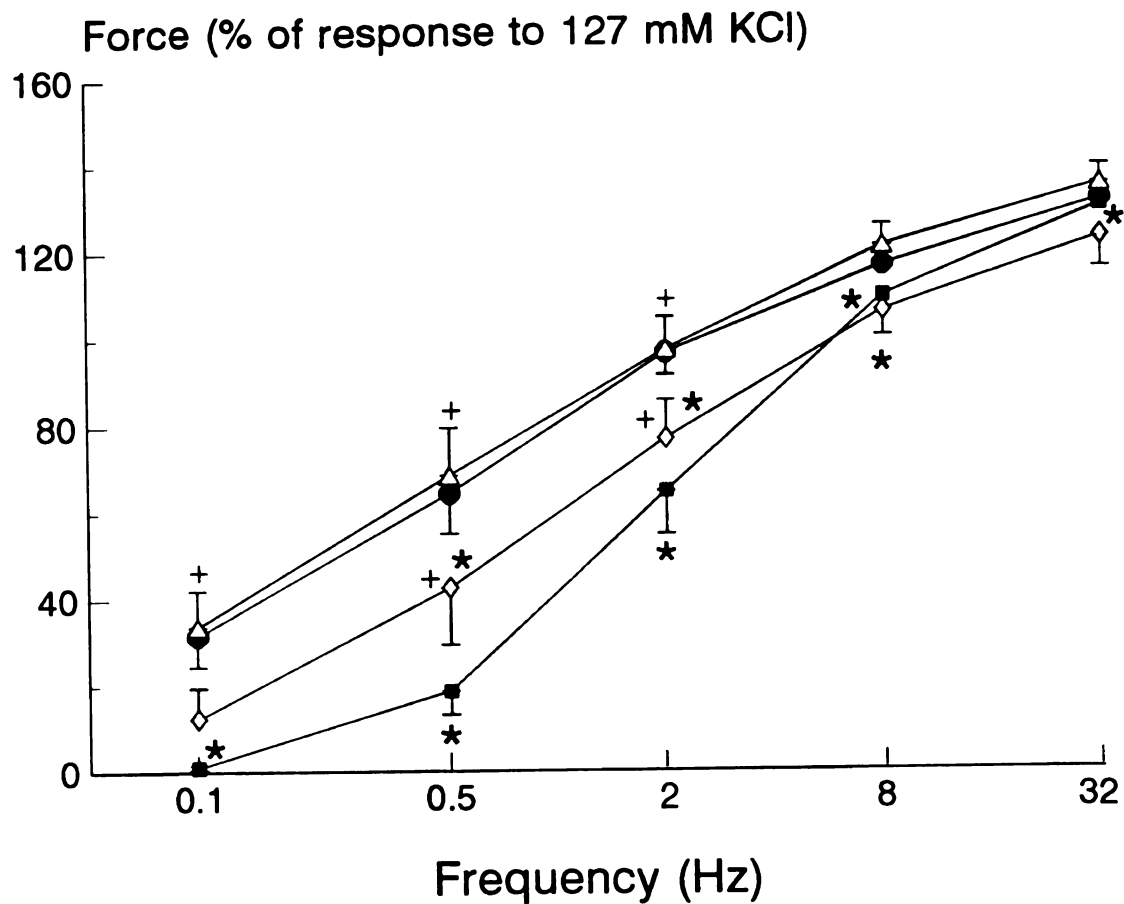


Fig. 6-2. Frequency-response curves of equine trachealis strips to electrical field stimulation (20 V, 0.5 ms). ○ Control; □ 10^{-6} M clonidine, $n = 6$; ◇ 10^{-6} M clonidine plus 10^{-7} M yohimbine, $n = 5$; △ 10^{-6} M clonidine plus 10^{-6} M yohimbine, $n = 4$. The inhibition of EFS-induced smooth muscle contraction by clonidine was concentration-dependently attenuated by yohimbine. * Significantly different from control, + significantly different from the 10^{-6} M clonidine.

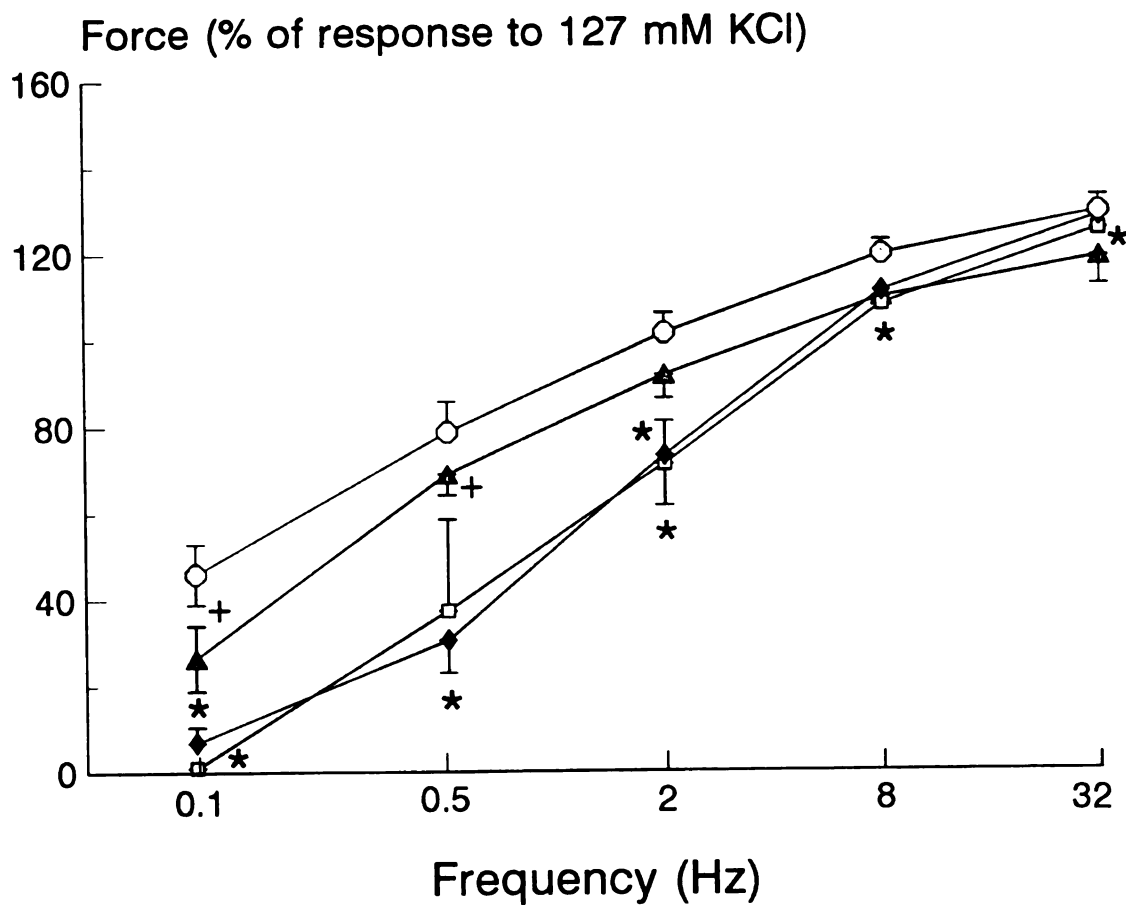


Fig. 6-3. Frequency-response curves of equine trachealis strips to electrical field stimulation (20 V, 0.5 ms). ○ control, ◇ 10^{-6} M clonidine alone, $n = 6$; □ 10^{-6} M prazosin plus 10^{-6} M clonidine, $n = 4$; △ 10^{-6} M idazoxan plus 10^{-6} M clonidine, $n = 3$. The clonidine-induced inhibition was prevented by pretreating the tissues with idazoxan but not with prazosin. * Significantly different from control, + significantly different from the 10^{-6} M clonidine.

