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SENSORY INNERVATION OF THE EQUINE LUNG:
DISTRIBUTION OF NERVE FIBERS AND TACHYKININ RECEPTORS

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SENSORY INNERVATION OF THE EQUINE LUNG: DISTRIBUTION OF NERVE FIBERS AND TACHYKININ RECEPTORS

By

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ABSTRACT

SENSORY INNERVATION OF THE EQUINE LUNG: DISTRIBUTION OF NERVE FIBERS AND TACHYKININ RECEPTORS

By

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To investigate the effector actions of sensory nerves in the normal equine lung, the anatomical structures involved (nerves and receptors) were studied using immunohistochemical methods to locate the nerves and quantitative [^{125}I] autoradiography to characterize the receptors. Nerves immunoreactive for substance P and calcitonin gene-related peptide (neurotransmitters of sensory nerves) were principally associated with respiratory epithelium, tracheobronchial glands, and vessels in the central airways of adult horses. In neonatal foals, immunoreactive nerves were abundant in both central and peripheral airways and were also associated with airway smooth muscle. Specific binding sites for [^{125}I]-Bolton Hunter substance P were most abundant over tracheobronchial glands, small bronchial vessels, and the respiratory epithelium in the lungs of adult horses, and present but less abundant over airway smooth muscle. Binding of [^{125}I]-Bolton Hunter substance P was completely inhibited by a specific neurokinin-1 receptor agonist, and slightly inhibited by specific neurokinin-2 and -3 receptor agonists, suggesting that the predominant tachykinin receptor type in the equine lung is the neurokinin-1 receptor.

This dissertation is dedicated to the three researchers who were instrumental for the completion of this dissertation:

Dr. Robert M. Bowker

Dr. N. Edward Robinson

Dr. Sorin I. Sonea

and to the one who started it all:

Dr. Christopher M. Brown

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CHAPTER 1

INTRODUCTION

Horses are generally used for athletic purposes so that even minor impairments of pulmonary function can have a serious effect on performance. Heaves, a syndrome with an allergic etiology (McPherson et al 1979b; McGorum et al 1993), is a common cause of pulmonary disease in stabled horses (McPherson et al 1978; McPherson et al 1979a). Horses affected with heaves cough, are hypoxemic, and have decreased exercise tolerance (McPherson et al 1978a); pulmonary resistance increases and pulmonary dynamic compliance decreases during an episode of heaves, reflecting airway obstruction due to bronchoconstriction and intraluminal accumulation of secretions and inflammatory debris (McPherson and Thomson 1983). These signs are reversed when allergens are removed from the environment of susceptible horses (Thomson and McPherson 1984).

In addition to being an important disease of horses, and thus of interest to veterinary clinicians and researchers, heaves is also one of the few naturally occurring models of asthma (Lowell, 1964). Both heaves and asthma are characterized by bronchoconstriction and airway hyperresponsiveness and inflammation (Derksen et al 1985), which are believed to be due to alterations

of neural function in the affected lungs (Barnes 1986a; De Jongste et al 1991; Broadstone et al 1991; Robinson 1993). Equine airways thus provide an excellent naturally occurring model of asthma.

The mechanisms underlying the increased airway tone and hyperreactivity present in the airways of heavey horses have been extensively investigated both in vivo (Murphy et al 1980; Scott et al 1988a; Scott et al 1988b; Scott et al 1991; Broadstone et al 1988; Gray et al 1989; Armstrong et al 1986) and in vitro (Broadstone et al 1991; LeBlanc et al 1991; Gray et al 1992). The bronchoconstriction and airway hyperresponsiveness typical of heaves are not due to an increased sensitivity to acetylcholine or an altered sensitivity to adrenergic agents of the airway smooth muscle itself (Broadstone et al 1991; Scott et al 1988a; Scott et al 1988b.; Scott et al 1991). Instead, in vitro experiments showed that electrical stimulation of nerves in the trachea and central bronchi resulted in more contraction in the airways of heavey horses than in those of normal horses (Broadstone et al 1991). This bronchoconstriction is mediated by cholinergic (parasympathetic) nerves since atropine, a muscarinic receptor antagonist, completely prevents neurally mediated airway smooth muscle contraction in tissues from normal and heavey animals (Broadstone et al 1991; LeBlanc et al 1991). These experiments indicate that cholinergic neurotransmission may be enhanced in the larger airways of heavey horses (Broadstone et al 1991). Enhancement of cholinergic neurotransmission has been shown to be mediated by tachykinins acting on presynaptic or

prejunctional cholinergic nerve terminals in rabbit and guinea pig airways (Tanaka and Grunstein 1986; Hall et al 1989; Watson et al 1993).

Tachykinins are a group of neuropeptides that include substance P (SP) and neurokinin A (NKA) (Solway and Leff 1991; Barnes et al 1991a). Tachykinins and an unrelated peptide, calcitonin gene-related peptide (CGRP), are believed to be the neurotransmitters of intrapulmonary C-fibers (Barnes et al 1991a; Barnes et al 1991b), as suggested by studies showing that tachykinins and CGRP are released from the lung following antidromic neural stimulation (Saria et al 1988), and that the effects of neural stimulation are mimicked by exogenous tachykinins (Takahashi et al 1993). It is possible that a portion of the heightened response to neural stimulation in heavey airways could be due to an enhancement of cholinergic neurotransmission by tachykinins released from afferent nerves within equine airways. Additionally, tachykinins and CGRP released from sensory nerves are believed to contribute to the airway inflammation characteristic of asthma (McDonald 1987; Holzer 1988; Solway and Leff 1991): the inflammation typical of heaves may also be mediated partly through the actions of sensory nerves. To determine whether this hypothesis has an anatomical basis in equine airways, we chose to investigate the existence and distribution of intrapulmonary afferent nerves in the equine lung.

In the last few years, the distribution of intrapulmonary afferent nerves has usually been investigated by detecting nerves immunoreactive for tachykinins (SP, NKA) and/or calcitonin gene-related peptide (CGRP) (Uddman

et al 1985; Uddman et al 1986; Lundberg et al 1984; Uchida et al 1987; Laitinen et al 1983; Nohr and Weihe 1991). The data gathered using non-immunological methods have been described extensively in several excellent reviews (Richardson 1979; Coleridge and Coleridge 1984; Coleridge and Coleridge 1986). In the lung, nerves containing SP and NKA (which are products of the same gene) also usually contain CGRP (Martling et al 1988; Nohr and Weihe 1991; Uddman et al 1986). Most intrapulmonary nerves immunoreactive for SP, NKA, and/or CGRP are C-fiber afferents, since capsaicin, which selectively affects unmyelinated sensory nerves (Jancsó et al 1967; Jancsó et al 1977), causes the disappearance of many or all nerves immunoreactive for these neuropeptides in the lung (Lundberg et al 1983b; Lundberg et al 1984; Cadieux et al 1986; Alving et al 1991; Solway and Leff 1991).

The distribution of nerves immunoreactive for SP, NKA, or CGRP has been studied most extensively in rodents (Martling et al 1988; Lundberg et al 1985; Uddman et al 1985; Cadieux et al 1986; Luts et al 1990; Keith and Ekman 1988; Nohr and Weihe 1991; Lundberg et al 1984; Uchida et al 1987; Laitinen et al 1983; Lundberg et al 1983b). The distribution of intrapulmonary afferent nerves has also been studied in cats (Nohr and Weihe 1991; Dey et al 1988; Lundberg et al 1984; Uddman et al 1985; Martling et al 1988), dogs (Nohr and Weihe 1991; Uddman et al 1985), and man (Laitinen et al 1983; Lundberg et al 1984; Uddman et al 1985; Palmer et al 1987; Martling et al 1988; Hislop et al 1990; Allen et al 1989). The general pattern of sensory

nerve distribution is similar in all species: nerves containing SP, NKA, or CGRP are usually present within and below the respiratory epithelium (Uddman et al 1985; Lundberg et al 1984; Uchida et al 1987; Laitinen et al 1983; Martling et al 1988; Keith and Ekman 1988; Luts et al 1990; Cadieux et al 1986; Palmer et al 1987; Dey et al 1988; Hislop et al 1990), around vessels (Uddman et al 1986; Uddman et al 1985; Martling et al 1988; Lundberg et al 1985; Luts et al 1990; Cadieux et al 1986; Palmer et al 1987; Lundberg et al 1984; Dey et al 1988; Hislop et al 1990; Allen et al 1989), in airway smooth muscle (Uchida et al 1987; Uddman et al 1985; Lundberg et al 1984; Martling et al 1988; Lundberg et al 1985; Luts et al 1990; Cadieux et al 1986; Palmer et al 1987; Laitinen et al 1983; Lundberg et al 1984; Dey et al 1988; Hislop et al 1990) and, sparsely, in alveolar walls (Uddman et al 1985; Cadieux et al 1986; Nohr and Weihe, 1991).