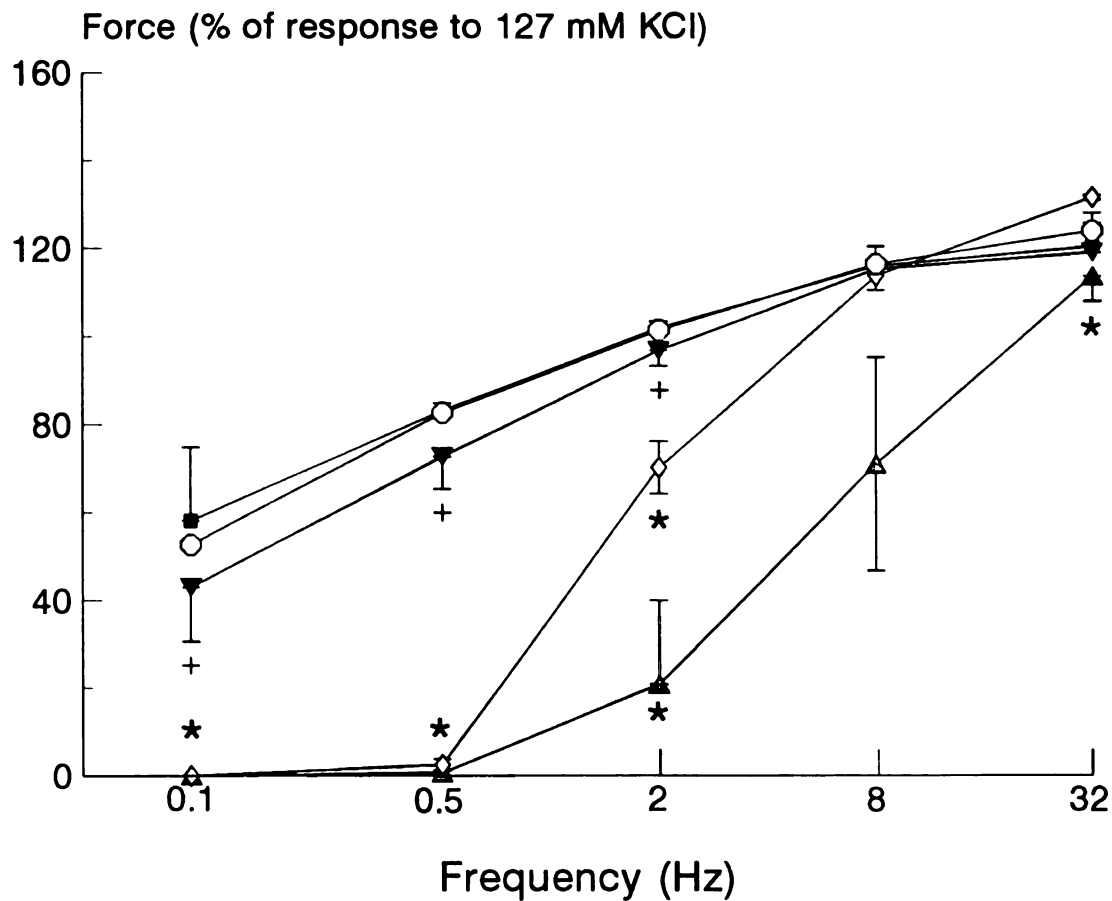


Fig. 6-4. Frequency-response curves of equine trachealis strips to electrical field stimulation (20 V, 0.5 ms). ○ control; □ 10^{-8} M UK 14,304, $n = 4$; ◇ 10^{-7} M UK 14,304, $n = 3$; △ 10^{-6} M UK 14,304, $n = 4$; ▽ 10^{-7} M idazoxan added 20 min before the addition of 10^{-7} M UK 14,304, $n = 3$. * Significantly different from control, + significantly different from the 10^{-7} M UK 14,304.

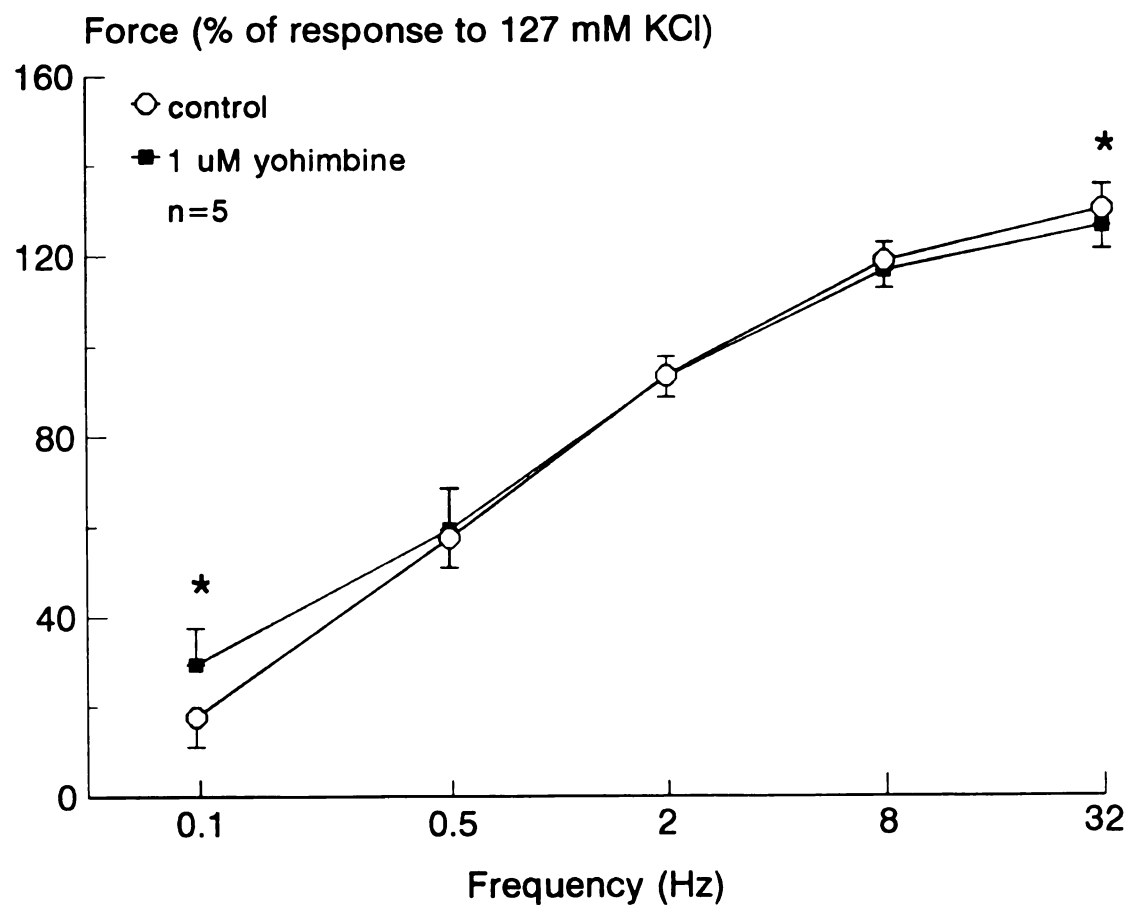


Fig. 6-5. Effect of yohimbine (10^{-6} M) on the contractile response of equine trachealis strips to electrical field stimulation (EFS, 20 V, 0.5 ms) in the absence of guanethidine. Yohimbine increased the response to 0.1 Hz and this increase was not affected by pretreating the tissues with guanethidine (10^{-5} M; data not shown). * Significant effect of yohimbine.

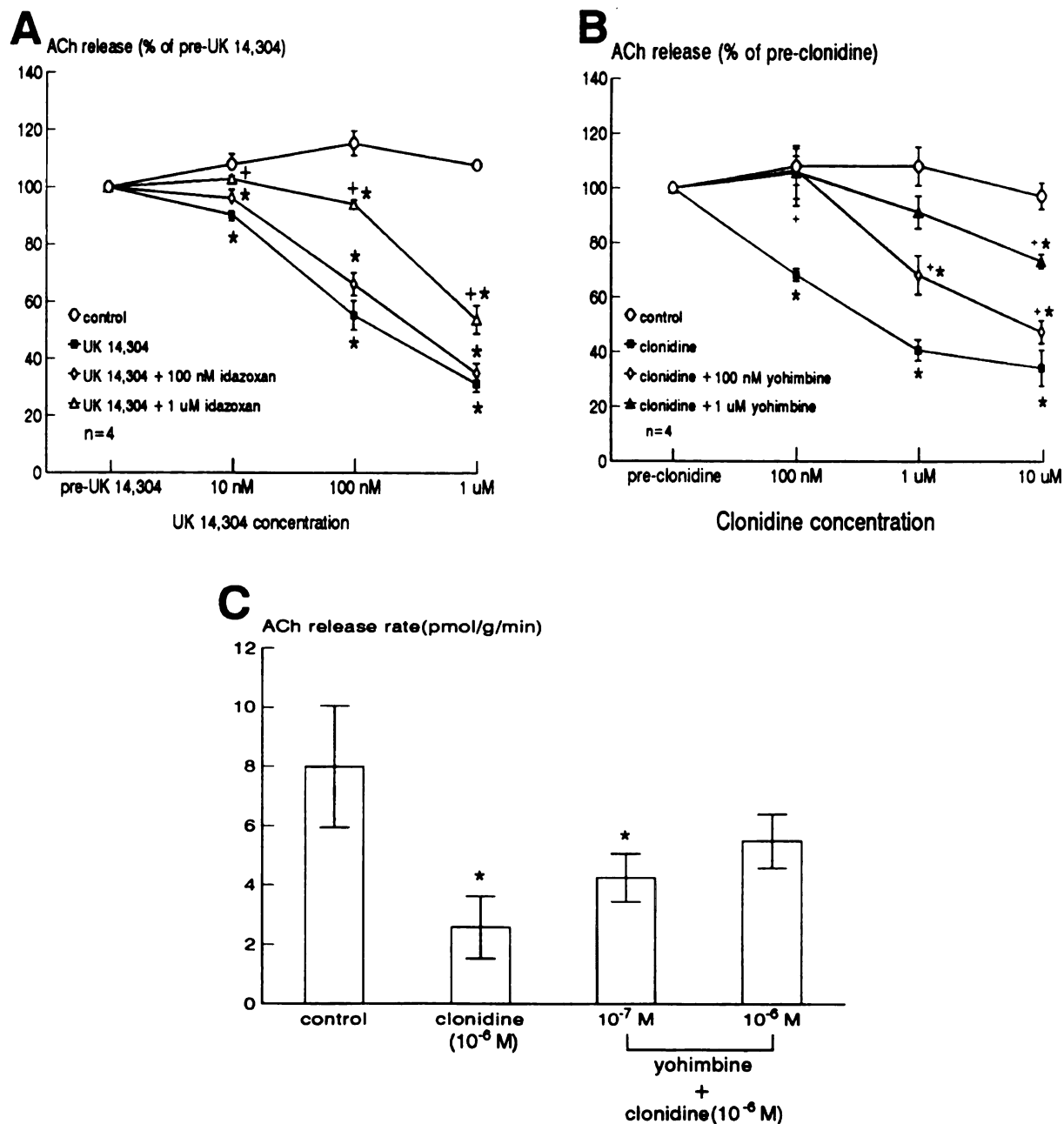


Fig. 6-6. Effects of α_2 -receptor agonists and antagonists on acetylcholine (ACh) release from equine trachealis strips in response to electrical field stimulation (EFS, 20 V, 0.5 ms, 0.5 Hz). * Significantly different from time control, + significantly different from UK 14,304 (A) or clonidine (B). A shows the inhibition of ACh release by UK 14,304 and the antagonizing effect of idazoxan. B shows the inhibition of ACh release by clonidine and the antagonizing effect of yohimbine. C gives the ACh release rate expressed as pmol/g/min at a clonidine concentration of 10⁻⁶ M.

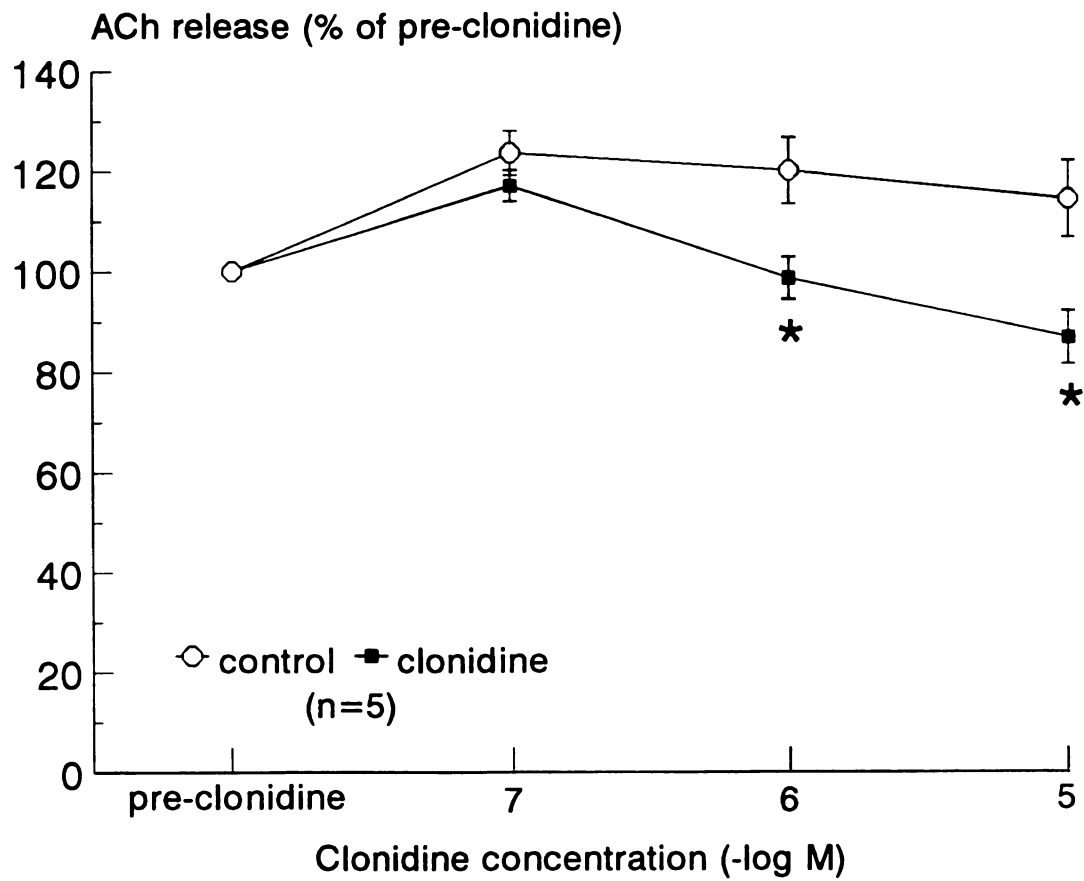


Fig. 6-7. Effect of clonidine on acetylcholine release from equine bronchial rings in response to electrical field stimulation (EFS, 20 V, 1 ms, 2 Hz). * Significantly different from time control.

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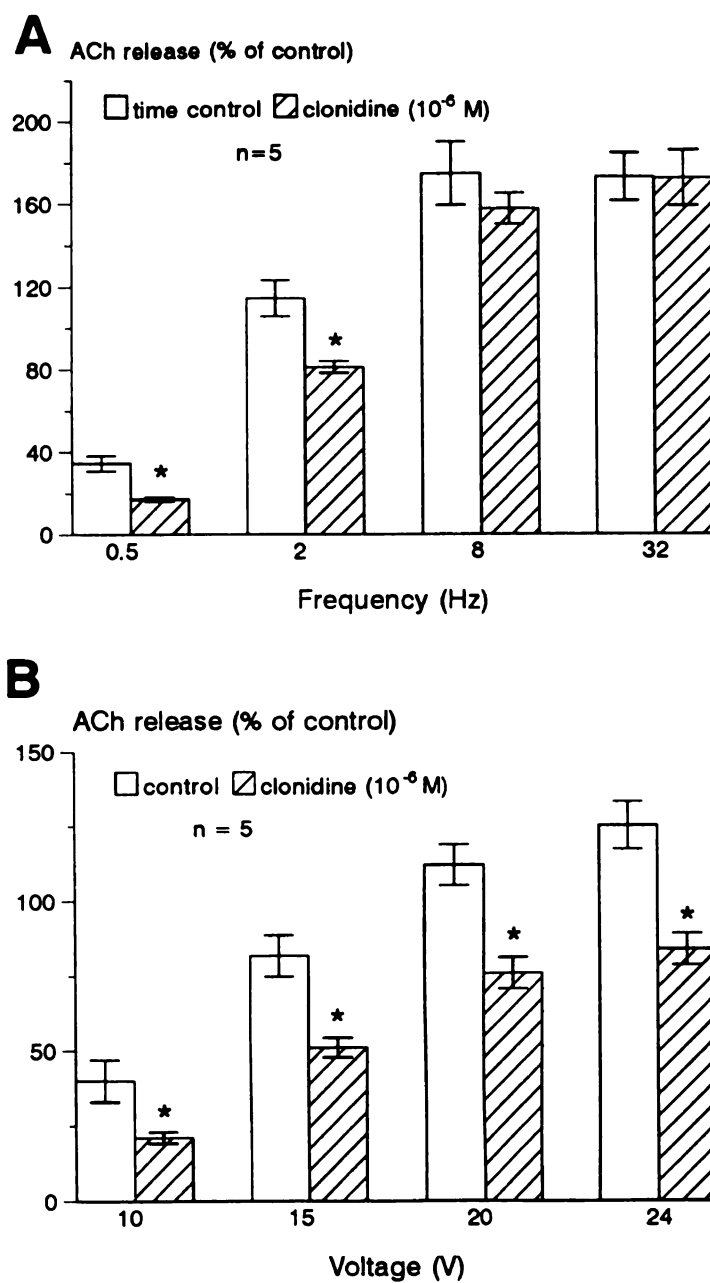


Fig. 6-8. Effect of frequency (A; 20 V, 0.5 ms) and voltage (B; 0.5 Hz, 0.5 ms) on clonidine-induced inhibition of acetylcholine (ACh) release from equine trachealis strips in response to electrical field stimulation. Data were normalized to an initial measurement of ACh release (2 Hz, 20 V, 0.5 ms) obtained prior to addition of drug. * Significant difference between time control and clonidine.