Although the general pattern of distribution of afferent nerves is similar in all mammals studied to date, marked species variations exist. Sensory nerve fibers are most abundant and widely distributed in rodents, particularly guinea pigs (Lundberg et al 1984), whereas they are relatively scarce and primarily concentrated in the central airways in man (Hislop et al 1990). The extent of the distribution of nerves containing SP, NKA, or CGRP in airway smooth muscle is also quite variable: in guinea pigs and rats, the airway smooth muscle contains numerous such nerves (Cadieux et al 1986; Lundberg et al 1984), whereas in human airways these nerves are scarce or non-existent (Laitinen et al 1983; Hislop et al 1990). Calcitonin gene-related peptide immunoreactive

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fibers are rarely seen within the epithelium in the human lung (Palmer et al 1987), whereas in other species they are common (Cadieux et al 1986; Luts et al 1990; Uddman et al 1985). Some of the reported differences between species, and between studies in the same species, might be due to the antibodies and techniques employed, which vary in specificity and sensitivity.

Immunoreactive nerve fibers generally are more numerous in the central airways and the trachea and less frequently seen in peripheral airways (Uddman et al 1985; Lundberg et al 1984; Uchida et al 1987; Laitinen et al 1983; Hislop et al 1990; Cadieux et al 1986). This has important implications for pulmonary function, since the neural control of airway tone is thus likely to vary with the location and size of the airway under study. Certainly, the trachea, main bronchi, and peripheral airways in the guinea pig lung react differently to neural stimulation, reflecting differences in the extent of their innervation (Manzini et al 1989; Lindén et al 1991). The greater maximal response to neural stimulation in the trachea of normal horses compared to third-generation bronchi (Broadstone et al 1991) and peripheral bronchi (LeBlanc et al 1991) might reflect a more extensive innervation of the trachea and central airways in the horse. In view of the much greater density of innervation in rodent lungs compared to human lungs, the rodent lung may not be a good model of the human lung in regard to neural modulation of pulmonary function. Unfortunately, systematic investigations of species differences, or of differences between the various regions of the lung, have not been carried out.

Sensory fibers in the lung are generally most numerous within and just below the respiratory epithelium, where they are ideally placed to detect noxious stimuli. These fibers can be activated by a wide variety of stimuli, initiating centrally mediated reflexes such as coughing, rapid, shallow breathing, and bronchoconstriction (Coleridge and Coleridge 1986; Solway and Leff 1991; Coleridge et al 1991). Simultaneously, sensory neurotransmitters are released within the lung following antidromic propagation of the neural impulse (local axon reflexes) (Holzer 1988; Solway and Leff 1991). Stimuli activating intrapulmonary C-fibers include inhaled irritants such as cigarette smoke, ammonia vapor, and SO_2, and mechanical irritants such as direct pressure, dust, dry air, or hypertonic solutions (Lundberg et al 1983a; Solway and Leff 1991; Coleridge and Coleridge 1986). Inflammatory mediators including bradykinin (Geppetti et al 1990; Saria et al 1988), histamine (Saria et al 1988; Ellis and Undem 1992), and prostacyclin (Mapp et al 1991) can also cause or enhance the release of sensory neurotransmitters in the lung. Furthermore, there is abundant evidence that allergens can, via the release of inflammatory mediators, cause the intrapulmonary release of sensory neurotransmitters (Alving 1991; Matsuse et al 1991; Kohrogi et al 1991; Ellis and Undem 1992), thereby accentuating the signs of allergic pulmonary disease.

Capsaicin, an extract of chili peppers, also causes the release of tachykinins and CGRP from C-fiber nerve terminals (Coleridge and Coleridge 1984; Saria et al 1988; Solway and Leff 1991; Holzer 1988), without affecting other nerves (Jancsó et al 1977; Jessell et al 1978; Solway and Leff 1991; Holzer

1988). This effect has been exploited extensively to study the actions of unmyelinated sensory nerves within the lung (Solway and Leff 1991; Holzer 1988; Coleridge and Coleridge 1984; Coleridge and Coleridge 1986).

Tachykinins and CGRP can have dramatic systemic and intrapulmonary effects, including vasodilation, systemic hypotension, plasma extravasation, bronchoconstriction, increased tracheobronchial secretions, and attraction and activation of mast cells and leukocytes (Solway and Leff 1991; Holzer 1988; Barnes et al 1991a; Barnes et al 1991b). These effects are mediated via specific receptors on target tissues such as airway smooth muscle, vessels, tracheobronchial glands, mast cells, and leucocytes (Solway and Leff 1991; Holzer 1988; Barnes et al 1991a; Barnes et al 1991b). Three major classes of tachykinin receptors have been identified to date, based on their relative affinities for SP, neurokinin A (NKA), and neurokinin B (NKB) (Quirion and Dam 1988) (Table 1). There is abundant pharmacological evidence for tachykinin receptors in airway smooth muscle, epithelium, and many other intrapulmonary tissues (Barnes et al 1991a; Regoli 1987). Calcitonin gene-related peptide acts on a distinct CGRP receptor present predominantly on vessels and alveolar walls (Mak and Barnes 1988).

Table 1. Characteristics of tachykinin receptors

	NK-1	NK-2	NK-3
relative affinities	SP)NKA)NKB	NKA)NKB)SP	NKB)NKA)SP
selective agonists	[Sar^9,$Met(O_2)^{11}$]-SP [Pro^9]-SP sulfone [βAla^4,Sar^9,$Met(O_2)^{11}$]-$SP_{(4-11)}$	[Nle^{10}]-$NKA_{(4-10)}$ $NKA_{(4-10)}$ [βAla^8]-$NKA_{(4-10)}$	senktide [$MePhe^7$]-NKB [βAsp^4,$MePhe^7$]-$NKB_{(4-10)}$

Systemically, tachykinins cause severe hypotension, tachycardia, vasodilation, and plasma extravasation (Hua et al 1984; Joos et al 1986). The effects on airway resistance are variable: marked increase in rodents (Hua et al 1984; Joos et al 1986), slight increase in cats (Andersson and Persson 1977), and little effect in man (Fuller et al 1987). These differences may reflect variations in receptor types or densities between species, or may only reflect the different dosages used. The in vivo infusion of CGRP causes similar cardiovascular effects but no edema or changes in pulmonary resistance (Abdelrahman et al 1992; Martling et al 1988; Gatto et al 1989; Matran et al 1989). Interestingly, in guinea pigs CGRP inhibits the SP-induced increase in pulmonary resistance (Gatto et al 1989).

In the vasculature, activation of NK1 receptors (Burcher et al 1991) causes a rapid but short-acting vasodilation (Piedimonte et al 1992; Laitinen et al 1987; Salonen et al 1988; Joos et al 1986; Andersson and Persson 1977; Fuller et al 1987; Saria et al 1988; Alving 1991; Worthen et al 1985).

Although tachykinins generally cause vasodilation, there are some notable exceptions, such as the NK2 receptor-mediated contraction of the endothelium-denuded rabbit pulmonary artery (Maggi et al 1990) and the NK3-receptor mediated contraction of the rat portal vein (Mastrangelo et al 1986). In general, CGRP synergistically enhances the vascular effects of tachykinin administration (Gamse and Saria 1985; Newbold and Brain 1993), perhaps by serving as a preferred substrate for the peptidases that degrade tachykinins (LeGreves et al 1985; LeGreves et al 1989). Calcitonin gene-related peptide is a potent dilator of arteries in its own right (Uddman et al 1986; Brain et al 1985), stimulating the release of the vasodilator nitric oxide (Abdelrahman et al 1992).

Activation of NK1 receptors in the rodent trachea and lungs causes neurogenic edema (Saria et al 1983; McDonald 1987; Solway and Leff 1991); NK2 receptors also contribute to this response in guinea pig airways (Eglezos et al 1991; Lei et al 1992; Rogers et al 1988; Tousignant et al 1993). In rats tachykinin receptors are predominantly localized in the trachea and central airways, which are the sites of neurogenic plasma extravasation (Sertl et al 1988).

In most species, tachykinins cause airway obstruction attributable to contraction of airway smooth muscle (Lundberg and Saria 1982), acting on receptors on airway smooth muscle or on cholinergic nerves. Activation of NK2 receptors causes bronchoconstriction in several species including man (Ellis and Undem 1990; Ireland et al 1991; Dion et al 1990; Maggi et al 1991);

in the guinea pig, NK1 receptor activation also contributes to bronchoconstriction (Ireland et al 1991; Maggi et al 1991). However, in rat and mouse, activation of NK1 receptors relaxes airway smooth muscle, an effect believed to be mediated via the release of prostanoids from the respiratory epithelium (Manzini 1992; Devillier et al 1992). Activation of presynaptic (Watson et al 1993) or prejunctional (Joos et al 1988; Hall et al 1989) tachykinin receptors on cholinergic nerves enhances acetylcholine release, which also causes bronchoconstriction. In contrast, CGRP generally has no effect on pulmonary resistance or airway smooth muscle tone (Martling et al 1988; Luts et al 1990; but see Palmer et al 1987). In fact, CGRP infusion in vivo inhibits the SP-induced increase in pulmonary resistance in guinea pigs (Gatto et al 1989).

The tachykinins have several pro-inflammatory effects. Substance P and NKA attract neutrophils and monocytes (Wiedermann et al 1989), stimulate lymphocyte proliferation (Payan et al 1984), and induce the release of inflammatory mediators from alveolar macrophages (Brunelleschi et al 1992). Substance P administration or sensory nerve activation causes release of histamine from mast cells (Ali et al 1986; Stead et al 1990). Although the breakdown products of CGRP are strong attractors of eosinophils, and may mediate eosinophil infiltration (Barnes et al 1991b), the effects of CGRP are generally anti-inflammatory (Barnes et al 1991b).

Obviously, activation of sensory nerves can have important effects on pulmonary function in both health and disease. To begin to understand the possible roles of intrapulmonary afferent nerves and their neurotransmitters in

the regulation of equine airway function, we investigated the distribution of nerves immunoreactive for SP and CGRP in the normal lungs of adult horses (Chapter 2). Central and peripheral airways were examined to try to detect regional variations of innervation. Since foals are particularly susceptible to pulmonary diseases (Beech 1985) in which the effector functions of C-fibers may be important, we also examined the distribution of SP and CGRP immunoreactive nerves in the lungs of neonatal foals (Part V). Furthermore, since tachykinins can mediate such a wide variety of effects, depending on the number and type of tachykinin receptor(s) present, we determined the distribution and type of tachykinin receptors present in central and peripheral airways of normal adult equine lungs using quantitative [^{125}I] receptor autoradiography (Chapter 4).

CHAPTER 2

SUBSTANCE P AND CALCITONIN GENE-RELATED PEPTIDE-LIKE IMMUNOREACTIVE NERVE FIBERS IN ADULT EQUINE LUNGS

Introduction

Heaves, a common disease of stabled adult horses, is characterized by airway obstruction following exposure of susceptible animals to dusty environments (McPherson et al 1978; Derksen et al 1985; Beech 1991). The bronchoconstriction present in heavey horses is reversed by atropine administration: the bronchoconstriction is therefore mediated by cholinergic nerves (Broadstone et al 1988; Broadstone et al 1991). In other species, pulmonary sensory nerves also contribute to the development of many of the signs of pulmonary disease such as inflammation and airway obstruction by modulating the release of acetylcholine from cholinergic nerves, or by acting directly on respiratory epithelium, vessels, airway smooth muscle, and tracheobronchial glands (Holzer 1988; Barnes et al 1991b; Solway and Leff 1991), but little is known about the effects of sensory nerve stimulation in equine airways.

In the species studied to date (rats, guinea pigs, dogs, and ferrets), sensory nerve endings in the respiratory epithelium can be activated by viruses, mycoplasma, smoke, ozone, or the release of specific inflammatory mediators

(Holzer 1988; Barnes et al 1991b; Solway and Leff 1991). The responses of sensory nerves to these stimuli are transmitted to the spinal cord and brain as well as to other branches of the same nerve fibers within the lung via local axon reflexes, resulting in the release of neurotransmitters both centrally and peripherally (Solway and Leff 1991). The neurotransmitters released by sensory nerves include one or more tachykinins such as substance P (SP) and neurokinin A, and usually also include calcitonin gene-related peptide (CGRP) (Solway and Leff 1991). The release of sensory neurotransmitters in the spinal cord and brain is responsible for reflexes such as coughing, increased tracheobronchial secretion, and laryngospasm (Holzer 1988; Solway and Leff 1991). In man and laboratory animals, the local pulmonary effects of tachykinins and CGRP include alterations of airway smooth muscle tone, vasodilation, edema formation, recruitment of neutrophils and other leukocytes, and histamine release from macrophages (Holzer 1988; Barnes et al 1991b; Solway and Leff 1991).

The potential contributions of sensory nerves to the regulation of pulmonary function in the equine lung are unknown at present. However, in view of the findings in other species, it is reasonable to suppose that the activation of pulmonary sensory nerves by irritants and inhaled allergens could lead to the development of the airway obstruction and hyperreactivity characteristic of heaves in horses.

As a first step in defining the role(s) of sensory nerves in the regulation of equine pulmonary function, we used immunohistochemical methods to

determine the distribution of pulmonary nerves containing SP or CGRP in adult equids with no clinical signs of pulmonary disease.

Materials and methods

In 13 adult horses and one adult pony, pulmonary tissue was collected from the areas shown in Fig 1. Table 1 describes the animals and lists the tissues examined for each animal; airway diameters are given in Table 2. None of the animals had clinical signs of pulmonary disease; the samples obtained appeared grossly and microscopically normal. Tissues obtained from these animals were used for several concurrent research projects in addition to this study.

The animals were killed with an intravenous injection of pentobarbital ($\geq$100 mg/kg). Samples were collected within 30 minutes of the animal's death and fixed by immersion in a 4% solution of paraformaldehyde buffered with 0.1 M sodium phosphate (pH as described subsequently); to improve the preservation of peripheral parts of the lung, the fixative was instilled via the bronchi into the peripheral portions of the lung prior to immersion in fixative. Tissues from animals 1 to 12 were fixed at pH $\approx$ 7.4, 4°C, for several days prior to immersion in 23% sucrose, 0.8% paraformaldehyde in 0.1 M sodium phosphate buffer (pH $\approx$ 7.4) for cryoprotection and long-term storage at 4°C. After determining that peptides such as peptide histidine isoleucine and vaso-active intestinal peptide were poorly recognized by specific antisera following prolonged fixation, the fixation protocol was modified. We discovered that the

immunohistochemical detection of SP or CGRP in respiratory tissue was not appreciably affected by the pH (within a range of 7 to 10) of the fixative nor by the duration of fixation (1 to several days), although the detection of some other peptides was optimal with fixation at pH ≈ 8 for 24 to 36 hours. To enhance the detection of other neuropeptides, the tissues from horses 13 and 14 were therefore fixed for 24 to 36 hours at pH ≈ 8, 4°C, prior to cryoprotection and storage as described previously.