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CHAPTER 7

ACH RELEASE FROM AIRWAY CHOLINERGIC NERVES IN HORSES WITH AIRWAY OBSTRUCTION (HEAVES)

Introduction

Some horses and ponies develop airway obstruction when stabled and fed dusty hay and enter remission at pasture. This type of airway obstruction is colloquially called "heaves." Muscarinic antagonists such as atropine markedly reduce the increased airway resistance in affected animals (Broadstone et al. 1988), indicating the involvement of a muscarinic mechanism. In in vitro studies, airway smooth muscle preparations from trachea and bronchi of heavey horses are hyporesponsive to exogenous ACh but hyperresponsive to EFS compared with those of normal horses (Broadstone et al. 1991), suggesting that ACh release from airway cholinergic nerves is probably increased in heavey horses. An increase in ACh release may contribute to the development of airway obstruction. Because this conclusion has been inferred by comparing the contractile response of airway smooth muscle to EFS and ACh, it needs to be verified by direct measurement of ACh release.

In early chapters of my dissertation, I reported that ACh release from horse airway cholinergic nerves is inhibited by activation of prejunctional muscarinic autoreceptors (Chapters 2 and 3) and α_2 -adrenoceptors (Chapter 6). Alterations in the

function of these receptors might result in increased ACh release. There is evidence to suggest that muscarinic autoreceptors on airway cholinergic nerves are probably dysfunctional in the lungs of asthmatics (Minette et al. 1989) and of guinea pigs infected with parainfluenza virus (Fryer et al. 1990) or challenged by ovalbumin (Fryer and Wills-Karp 1991). It is possible that other prejunctional receptors such as the α_2 -adrenoceptors may be also affected in diseases characterized by airway inflammation and obstruction.

Prostaglandin E_2 inhibits ACh release from canine airway cholinergic nerves (Deckers et al. 1989a). In the horse, however, PGE_2 lacks such an effect in both the trachea and bronchi (Chapter 5). It was of interest to know if horses with heaves respond differently to PGE_2 .

The present study was conducted to answer the following questions: 1) Is ACh release from airway cholinergic nerves of the heavy horse increased under the in vitro conditions? 2) Are muscarinic autoreceptors or prejunctional α_2 -adrenoceptors on airway cholinergic nerves dysfunctional in the heavy horse? 3) Does PGE_2 modulate ACh release from airway cholinergic nerves in heavy horse?

Materials and methods

Animals

Airway tissues were obtained from nine normal (weighing 387.4 ± 24.0 kg) and five heavy (weighing 406.2 ± 29.8 kg) adult horses of either sex. The normal horses had no clinical signs of pulmonary disease and, post mortem, their lungs were normal in gross appearance. The heavy horses had a history of heaves. Several days before

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the experiment, they were transferred from pasture to straw-bedded stalls where they were fed dusty hay. They remained in the stall until clinical signs of airway obstruction, such as flared nostrils and forced abdominal effort during expiration, appeared. The presence of airway obstruction was confirmed by pulmonary function measurements. Results of previous studies indicate that normal horses in the barn environment have a R_L of 0.87 ± 0.10 cm H₂O/L/sec, a $\Delta P_{pl_{max}}$ of 6.7 ± 0.4 cm H₂O, and a C_{dyn} of 1.26 ± 0.15 L/cm H₂O (Gray et al. 1992a). The heavy horses used in the present study had on average a R_L of 2.6 ± 0.3 cm H₂O/L/sec, a $\Delta P_{pl_{max}}$ of 36.6 ± 6.2 cm H₂O, and a C_{dyn} of 0.35 ± 0.08 L/cm H₂O during airway obstruction. The strong muscarinic component to airway obstruction was confirmed by administration of atropine (0.2 mg/kg), which dilated the airways in all the animals. Pulmonary resistance, $\Delta P_{pl_{max}}$, and C_{dyn} changed to 1.2 ± 0.3 cm H₂O/L/sec ($P < 0.05$), 15.6 ± 2.9 cm H₂O ($P < 0.05$), and 0.74 ± 0.18 L/cm H₂O ($P > 0.05$), respectively, within 5 to 15 min of atropine administration. In vitro experiments were conducted at least 3 days after the atropine trial.

Preparation of tissues

Animals were euthanized by intravenous injection of an overdose of pentobarbital sodium. Trachealis strip bundles with epithelium (each consisting of four strips of 2 mm wide and 17–19 mm long) were prepared from the dorsal membrane of the tracheal segment between the 16th and 25th cartilaginous rings above the carina using the technique described in Chapter 2. Bronchial segments (3–5 mm outside diameter, ~15 mm long) with epithelium were prepared from the left or right lung. The tissue

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preparations were then suspended in 2-ml tissue baths (Radnoti Glass Technology, Inc., Monrovia, CA). Each bath had a pair of parallel platinum wire electrodes built against the wall in the vertical direction. For trachealis strip bundles, one end of the preparation was fixed to the tip of a glass tissue holder. The suture on the other end was attached to a steel bar through a piece of elastic. The tension applied to the tissue was well within the range of 2–20 grams, which has been determined to have no influence on ACh release from airway cholinergic nerves (Chapter 2). The bronchial tubes were gently guided onto the shorter arm of an "L"-shaped glass tissue holder. To prevent the bronchial tube from floating above the surface of the bath solution, it was tied to the hook at the end of the tissue holder. Tissue baths contained Krebs-Henseleit solution (composition in mM: NaCl 118.4, NaHCO₃ 25.0, dextrose 11.7, KCl 4.7, CaCl₂·2H₂O 2.6, MgSO₄·7H₂O 1.19, KH₂PO₄ 1.16), which was maintained at 38°C and bubbled with 95% O₂/5% CO₂.

Protocols

Tissues were equilibrated in the baths for 2–3 hours, during which time the bath solution was replaced at 10- to 20-min intervals. This extended equilibration period was necessary in order to reduce the amount of foam formed during bubbling. In a 2-ml bath, foam formation results in overflow of bath solution so that ACh release cannot be accurately determined.

In order to determine if muscarinic autoreceptors are defective in the heavy horse, one trachealis strip bundle and one bronchial segment were prepared from each control or heavy horse. After incubation with 10 μM neostigmine for 60 min, 5 periods

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of 20-min EFS (20 V, 4 Hz, 2 ms) were applied to the tissues at 30-min intervals. The first period was applied in the absence of atropine. During the remaining 4 periods, tissues were stimulated in the presence of logarithmically increasing concentrations of atropine ($10^{-8} \sim 10^{-5}$ M) in addition to 10 μ M neostigmine. EFS was applied after incubation with each concentration of atropine for approximately 25 min. To make sure that the effect of atropine was not a time-related response, one bronchial segment was prepared from each normal and heavey horse and one trachealis strip bundle was also prepared from each heavey horse. Except for the omission of atropine, these tissue preparations otherwise received the same treatment as those used to study the effect of atropine. A time control experiment was not conducted for trachealis tissues of the control horse because I have previously demonstrated that ACh release is constant over up to 6 EFS periods in the absence of atropine (Chapter 3).

In trachealis tissues used to examine the effect of atropine, the ACh release data measured during the first EFS period were also used to determine if ACh release is increased in the heavey horse. In an effort to eliminate the possible influences of edema, infiltration of inflammatory cells, or muscular hypertrophy on the interpretation of our results, ACh release was expressed in units of pmol/g tissue dry weight/min, pmol/mm² surface area of the trachealis/min, as well as pmol/g tissue wet weight/min prior to comparisons between the control and heavey horses. For the purpose of calculating the epithelial surface area of the trachealis, the length of each trachealis strip bundle was measured carefully. The surface area of each bundle was calculated as the product of the length of the bundle, the width of each strip, and the number of strips in each bundle.

To determine if prejunctional α_2 -adrenoceptors are dysfunctional in the airways of the heavy horse, one pair of trachealis strip bundles and one pair of bronchial segments were prepared from each heavy horse. After incubation with neostigmine (1 μ M), atropine (0.1 μ M), and guanethidine (10 μ M) for 60 min, 4 periods of 15-min EFS (20 V, 0.5 Hz, 0.5 ms for trachea; 20 V, 2 Hz, 1 ms for bronchi) were applied to the tissues at 30-min intervals. The first period was applied in the absence of clonidine. During the remaining 3 periods, one preparation of each pair was stimulated in the presence of logarithmically increasing concentrations of clonidine (10^{-7} – 10^{-5} M) in addition to neostigmine, atropine, and guanethidine, whereas the other preparation did not receive clonidine and served as time control. The tissues were incubated with each concentration of clonidine for approximately 25 min before stimulation. Historical data from normal horses (Chapter 6) were cited for comparing the effect of clonidine between heavy and normal animals.