The immunohistochemical procedure has been described in detail elsewhere (Sonea et al 1993). In brief, cryoprotected tissues were embedded in 1% agarose at 42°C from which free-floating 40 μm thick sections were obtained on a freezing stabe microtome; the most peripheral portions of the lung were fragile and were sectioned at 60 μm. After rinsing the sections in 0.1M sodium phosphate buffer (PB; pH = 7.4), endogenous peroxidases were quenched with 100% methanol, and the sections were rinsed again in PB followed by 2 or 3 rinses in 1% non-immunized goat serum and 0.1% Triton-X 100 in PB (1% NGS). Sections were then incubated for 30 minutes in 3% non-immunized goat serum, 0.1% Triton-X 100, and 360 mg gelatin per 100 ml of PB (3% NGS); all incubations were at room temperature unless otherwise indicated. The sections were then incubated with rabbit anti-SP (1:8000) (Peninsula) or anti-CGRP (1:16000) antiserum (Peninsula) in 1% NGS, for 72 hours at room temperature, or overnight at 37°C. After washing with 1% NGS and a 30-minute incubation in 3% NGS, the sections were then placed in anti-rabbit IgG biotinylated goat antiserum (Vector Laboratories or Zymed Laboratories) in

1% NGS (1:200) for 12 to 24 hours at room temperature, or 2 hours at 37°C. Sections were rinsed again with 1% NGS, incubated in 3% NGS for 30 minutes, and washed with PB containing 0.9% NaCl prior to placement in an avidin-peroxidase (Vector Laboratories) or streptavidin-peroxidase (Zymed Laboratories) complex in PB containing 0.9% NaCl (1:200) for 12 to 24 hours at room temperature, or 2 hours at 37°C. The location of the antigen-immunoglobulin-peroxidase complex was revealed using nickel intensified 3,3' diaminobenzidine tetrahydrochloride as the chromagen (Bowker 1986). The final result was a black insoluble deposit at the site of the antigen-antibody-peroxidase complex.

Control experiments included pre-incubation of each antibody with its respective antigen (100 μg/ml CGRP [Sigma] or 500 μg/ml SP [Sigma]) prior to incubation with the tissue, and substitution of non-immunized rabbit serum for the primary antibody. Specific staining of neural elements was not observed in either control experiment. Since the antigens detected in equine pulmonary tissue may have been very similar but not identical to SP or CGRP, we used the terms SP- or CGRP-like immunoreactive (-LI) to describe the peptides recognized by the specific antisera in this study.

Most immunostained sections were counterstained with methylene blue, azure B, and eosin Y prior to examination with a light microscope; some sections were not counterstained to enhance the photographic appearance of immunoreactive nerves.

Results

The black, beaded immunoreactive nerve fibers were clearly visible in both counterstained and non-counterstained sections. Appreciable differences were not noticed between the results obtained with avidin-peroxidase or streptavidin-peroxidase, or between those obtained following prolonged room temperature incubations and those obtained following the more brief 37°C incubations. The distributions of SP-LI and CGRP-LI nerve fibers were very similar, although the staining of SP-LI was nearly always fainter and SP-LI nerve fibers appeared less numerous when compared to CGRP-LI fibers in adjacent, non-serial sections (Fig 2). Within the examined population, age, sex, or breed did not influence the frequency with which SP- or CGRP-LI nerves were detected, although individual animals varied widely: immunoreactive nerves were abundant in horses 4, 7, and 14, scarce in horses 2, 5 and, 13, and intermediate in the others. In all horses, immunoreactive nerves were most common in the larger airways and rarely seen in the pulmonary parenchyma.

Respiratory epithelium—Within the respiratory epithelium, immunoreactive nerves were most numerous in the trachea, first- and third-generation bronchi, and near bronchial bifurcations in cranial and caudal smallbronchi (see Fig 1). In these areas, SP- and CGRP-LI nerve fibers often formed very dense networks just below the epithelium (Fig 3), at the level of the basal lamina, from which fine, beaded fibers spread into the respiratory epithelium, at times appearing to reach the lumen of the airway (Fig 4). In these airways, although the immunoreactive networks were not present in every field of view at a

magnification of 100X, immunoreactive nerve fibers were nearly always present within the respiratory epithelium in the same views. In un-branched segments of cranial and caudal small bronchi with an inner diameter ≥ 4 mm (with the exception of the large caudo-dorsal bronchus; see Fig 1 for sampling site), the neural networks below the epithelium were usually less dense and were separated by large areas devoid of SP- or CGRP-LI nerve fibers. Immunoreactive networks or intra-epithelial nerve fibers were only rarely detected (usually near bronchial bifurcations) in bronchi smaller than 4 mm diameter, and never in non-cartilaginous bronchioles.

Vasculature—In the connective tissues surrounding the airways, immunoreactive nerves wound through the adventitial layer of 50 to 2000 μm inner diameter bronchial arteries (Fig 5). Bronchial veins were more sparsely innervated. Large (≥ 1 mm diameter) pulmonary vessels accompanying bronchi only contained a few SP- or CGRP-LI nerve fibers in the adventitial layer, usually associated with the *vasa vasorum* (Fig 6). In the parenchyma, the pulmonary arteries and veins were sometime difficult to distinguish; when it was possible to clearly identify the vessels, pulmonary arteries were innervated, whereas pulmonary veins were not (Fig 7).

Immunoreactive nerves also surrounded thin-walled venous sinusoids (30 to 155 μm diameter) near the luminal surface of the respiratory smooth muscle of the trachea and the first- and third-generation bronchi (Fig 8). Immunoreactive nerve fibers often accompanied the numerous small (4 to 15 μm diameter) vessels just below the respiratory epithelium within the lamina propria of the

trachea and larger cartilaginous bronchi (≥ 4 mm diameter) (Fig 9); the same nerve fibers appeared to form the networks at the level of the basal lamina described earlier.

Airway smooth muscle—Substance P- or CGRP-LI nerve fibers rarely innervated airway smooth muscle. In 12 of the 14 adult animals studied, immunoreactive nerve fibers coursed in small fascicles (usually 2 to 6 fibers) on the surface of muscle bundles (Fig 10), often accompanying small vessels, but did not branch on the surface of the airway smooth muscle. However, branching SP and CGRP-LI nerve fibers unaccompanied by vessels were seen on the surface of the airway smooth muscle of cartilaginous bronchi in the remaining 2 animals (#4 and #7) (Fig 11).

Tracheobronchial glands—A variable proportion of the tracheobronchial glands present in the larger airways contained SP- or CGRP-LI nerve fibers (Fig 9). Due to the section thickness it was difficult to determine whether the nerve fibers accompanied vessels within the glands or innervated the gland itself. Immunoreactive nerves were seen in close apposition to the ducts of tracheobronchial glands within the lamina propria (Fig 12).

Lymph nodes—Substance P- or CGRP-LI nerve fibers accompanied blood and lymphatic vessels in the middle tracheobronchial lymph nodes; a few isolated nerve fibers unaccompanied by a vessel were seen within the parenchyma of the nodes (Fig 13).

Neural structures—Occasionally, small tangles of immunoreactive nerves were seen at the periphery of bronchial cartilages or at the attachment of the

trachealis to the tracheal cartilage (Fig 14). These nerve fibers appeared to encircle a spindle-shaped structure and may therefore represent a type of sensory receptor.

Regional distribution—Nerve fibers immunoreactive for SP or CGRP were very rarely detected in the lamina propria of the sampled large caudo-dorsal bronchi (see Fig 1), although they were abundant in the lamina propria of the third-generation bronchi collected and processed simultaneously (Fig 15). When present, the rare immunoreactive nerves in the lamina propria of the caudo-dorsal bronchus had a distribution similar to that of the third-generation bronchi. The sampling site of the caudo-dorsal bronchus had been chosen to yield an airway with an inner diameter similar to that of third-generation bronchi (Table 2): these differences were therefore not due to differing airway diameters. However, the samples of caudo-dorsal bronchus selected were free of branch-points for 1 cm or more, whereas the third-generation bronchi were short and branched soon after arising.

Discussion

Our study clearly demonstrates the presence of nerves immunoreactive for two sensory neuropeptides, SP and CGRP, within the lungs of adult equids. The distributions of SP-LI and CGRP-LI nerves were very similar in adjacent sections of equine pulmonary tissue. Tachykinins and CGRP are colocalized within most pulmonary sensory nerves in the lungs of other species (Martling et al 1988). It is therefore possible that SP-LI nerve fibers in the equine lung

contained CGRP, although the reverse may not always have held true since CGRP-LI nerves seemed more abundant than SP-LI nerves in many adjacent, non-serial sections.

In this study, immunoreactive nerves were prominently associated with the respiratory epithelium and the vasculature. This distribution is identical to that of nerve immunoreactive for another tachykinin, neurokinin A (Sonea and Bowker 1991d) and to that reported in a separate study of 5 adult horses with healthy lungs (Hillman et al 1992). Sensory innervation in the equine lung is thus similar to that of other mammals such as rats, guinea pigs, dogs, cats, and man, although there are some species specific differences in the extent of innervation (Nohr & Weihe 1991; Lundberg et al 1984; Martling et al 1987b).

Respiratory epithelium—The presence of numerous fine SP- and CGRP-LI nerve fibers within the airway epithelium of the larger airways, particularly near bifurcations, indicates that sensory nerves are well placed to detect inhaled noxious stimuli in adult equids. Inhalation of allergens by horses susceptible to heaves or the destruction of respiratory epithelium by viral agents could therefore cause the activation of sensory nerves in the respiratory epithelium and the subsequent intrapulmonary release of SP and CGRP. Stimulation of sensory nerve fibers has been shown in other species to lead to local and central release of tachykinins and CGRP, followed by coughing, bronchoconstriction, and local inflammation (Lundberg et al 1991; Holzer 1988; Barnes et al 1991b; Solway and Leff 1991).

Furthermore, the damage or loss of respiratory epithelium noted in heaves (Kaup et al 1990) or viral infections (influenza, equine herpesvirus I) (Powell 1991) may enhance and prolong the actions of tachykinins and CGRP, since the epithelium is the principal source of neutral endopeptidases, the enzymes responsible for the degradation of these neuropeptides (Nadel and Borson 1991). Inhibition of neutral endopeptidases potentiates the tachykinin-induced increase in pulmonary resistance in guinea pigs (Thompson and Sheppard 1988), and is believed to be one of the mechanisms whereby influenza and other common pathogens enhance the inflammatory response in the lung (Nadel and Borson 1991; McDonald 1992).

Vasculature—The extensive network of small vessels in the lamina propria of the bronchi and trachea carry nutrients and oxygen to the respiratory epithelium (McFadden 1992). These vessels are involved in thermoregulation and are also the source of the fluids secreted by the respiratory epithelium during exercise to counteract the dehydration of the airways (McFadden 1992).

The neural control of the tone of equine bronchial vessels appears complex: nerves containing adrenergic and several peptidergic neurotransmitters have been described in close apposition to the numerous small vessels in the lamina propria (Sonea and Bowker 1991d; Sonea et al 1991c; Sonea et al 1993). Of the neurotransmitters detected in our studies of equine airways, several are vasodilators in other species: SP, CGRP, vasoactive intestinal peptide, and peptide histidine isoleucine (Barnes et al 1991a; Barnes et al 1991b). In the equine lung, the contribution of SP- and CGRP-LI nerves to the

neural control of vascular tone in the lamina propria may be substantial. This hypothesis is supported by the presence of high concentrations of SP binding sites on these vessels (see Chapter 4).

Should stimulation of sensory nerves within equine airways lead to the release of tachykinins and CGRP in the equine lung via local axon reflexes, these neurotransmitters are likely to cause vasodilation of the bronchial vessels in the lamina propria. In turn, vasodilation of these vessels might result in congestion of the lamina propria and changes in its thickness. As demonstrated in sheep, congestion and dilation of bronchial vessels can substantially reduce airway diameter (Mariassy et al 1991; Lockhart et al 1992). Vascular congestion due to the local release of SP and CGRP may contribute to the airway obstruction characteristic of heaves and other equine pulmonary diseases. In support of this hypothesis, it has been noted that the impairment of pulmonary function observed in heavey horses (McPherson et al 1978a; Derksen et al 1985) is not completely reversed by bronchodilators (Murphy et al 1980; Broadstone et al 1988), indicating that factors other than brochoconstriction contribute to airway obstruction in this syndrome.

In many species, SP also causes increased vascular permeability of tracheobronchial vessels (Rogers et al 1988; Eglezos et al 1991), leading to edema formation. In the lung, tachykinin-mediated plasma extravasation has only been reported in rodents (Rogers et al, 1988; Lembeck and Holzer 1979) and has not been investigated in horses.

Airway smooth muscle—The relative scarcity of SP- or CGRP-LI nerve fibers closely associated with airway smooth muscle observed in this study may indicate that airway smooth muscle tone is not the primary target of the efferent actions of sensory nerves in the adult horse. Preliminary experiments indicate that SP causes little or no bronchoconstriction in small (3 to 5 mm diameter) airways of adult horses (Le Ninivin et al 1993; LeBlanc, personal communication). The weak bronchoconstrictive effects of tachykinins may reflect the small number of SP binding sites in the airway smooth muscle of normal adult horses (see Chapter 4). Tachykinins or CGRP may still increase airway smooth muscle tone indirectly, either by causing the release of inflammatory mediators from macrophages and leukocytes, or by modulation of cholinergic neurotransmission (Hall et al 1989).

Regional distribution—The primary function of epithelial afferent nerves in the lung is probably the detection of inhaled noxious substances which, via both local axon reflexes and centrally mediated reflexes, would initiate protective mechanisms such as increased tracheobronchial secretion, bronchospasm, rapid, shallow breathing, and perhaps coughing (Solway and Leff 1991; Coleridge and Coleridge 1986). In adult horses, the scarcity of sensory nerves in the large caudo-dorsal bronchi may signify that the protective responses of these airways and the lung supplied by them are reduced in comparison to the responses of the trachea and first- and third-generation bronchi. It is thus possible that the scarcity of sensory nerves in the caudo-dorsal bronchi may increase the chances of damage to the caudo-dorsal areas

of the lung. In this respect, it is interesting to note that the area of predilection for exercise-induced hemorrhage is the caudo-dorsal lung (O'Callaghan et al 1987), which is ventilated by the caudo-dorsal bronchus sampled in this study.

Summary

1. Sensory nerves in the equine lung are well distributed to detect inhaled irritants: they are predominantly situated in the respiratory epithelium of central airways and at bronchial bifurcations.

2. The activation of intra-epithelial nerves in the equine lung may affect other tissues, since afferent nerves were also closely apposed to the small bronchial vessels in the lamina propria, and to tracheobronchial glands. The release of neurotransmitters via local axon reflexes onto these structures could cause vasodilation and increased tracheobronchial secretion, contributing to the inflammatory response of the lung to inhaled irritants.

3. Nerves immunoreactive for SP or CGRP were closely apposed to airway smooth muscle in only 2 of 14 horses studied, suggesting that in most adult horses activation of afferent nerves is unlikely to directly cause broncho-constriction in this species.

Table 1—Description of experimental animals and tissues examined

Horse	Age*	Sex+	Breed	Tissues examined	Comments
1	10	G	Thoroughbred	T, 1B, 3B, CDL	Pulmonary function tests normal
2	3	G	Standardbred	T, 3B, CDL	Pulmonary function tests normal
3	16	F	Thoroughbred	T, 1B, 3B, CDSB, CDL, LN	
4	20	F	Arabian	T, 1B, 3B, CDSB, CDL	Pulmonary function tests normal
5	26	F	Arabian	T, 3B, CDL	Pulmonary function tests normal
6	7	F	grade	T, 1B, 3B, CDSB, CDL	Pulmonary function tests normal
7	17	F	grade pony	T, 1B, 3B, CDSB, CDL	
8	5	G	Standardbred	3B, CRSB	CGRP only
9	6	G	Palomino	3B, CRSB	CGRP only
10	4	F	Arabian	3B, CRSB, DB	
11	8	F	Arabian	CRSB, CRL, CDSB, CDL	
12	Aged	F	Quarterhorse	T, 3B, DB	
13	9	G	Arabian cross	3B, CRSB, DB, CDSB	
14	3	F	Arabian cross	3B, CRSB, DB, CDSB	

* Ages in years (y) when available; aged = over 20 years old, exact age undetermined.

+ F = female, G = gelding (castrated male).

Abbreviations correspond to those of Fig 1: T = trachealis muscle (cervical or intrathoracic); 1B = first-generation bronchi; 3B = third-generation bronchi; CRSB = cranial small bronchi; CRL = cranial peripheral lung; DB = caudo-dorsal bronchus at approximately the junction of the caudal and middle thirds of the lung; CDSB = caudo-dorsal small bronchi; CDL = caudal peripheral lung; LN = middle tracheobronchial lymph nodes.