Four bronchial segments were prepared from each heavy horse to determine the effect of exogenous PGE₂ (10^{-9} – 10^{-7} M) and the effect of indomethacin (3×10^{-6} M), a cyclooxygenase inhibitor, on ACh release. Experiments were not performed with trachealis tissues because there were only four tissue baths available for this part of the study, and histological lesions are more prominent in the bronchi than trachea of the heavy horse (Breeze 1979). The protocols were the same as those used for the bronchial segments of the normal horse (Chapter 5). Briefly, four 12-min periods of EFS were applied to the tissues. Period 1 was applied in the absence of PGE₂ and indomethacin. During the remaining three periods, PGE₂, PGE₂ vehicle, indomethacin, and indomethacin vehicle were added separately to the four baths. The concentrations

of indomethacin (3×10^{-6} M) and its vehicle were constant during the three periods, whereas the concentrations of PGE₂ (10^{-9} – 10^{-7} M) and its vehicle were increased logarithmically over successive periods. For details of the protocols, please refer to Chapter 5.

Tissue bath liquid was collected after EFS for ACh measurement. The bath solution was replaced before the start and at the end of each EFS period. Each time the bath solution was replaced, the tissue bath was flushed 2–3 times with Krebs-Henseleit solution.

The wet weight of each tissue preparation was determined at the end of the experiment after blotting with filter paper. For tissues used to compare ACh release rate between normal and heavy horses, the dry weight was also determined after keeping the tissues in a desiccator containing anhydrous CaCl₂ for at least one week, which was adequate for the tissue to dry thoroughly.

ACh analysis

Collected samples were filtered through 0.2 μ nylon membrane filters (Acrodiscs 13, Gelman Sciences, Ann Arbor, MI). The ACh content was determined by HPLC coupled with electrochemical detection. The technique was described in Chapter 2. The following modifications were made in this study. 1) Some pieces of equipment were replaced: electrochemical detector (Coulchem II, ESA Inc., Bedford, MA); ACh and choline analytical column (ESA Inc.); autosampler (ISS 200, Perkin-Elmer, Norwalk, CT). 2) The mobile phase (water solution) contained 100 mM Na₂HPO₄, 0.5 mM tetramethylammonium chloride, 2 mM 1-octanesulfonic acid, and 0.005% reagent MB

(ESA) with pH adjusted to 8.0 with H_3PO_4 . At a flow rate of 0.35 ml/min, the retention times for choline and ACh were 3.9 and 6.2 min, respectively. 3) 25 μl of calibration standard or bath solution was injected. The standard was injected every 4 samples and the ACh amount in the samples was calculated based on the bracketed calibrations. The lower limit of determination was 50 femtomoles. 4) To prevent the breakdown of ACh molecules at room temperature as observed previously (Chapter 2), samples were kept refrigerated ($1-3^\circ\text{C}$) in the autosampler by a refrigerated bath circulator (RTE-110D, NESLAB Instruments, Inc., Portsmouth, NH) during the analysis, which lasted for less than 12 hours.

Drugs

Neostigmine methylsulfate, atropine sulfate, clonidine hydrochloride, and guanethidine monosulfate (Sigma Chemical Co., St. Louis, MO.) were dissolved in and diluted with water. PGE_2 (Cayman Chemical Company, Ann Arbor, MI) was first dissolved with methanol (1 mg/ml) and subsequently diluted to 10^{-5} M with distilled water. The 10^{-5} M PGE_2 was stored as a stock solution at -15°C and diluted with water before use. Indomethacin (Sigma) was dissolved in solutions of equimolar sodium carbonate. The concentrations of all drugs are expressed as their final bath concentrations.

Statistical analysis

To examine the effect of clonidine, PGE_2 , and indomethacin, ACh release in each bath during the first EFS period was regarded as 100% and that during subsequent

periods was expressed as a percentage of this value. The ACh release during each period was compared between the control and drug-treated tissues by an unpaired *t*-test. To determine if ACh release rate differed in trachealis tissues of the control and heavy horse, the release data were compared between the two groups by an unpaired *t*-test. To investigate if muscarinic autoreceptors are dysfunctional in the heavy horse, ACh release during the first EFS period was regarded as 1, and that during subsequent periods was calculated as multiples of 1. The calculated values were compared between the heavy and control horses by an unpaired *t*-test. All values were expressed as mean $\pm$ SE. $P < 0.05$ was considered statistically significant. Except when otherwise specified, *n* equals the number of animals.

Results

All samples contained a measurable amount of ACh. ACh content remained constant during the period when samples were kept in the autosampler. The content measured after 12 hours varied from the original value by no more than -5% or +1% (average -1.23%, *n* = 13).

In the presence of neostigmine alone, the rate of EFS-induced ACh release from the tracheal preparations was the same in the control and heavy horses. The values were 11.4 ± 2.0 versus 14.1 ± 3.0 pmol/g wet weight/min, 53.0 ± 8.7 versus 65.4 ± 13.9 pmol/g dry weight/min, and 1.38 ± 0.22 versus 1.68 ± 0.32 pmol/cm² epithelial surface area of trachealis/min for tissues of normal (*n* = 5) and heavy (*n* = 5) horses, respectively.

Atropine augmented ACh release in both the trachea and bronchi. Chromatograms of a few samples collected from a single tissue bath showing the effect of atropine on ACh release are displayed in Fig. 7-1. The magnitude of augmentation was similar between heavy and control horses (Fig. 7-2). ACh release rate was constant over the five stimulation periods in the absence of atropine (data not shown).

In the heavy horse trachea, clonidine did not inhibit ACh release significantly at 10^{-7} M (Fig. 7-3A) as it did in the trachea of normal horses (Fig. 6-6B). At 10^{-6} and 10^{-5} M concentrations, clonidine inhibited ACh significantly (Fig. 7-3A). However, the magnitude of inhibition was less than that observed in the normal horses (about 30% less at 10^{-6} M clonidine) (Figure 6-6B), although the difference was not statistically significant ($P = 0.06$ at 10^{-6} M clonidine).

In the heavy horse bronchi, clonidine did not inhibit ACh release significantly (Fig. 7-3B). This result is in contrast to its significant inhibitory effect in the normal horse bronchi (Fig. 6-7). However, when the magnitude of inhibition was compared, there was no significant difference between the normal and heavy horses.

Neither PGE₂ (Fig. 7-4A) nor indomethacin (Fig. 7-4B) had any effect on ACh release in the bronchial tissues. The results were similar to those obtained in the normal horses (Fig. 5-7B and Fig. 5-5B)

The wet weight of each tissue preparation averaged 192.6 ± 7.1 , 180.6 ± 27.4 , 178.7 ± 7.3 , and 201.2 ± 14.0 mg for control trachea ($n = 5$ bundles), control bronchi ($n = 10$ segments), heavy trachea ($n = 9$ bundles), and heavy bronchi ($n = 40$ segments), respectively. In trachealis tissues whose dry weight was determined, the

dry/wet weight ratio was 0.214 ± 0.003 ($n = 5$ bundles) and 0.214 ± 0.002 ($n = 5$ bundles) in the normal and heavy horses, respectively.

Discussion

In the present study, I compared ACh release from airway cholinergic nerves of trachealis preparations of normal and heavy horses. To do this, I first needed to decide how to normalize my data. In studies described in the earlier chapters, I expressed the ACh release data as pmol/g wet weight of tissue/min. Because the airways of heavy horses may have edema, infiltration of inflammatory cells, or muscular hypertrophy, comparison of data expressed in this unit may be unable to reflect the real situation. I therefore also normalized the ACh release data by the dry weight and surface area of the trachealis. Normalization by dry weight would remove the possible influence of edema. Because the surface area of the trachea is unlikely to change significantly in the heavy horse, normalization by the surface area would remove the possible influence of muscular hypertrophy and infiltration of inflammatory cells, which may affect both the wet and dry weight.

No matter how I normalized my ACh release data, all the evidence indicates that the ACh release rate was similar in the trachea between the two groups of horses. This result is in contradiction to the conclusion of a previous study that suggests an increased ACh release from airway cholinergic nerves in the heavy horse (Broadstone et al. 1991). The conclusion of the previous study was inferred by comparing the contractile responses of airway smooth muscle to exogenous ACh and to EFS that releases endogenous ACh. To use this latter approach, it is necessary to assume that exogenous

and endogenous ACh behave similarly. However, because the amount of the neurally released ACh is much less than the exogenously administered ACh, contractions induced by EFS are more likely to be influenced by modulators of muscle contractions than contractions induced by exogenous ACh. Besides, various neurotransmitters may be released from all kinds of nerves during EFS and may therefore affect the response to EFS but not to exogenous ACh. Furthermore, if a factor has an effect on gap junctions, it may influence the response to EFS but not to exogenous ACh. For these reasons, the indirect approach may not always be able to provide a correct conclusion. I therefore believe the evidence obtained in the present study.