Table 2—Inner diameters of sampled airways

Airway examined	n	Inner diameter (mm) ± SD
3B	10	9.5 ± 2.0
DB	5	10.1 ± 2.4
CDSB	11	3.1 ± 0.8
CRSB	7	3.0 ± 0.9

Abbreviations correspond to those of Fig 1: 3B = third-generation bronchi; CRSB = cranial small bronchi; DB = caudo-dorsal bronchus at approximately the junction of the caudal and middle thirds of the lung; CDSB = caudo-dorsal small bronchi.

The diameters of the trachea and first-generation bronchi were not recorded; the diameters of some airways were not measured prior to sectioning and are therefore not included in this table.

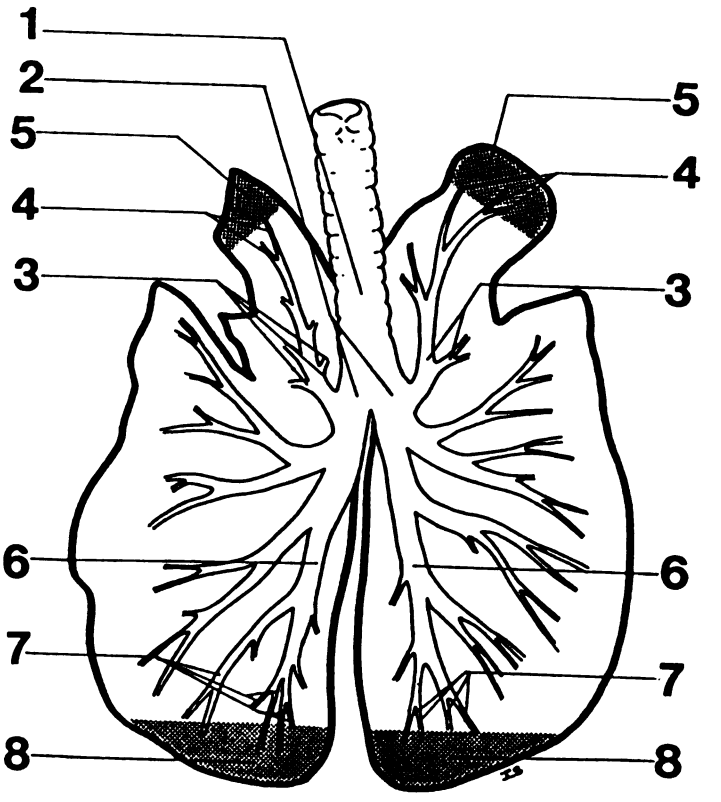
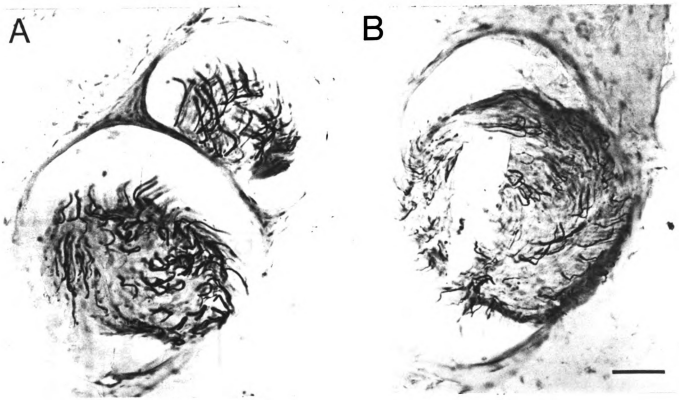


Figure 1. Schematic representation of the equine lung indicating sampling sites. For clarity, the accessory lobe, which was not sampled, is omitted. 1 = trachea (T; intrathoracic or cervical); 2 = first-generation bronchi (1B); 3 = third-generation bronchi(3B); 4 = cranial small bronchi (CRSB); 5 = cranial peripheral lung (CRL); 6 = largest caudo-dorsal bronchus at approximately the junction of the caudal and middle thirds of the lung (DB); 7 = caudo-dorsal small bronchi (CDSB); 8 = caudal peripheral lung (CDL).

Figure 2. Similar distribution of SP- and CGRP-LI nerves in the adult equine lung. Black immunoreactive peribronchial nerves in adjacent, non-serial sections of a third-generation bronchus stained for (A) CGRP and (B) SP. Note the more intense immunostaining obtained with the CGRP antiserum; SP immunoreactive nerve fibers were clearly less numerous than CGRP immunoreactive nerve fibers in this peribronchial nerve bundle. Counterstained with methylene blue, azure B, and eosin Y (MBAE). Bar = 50 μm.

Figure 2.



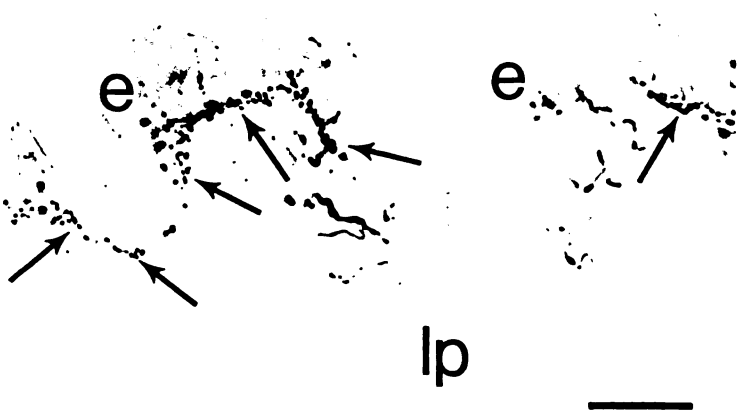


Figure 3. Sub-epithelial networks of CGRP-LI nerve fibers. Networks of CGRP-LI nerve fibers (arrows) just below the epithelium of the trachea: note that the networks are not continuous in this plane of section. Respiratory epithelium (e); lamina propria (lp). Not counterstained to allow better visualization of the immunoreactive nerve fibers. Bar = 50 μm.

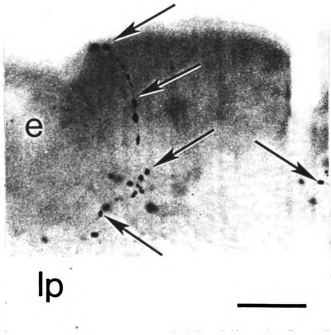


Figure 4. Intra-epithelial CGRP-LI nerve fibers. CGRP-LI nerve fibers (arrows) within the epithelium (e) of a third-generation bronchus. Note that a fiber appears to reach the lumen of the airway, where inhaled stimuli might activate it more readily. Lamina propria (lp). Not counterstained. Bar = 25 μm.

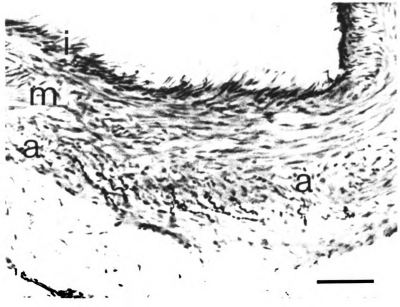


Figure 5. CGRP-LI nerve fibers in the adventitia of a bronchial artery. CGRP-LI nerve fibers in the adventitial (a) layer of a bronchial artery accompanying 4 mm inner diameter bronchus. Immunoreactive nerves are not apparent in the muscular (m) or intimal (i) layers. Counterstained with MBAE. Bar = 50 μm.

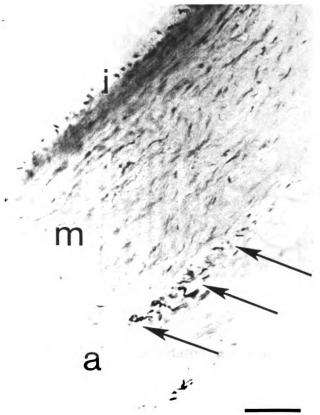


Figure 6. CGRP-LI nerve fibers in the adventitial layer of a pulmonary artery. CGRP-LI nerve fibers accompanying a *vasa vasorum* in the adventitial layer (a) of a 2-mm inner diameter pulmonary artery near a 3-mm inner diameter bronchus. Muscular layer (m); intimal layer (i). Counterstained with MBAE. Bar = 50 μm.