In the heavy horse, blockade of muscarinic receptors by intravenous administration of atropine markedly reduces airway resistance during acute exacerbations of airway obstruction. However, atropine does not have such an effect in the control horse or in the heavy horse during remission (Broadstone et al. 1988). The inhibitory effect of atropine on airway resistance in the heavy horse may be due to either an increased sensitivity of airway smooth muscle to ACh or an increased local ACh concentration or both. In vivo, the airway of heavy horses is hyperresponsive to aerosol of methacholine (Armstrong et al. 1986), suggesting that increased sensitivity of airway smooth muscle to neurally released ACh is a possible cause for the development of airway obstruction and, therefore, for the response to atropine. However, isolated airway smooth muscle preparations from heavy horses are not hyperresponsive to ACh in the tissue bath (Broadstone et al. 1991), suggesting that airway smooth muscle may behave differently in its neurohumoral environment in vivo. In the present study, ACh release reflects only changes in efferent nerve function. I observed that ACh release from cholinergic nerves

innervating the trachea was not increased in the heavy horse under the in vitro conditions. However, I could not exclude the possibility that ACh release is increased in vivo, particularly if the increase is due to reflex activation or increased central firing. In addition, an increase in ACh release may happen only in peripheral airways where airway inflammation is most severe.

Muscarinic autoreceptors are potent inhibitory receptors on airway cholinergic nerves. The neurotransmitter ACh can act on presynaptic muscarinic autoreceptors to inhibit further ACh release (Chapters 2, 3). When the receptors are blocked, ACh release increases. In the present study, the non-selective muscarinic antagonist atropine augmented ACh release to the same extent in the normal and heavy horses, suggesting that the autoreceptors are not defective in heavy horses. This observation is in contrast to those made in humans with asthma (Minette et al. 1989) and in guinea pigs infected with parainfluenza virus (Fryer et al. 1990) or challenged by ovalbumin (Fryer and Wills-Karp, 1991). In guinea pigs, antigen challenge is associated with a large influx of inflammatory cells, especially eosinophils, into the airway. Eosinophil proteins, such as major basic protein, are antagonists for M_2 receptors (Jacoby et al. 1992). Although heaves is also a type of allergic pulmonary disease, the inflammatory cells in the lungs are predominantly neutrophils and few eosinophils are present, as revealed by bronchoalveolar lavage (Derksen et al. 1985c). If the presence of a large number of eosinophils is necessary for the dysfunction of muscarinic autoreceptors, lack of numerous eosinophils in the lungs may explain why the function of the autoreceptors is not altered in the heavy horse. Furthermore, the subtype of the muscarinic autoreceptors in the horse airways is not clear. Although the M_2 -selective muscarinic antagonist gallamine

potentiates the contractile response of airway smooth muscle to EFS in guinea pigs (Fryer and MacLagan 1984), cats (Blaber et al. 1985), and dogs (Ito and Yoshitomi 1988), which has been attributed to the blockade of muscarinic autoreceptors, it does not have such an effect in the horse (Yu et al. 1992a), suggesting that there are no gallamine-sensitive functional inhibitory muscarinic receptors on the cholinergic nerve terminals. If only the gallamine-sensitive muscarinic receptors are affected by the airway inflammation, the autoreceptors should then have a normal function in the heavy horse because the autoreceptors on horse airway cholinergic nerves probably belong to another subtype.

The inhibitory effect of the α_2 -adrenoceptor agonist clonidine on ACh release was remarkably yet insignificantly less in the heavy horse trachea than in the normal horse trachea (Chapter 6). In the bronchi, clonidine had no significant effect on ACh release in the heavy horse as observed in the present study, but it inhibited ACh release significantly in the normal horse (Chapter 6). However, the magnitude of inhibition in the bronchi was not significantly different between the normal and heavy horses. Therefore, the difference in prejunctional α_2 -adrenoceptor function between normal and heavy horses is unlikely to be substantial even if it truly exists. In heavy horses, xylazine causes bronchodilation during acute exacerbations of heaves (Broadstone et al. 1992). Since xylazine is an α_2 -agonist, the bronchodilatory response to xylazine may suggest the presence of functional α_2 -receptors on airway cholinergic nerves.

My results obtained with PGE₂ and indomethacin in the heavy horse were similar to those obtained in the normal horse (Chapter 5). Neither PGE₂ nor indomethacin had any effect on ACh release. Therefore, although the airway mucosal PGE₂ production is

decreased in heavy horses (Gray et al. 1992a), such a change in PGE₂ production would not augment ACh release from airway cholinergic nerves.

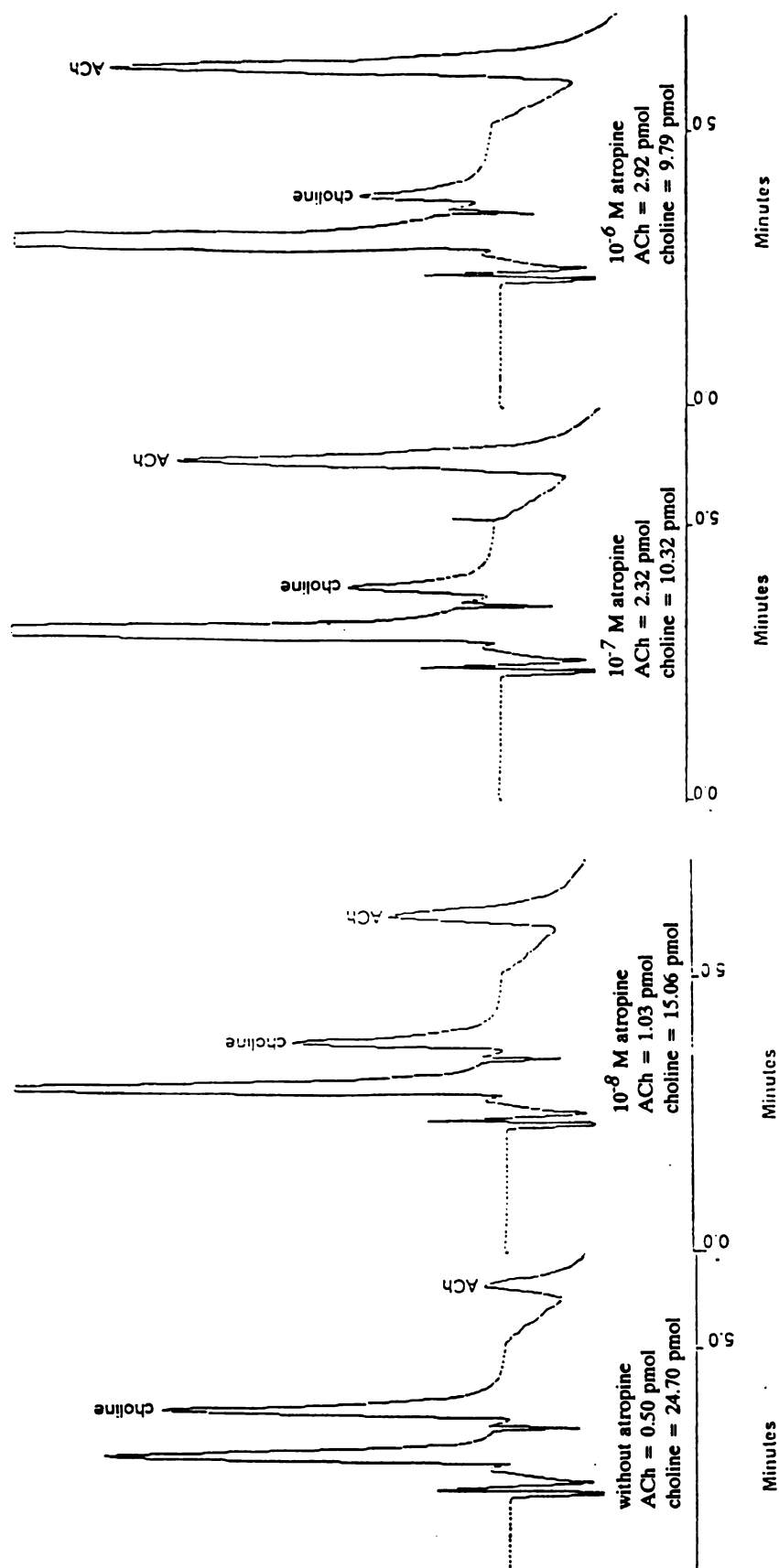


Fig. 7-1. Typical chromatograms showing the effect of atropine on ACh release from cholinergic nerves innervating horse trachea. The sensitivity of the electrochemical detector was set at 100 nA during the first 5 min of each run and at 2 nA thereafter. Twenty-five μ l tissue bath liquid was injected for each sample. Atropine concentration-dependently augmented ACh release.