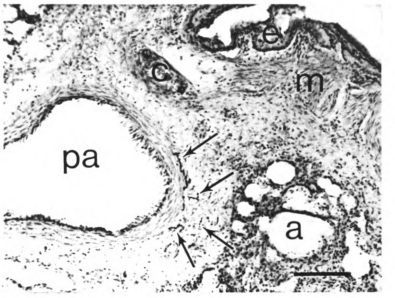


Figure 7. CGRP-LI nerve fibers accompanying a peripheral pulmonary artery. Peripheral pulmonary artery (pa) near a 350-μm inner diameter bronchus. Note the CGRP-LI nerve fibers (arrows) in the adventitial layer. Alveoli (a); bronchial cartilage (c); airway smooth muscle (m); bronchial epithelium (e). Counterstained with MBAE. Bar = 50 μm.

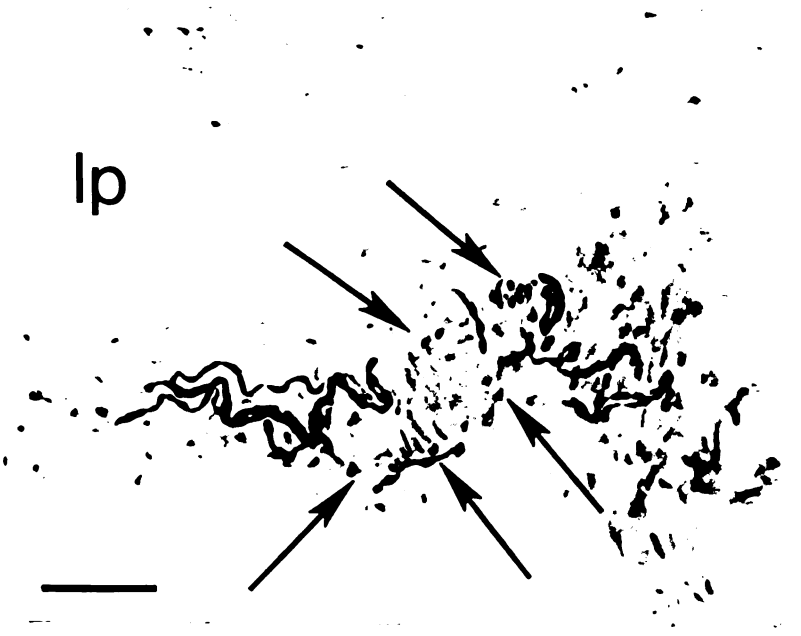


Figure 8. CGRP-LI nerve fibers closely associated with a venous sinusoid. CGRP-LI nerve fibers surrounding a small venous sinusoid (arrows) in the lamina propria (lp) of the trachea. Counterstained with MBAE. Bar = 50 μm.

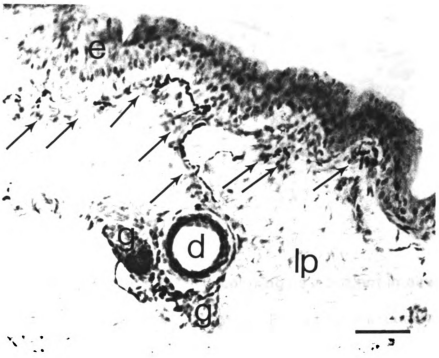


Figure 9. CGRP-LI nerve fibers in the lamina propria of the trachea. CGRP-LI nerve fibers in the lamina propria (lp) of the trachea. The nerve fibers follow the course of the small vessels in the lamina propria (arrows); they can also be seen within a tracheobronchial gland (g). Epithelium (e); gland duct (d). Counterstained with MBAE. Bar = 50 μm.

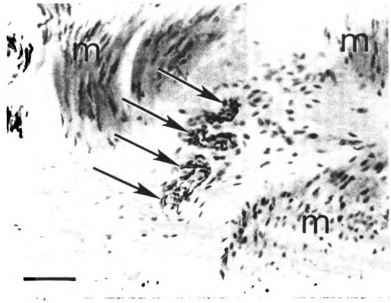


Figure 10. Example of nerve fascicle coursing near but not in airway smooth muscle. In 12 of the 14 horses sampled, SP or CGRP immunoreactive nerve fibers did not branch on airway smooth muscle. In this photomicrograph, SP immunoreactive nerve fibers are present in fascicles (arrows) between smooth muscle fibers (m) in the trachea. Counterstained with MBAE. Bar = 50 μm.

Figure 11. Example of innervated airway smooth muscle. Tachykinin or CGRP immunoreactive nerve fibers appeared to innervate airway smooth muscle in 2 of the 14 adult horses sampled. Black SP immunoreactive nerve fibers associated with the airway smooth muscle (m) of a third-generation bronchus of horse #7. Counterstained with MBAE. Bar = 50 μm.

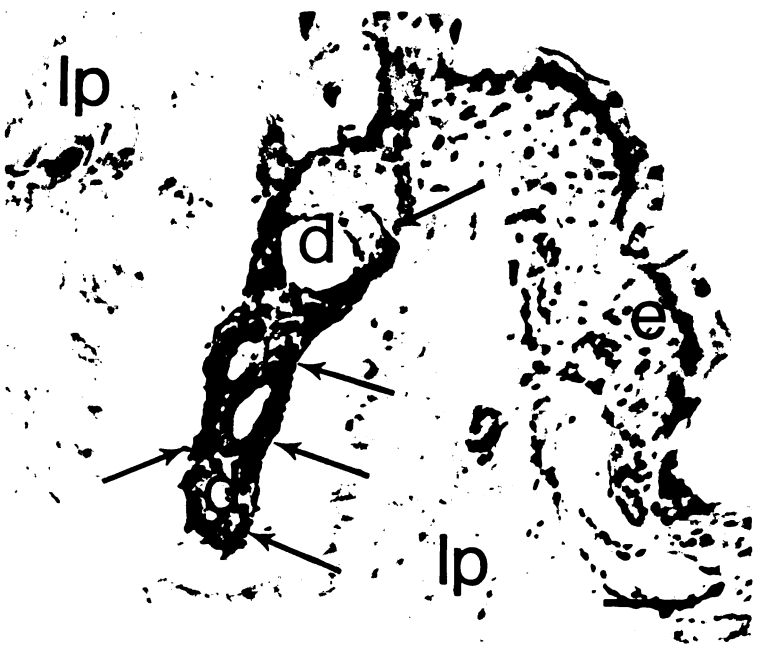


Figure 12. CGRP-LI nerve fibers in the duct of a tracheobronchial gland. Duct (d) of a tracheobronchial gland: note the CGRP-LI nerve fibers (arrows) in the walls of the duct. Epithelium (e); lamina propria (lp). Counterstained with MBAE. Bar = 50 μm.

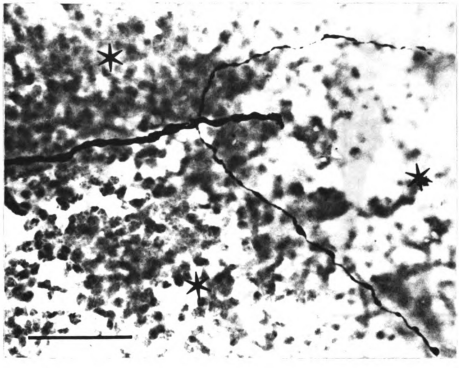


Figure 13. CGRP-LI nerve fibers in a middle tracheobronchial lymph node. CGRP-LI nerve fibers coursing through a middle tracheobronchial lymph node (all visible cells are lymphocytes); no vessels were present in this and adjacent sections. Counterstained with MBAE. Bar = 50 μm.