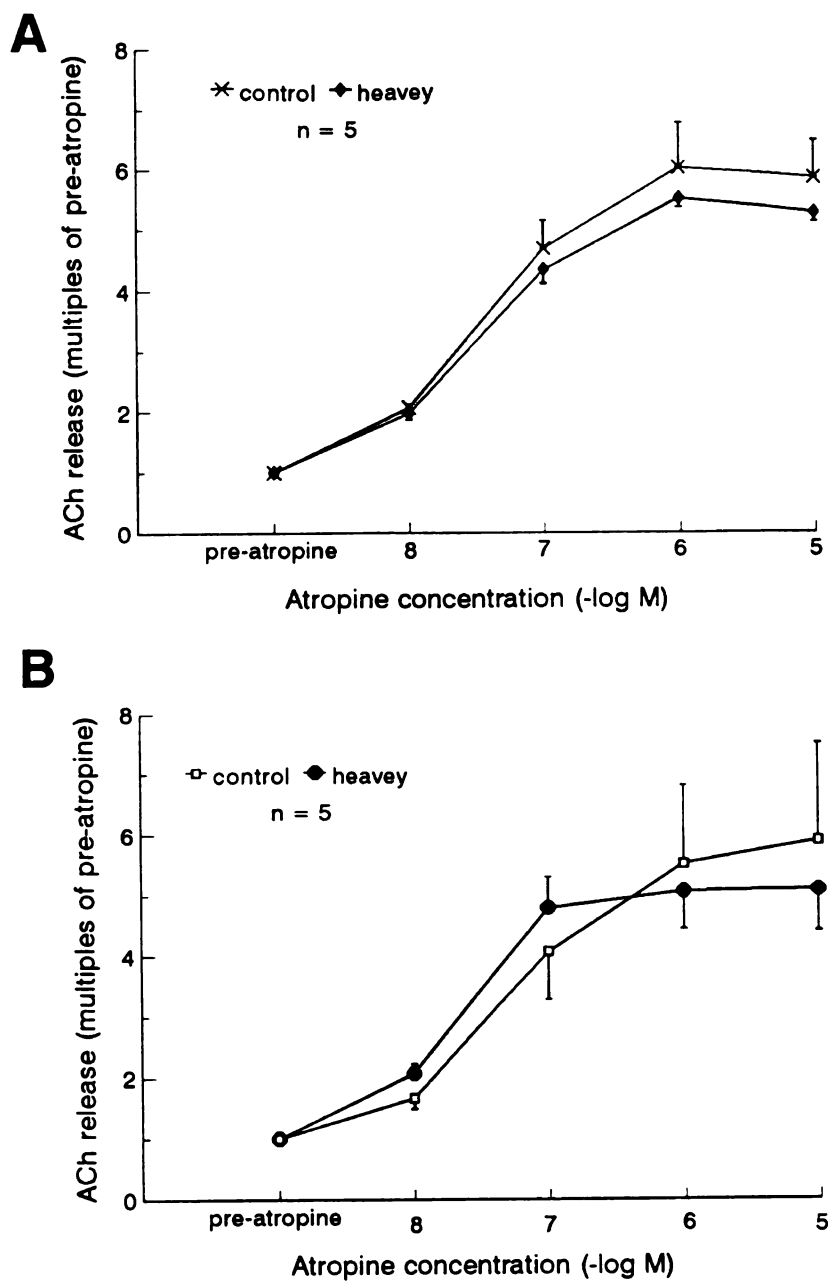


Fig. 7-2. Effect of atropine (10^{-8} – 10^{-5} M) on ACh release from cholinergic nerves innervating the trachea (A) and bronchi (B) of normal and heavy horses. Acetylcholine release rate during the first EFS period was regarded as 1, and the rate during subsequent periods was calculated as multiples of the rate of period 1. There was no difference in the augmenting effect of atropine between normal and heavy horses.

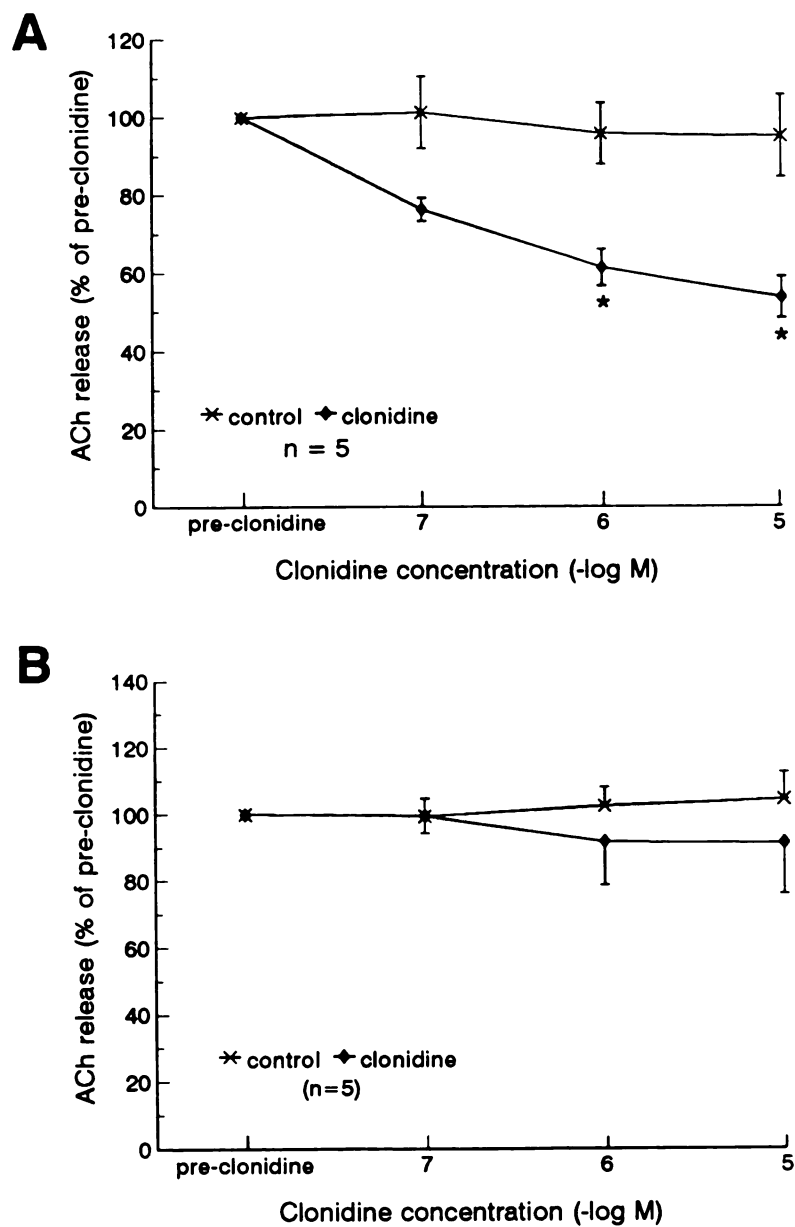


Fig. 7-3. Effect of clonidine (10^{-7} – 10^{-5} M) on ACh release from cholinergic nerves innervating the trachea (A) and bronchi (B) of heavy horses. Clonidine inhibited ACh release concentration-dependently in the trachea but was without an effect in the bronchi.

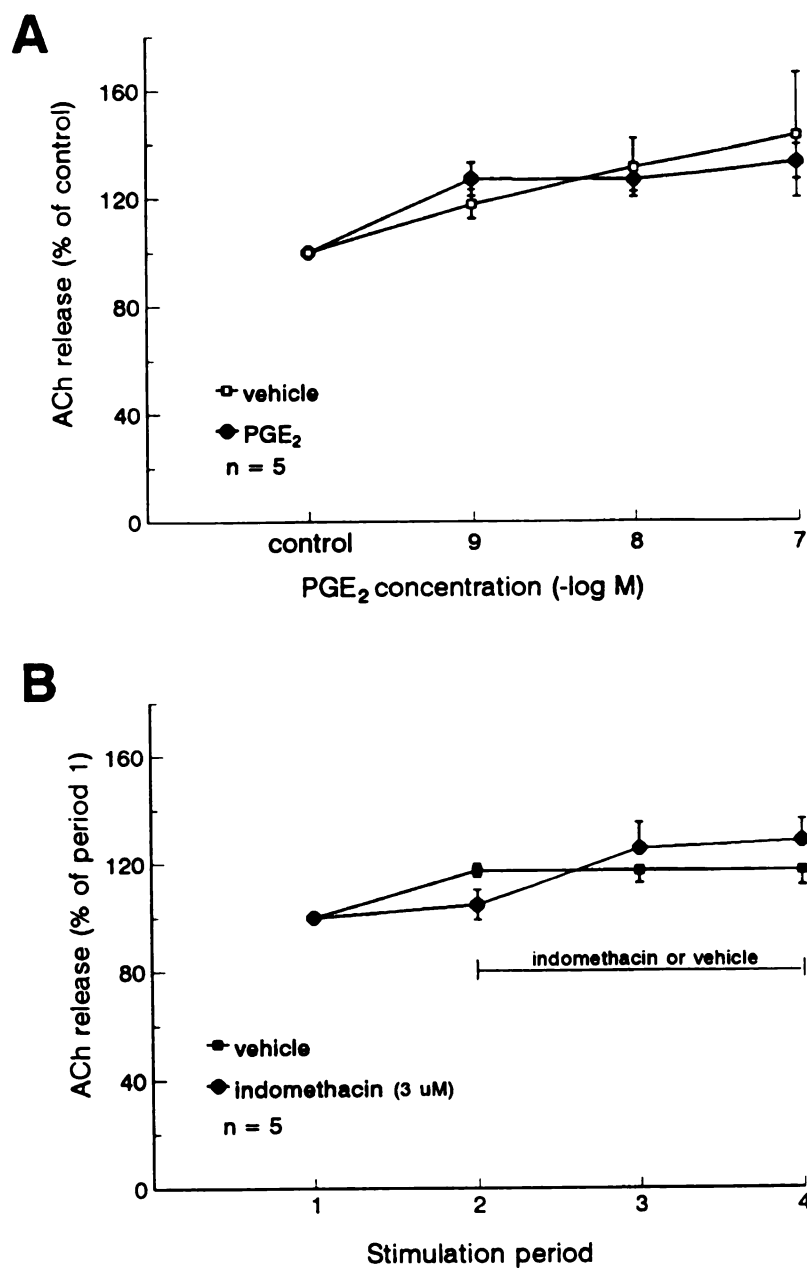


Fig. 7-4. Effects of PGE₂ (10^{-9} – 10^{-7} M) (A) and indomethacin (3×10^{-6} M) (B) on ACh release from cholinergic nerves innervating the heavy horse bronchi. Neither PGE₂ nor indomethacin showed any effect on ACh release.

CHAPTER 8

SUMMARY AND CONCLUSIONS

During my dissertation research, I studied prejunctional modulation of equine airway cholinergic neurotransmission. My interest in equine airways was stimulated by the observation that there is a predominant muscarinic component to airway obstruction in the equine airway disease known as heaves.

In order to obtain direct evidence of airway cholinergic neurotransmission, I adapted an ACh analysis technique to measure EFS-induced ACh release from isolated equine airway smooth muscle preparations suspended in 2-ml tissue baths. With this technique, I first studied the effects of stimulation intensity and muscle preload on ACh release (Chapter 2) because such information was important for the proper design of later experiments. In the absence of EFS, ACh content in the incubation solution was hardly detectable. Acetylcholine release increased sharply in response to EFS. Tetrodotoxin was able to abolish the EFS-induced ACh release, indicating the cholinergic neural origin of ACh. Acetylcholine release was dependent on stimulation intensity. Increasing frequency (0.5–16 Hz) and voltage (5–20 V) increased ACh release; increasing pulse duration (0.5–3 ms) had only a minor effect. Alterations in muscle preload (2–20 g) had no effect on ACh release. ACh release rate was constant within each and over different stimulation periods.

My observation that atropine augmented ACh release (Chapter 2) indicates the presence of muscarinic autoreceptors on horse airway cholinergic nerves. In order to determine which subtype of muscarinic receptors the autoreceptors belong to, the effects of several selective muscarinic antagonists as well as the nonselective muscarinic antagonist atropine were compared (Chapter 3). Although all muscarinic antagonists augmented ACh release, neither hexahydrosiladifenidol (HHSiD, M_3 -selective), pirenzepine (M_1 -selective), nor AF-DX 116 (M_2 -selective) was as potent or augmented ACh release as much as atropine. Hexahydrosiladifenidol inhibited muscle contraction more potently than ACh release, indicating that autoreceptors are not M_3 . Autoreceptors are unlikely to be M_1 because the augmenting effect of pirenzepine emerged at a concentration 240 times its K_d for M_1 receptors. Evidence was inadequate either to confirm or deny M_2 receptors as muscarinic autoreceptors. Although the concentration at which AF-DX began to augment ACh release was only 21 times its K_d for M_2 receptors, the maximal effect of AF-DX 116 was no greater than that of HHSiD or pirenzepine.

To determine if PGE_2 is important in regulating ACh release from equine airway cholinergic nerves, I conducted two studies. In ponies, I studied the effects of exogenous PGE_2 and endogenous prostanoids on ACh release by comparing the contractile response of trachealis strips to EFS and ACh (Chapter 4). Although exogenous PGE_2 inhibited the contractile response to both EFS and ACh in a concentration-dependent manner, the concentration required to inhibit the response to EFS (10 nM) was less than that required to inhibit the response to ACh (0.1 μ M). Cyclooxygenase inhibition with aspirin (10^{-5} – 10^{-3} M) or meclofenamate (10^{-8} – 10^{-4} M) had no effect on either the response to EFS

or to ACh even though PGE₂ production was inhibited. My results suggest that exogenous PGE₂ inhibit airway cholinergic neurotransmission but endogenous prostanoids do not have such an effect.

In horses, the effects of exogenous PGE₂ and endogenous prostanoids on ACh release were determined by direct measurement of ACh release (Chapter 5). Neither exogenous PGE₂ (10^{-9} – 10^{-7} M) nor endogenous prostanoids were demonstrated to have an inhibitory effect on ACh release in both the tracheal and bronchial preparations. In contrast, I demonstrated that both exogenous PGE₂ (10^{-9} – 10^{-7} M) and endogenous prostanoids inhibit ACh release from canine airway cholinergic nerves.

In order to determine the presence and function of α_2 -adrenoceptors on horse airway cholinergic nerves, I studied the effects of several α_2 -receptors agonists and antagonists on ACh release (Chapter 6). The α_2 -receptor agonists clonidine (10^{-7} – 10^{-5} M) and UK 14,304 (10^{-8} – 10^{-6} M) concentration-dependently inhibited ACh release and this inhibition was attenuated by the α_2 -receptor antagonists yohimbine and idazoxan. These results indicate that α_2 -adrenoceptors exist on horse airway cholinergic nerves and activation of these receptors inhibits cholinergic neurotransmission.

I then turned my attention to horses with heaves in order to determine if ACh release from airway cholinergic nerves is increased and if the prejunctional muscarinic autoreceptors and α_2 -adrenoceptors are dysfunctional under in vitro conditions. I observed that ACh release rate from cholinergic nerves innervating the trachealis was not higher in heavey than normal horses. Blockade of muscarinic autoreceptors by atropine (10^{-8} – 10^{-5} M) augmented ACh release to the same extent in normal and heavey horses, suggesting that there is no dysfunction of muscarinic autoreceptors in the heavey horse.

Although clonidine inhibited ACh release in both the trachea and bronchi of normal horses, it inhibited ACh release only in the trachea in horses with heaves. Furthermore, the effect of clonidine in the trachea was less in the heavy horse than in the normal horse. The results suggest that the prejunctional α_2 -inhibitory mechanism is probably dysfunctional. The effect of exogenous PGE₂ and endogenous prostanoids on ACh release was only examined in the bronchi. As was true in the normal horses, neither of them had any effect on ACh release. Lack of an effect of endogenous prostanoids on airway cholinergic neurotransmission suggests that the reduced airway mucosal PGE₂ production of heavy horses (Gray et al. 1992) has no influence on ACh release from airway cholinergic nerves.

For technical reasons, direct measurements of ACh release from airway cholinergic nerves became possible only a few years ago. Traditionally, information about ACh release from airway cholinergic nerves was inferred from comparisons of the contractile response of airway smooth muscle to exogenous ACh and to neural activation that releases endogenous ACh. Although conclusions obtained by the two different approaches are often compatible, such as the case of prejunctional inhibitory α_2 -adrenoceptors on horse tracheal cholinergic nerves (Chapter 6) and the effect of PGE₂ on ACh release in canine airways (Deckers et al. 1989; Waters et al. 1984), I met with several occasions when the two approaches led to different conclusions. First, cyclooxygenase inhibition markedly augments the contractile response of horse bronchial smooth muscle to EFS with only a slight effect on the response to exogenous ACh, suggesting that endogenous prostanoids inhibit airway cholinergic neurotransmission (Yu et al. in press a), but direct ACh measurements indicate that ACh release did not increase

following administration of cyclooxygenase inhibitors (Chapter 5). Second, although activation of prejunctional α_2 -adrenoceptors by clonidine (10^{-6} and 10^{-5} M) inhibits ACh release from cholinergic nerves in horse bronchi (Chapter 6), comparison of the response of bronchial smooth muscle to EFS and ACh was unable to demonstrate such an effect of clonidine over the same concentration range (LeBlanc et al. 1993). Third, although comparison of the response of airway smooth muscle to EFS and exogenous ACh suggests that ACh release from airway cholinergic nerves innervating trachealis of heavy horses is increased under the in vitro conditions (Broadstone et al. 1991), direct measurement of ACh release could not confirm this finding (Chapter 7). The specific causes for these discrepancies are unclear. As I indicated in the introduction of my dissertation, there are some potential problems in evaluating ACh release or prejunctional modulation by comparing the response of airway smooth muscle to EFS and ACh. It remains to be determined when it is appropriate to use the indirect approach to study prejunctional modulation. The above-mentioned discrepancies highlight the importance of direct measurement of ACh release.

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