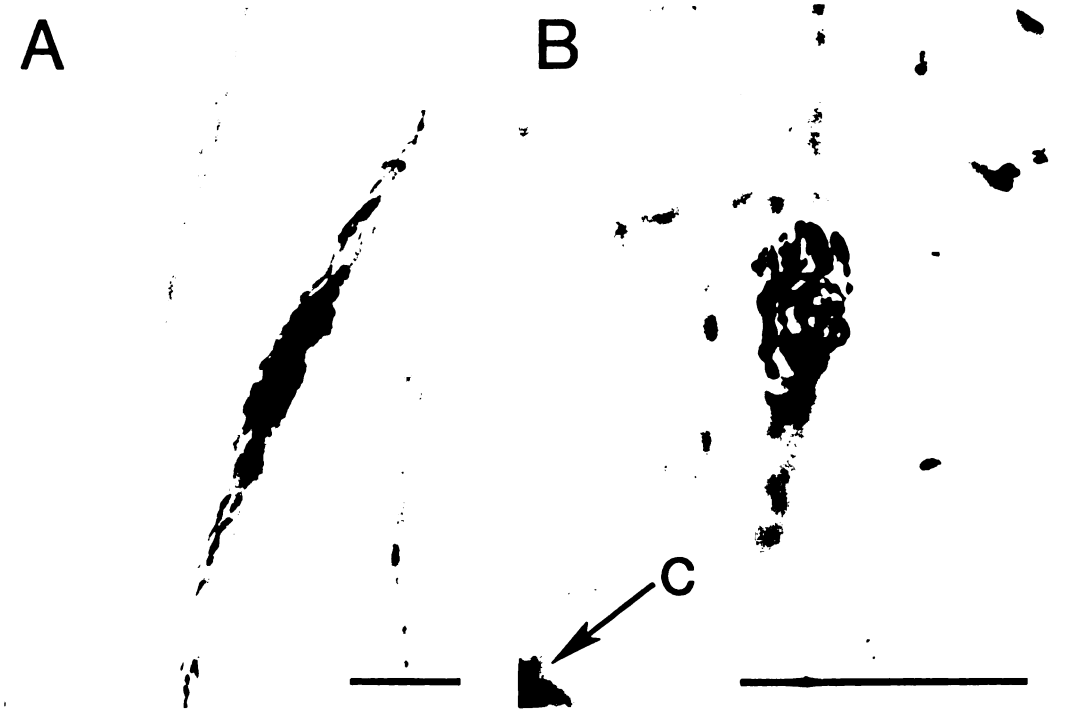
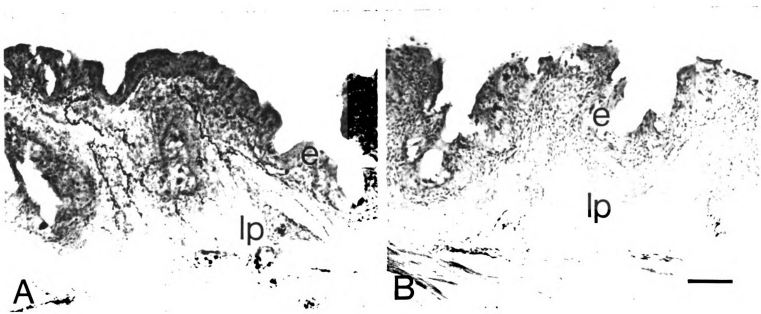


Figure 14. Possible sensory receptors. (A) At the insertion of the trachealis muscle on the tracheal cartilage, a spindle-shaped bundle of nerves immunoreactive for CGRP may represent a stretch receptor. Bar = 50 μm. (B) Cross-section of a similar CGRP immunoreactive nerve bundle near the cartilage (c) of a 3-mm diameter bronchus. Counterstained with MBAE. Bar = 50 μm.

Figure 15. Comparison of CGRP-LI nerves in a third-generation bronchus and a dorsal bronchus. Simultaneously processed third-generation (A) and dorsal (B) bronchi from the same lung, exposed to CGRP antiserum. Note the numerous CGRP-LI nerve fibers in the lamina propria of the third-generation bronchus (A) and the complete absence of similar nerve fibers in the dorsal bronchus (B). Epithelium (e); lamina propria (lp). Counterstained with MBAE. Bar = 100 μm.

Figure 15.



CHAPTER 3

DISTRIBUTION OF SP- AND CGRP-LIKE IMMUNOREACTIVE NERVE FIBERS IN THE LOWER RESPIRATORY TRACT OF NEONATAL FOALS: EVIDENCE FOR LOSS DURING DEVELOPMENT

Introduction

As shown in section IV, the lungs of adult equids contain nerves immunoreactive for SP and CGRP which, given their distribution, may contribute in the regulation of pulmonary function in health and disease in mature horses. Because these peptides may also have important effects on neonatal pulmonary function, and pulmonary disease is a common cause of morbidity and mortality in neonatal foals (Beech, 1985), we sought to determine the location of nerves containing SP (chosen to represent the tachykinins) and CGRP in the neonatal equine lung using immunohistochemistry.

Materials and methods

Animals—Seven neonatal foals and one adult horse were used in this study. The foals and the adult horse were killed with an intravenous injection of pentobarbital ($\geq$ 100 mg/kg). Pulmonary tissue was collected from the areas

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shown in Fig 1: the sampling sites resembled those chosen for the adult lungs. The foals were part of a study that did not involve the lungs. Table 1 describes the foals and lists the tissues examined for each animal; airway diameters are listed in Table 2. Foals #1, 3, 4, 5, and 7 had macroscopically normal lungs; foals #2 and 6 had a few 0.5- to 2-cm diameter abnormal areas (not sampled) within predominantly healthy-appearing lungs. To verify that any differences between the data obtained in this study and the data obtained in Chapter 2 were not due to differences in processing or technique, pulmonary tissue obtained from that study (horse #14) was processed with the foal tissue.

Immunohistochemistry—The procedure is described in detail in Chapter 2. Tissues from foals #1 and 2 were fixed at pH ≈ 7.4, 4°C, for several days, then immersed in 23% sucrose, 0.8% paraformaldehyde in 0.1 M sodium phosphate buffer (pH ≈ 7.4) for cryoprotection and long-term storage. Pulmonary tissue from foals #3 to 7 was fixed for 24 to 36 hours at pH ≈ 8, 4°C prior to cryoprotection. In this study, both fixation protocols gave similar results.

Results

The black, beaded immunoreactive nerve fibers were clearly visible in both counterstained and non-counterstained sections. The distributions of SP-LI and CGRP-LI nerve fibers were generally similar in adjacent, non-serial

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sections of the same tissues (Fig 2), although CGRP-LI nerve fibers were more prominently stained.

Immunoreactive nerves were present in all the sampled areas of the neonatal lung. Substance P- and CGRP-LI nerve fibers were most abundant in and around the larger sampled airways such as the third-generation bronchi (3B, see Fig 1), but were also frequently seen in smaller bronchi and non-cartilaginous bronchioles. Although immunoreactive nerve fibers were somewhat sparser in the caudal part of the lung when compared to the cranial part, the distribution was very similar in all sampled areas of the lung.

Nerves immunoreactive for SP- or CGRP-LI could be seen in the airway smooth muscle of all the intrathoracic airways sampled, from the large, cartilaginous 3B and caudo-dorsal bronchus (DB; see Fig 1), to the smallest bronchioles (Fig 3). Although some of these nerves may have accompanied vessels out of the plane of section, the majority appeared to be exclusively associated with respiratory smooth muscle.

The larger pulmonary arteries and veins ($\geq$ 3 mm diameter) contained numerous SP- and CGRP-LI nerve fibers in the adventitial layer, usually associated with *vasa vasorum* (Fig 4); none were detected in the muscularis or intima. Smaller pulmonary arteries (2 mm $\geq$ $\geq$ 40 μm inner diameter), including parenchymal ones, were similarly innervated (Fig 5), in contrast to the identifiable medium to small pulmonary veins ($\leq$ 1.5 mm diameter), which contained far fewer SP- or CGRP-LI nerve fibers (not shown).

Immunoreactive nerve fibers were frequently seen in the adventitial layer of bronchial arteries and veins within the peribronchial sheath (Fig 6). Within the lamina propria, immunoreactive nerves accompanied the numerous small (4- to 15-μm diameter) bronchial vessels that branched profusely just below the epithelium in cartilaginous airways (Fig 7). Very few venous sinusoids were observed in the lamina propria of the larger bronchi (3B and DB) of foals: when present, their thin walls contained one or more SP- or CGRP-LI nerve fibers (not shown).

In all the cartilaginous airways examined, SP- and CGRP-LI nerve fibers formed a very dense network at the level of the basal lamina from which fine, beaded fibers spread into the respiratory epithelium (Fig 7), at times appearing to reach the lumen of the airway. The networks of nerve fibers were most extensive near bronchial bifurcations, where they were almost continous in every field of view.

Tracheobronchial glands were seen in cartilaginous airways of all sizes but were most abundant in 3B. Only large tracheobronchial glands (over 200 μm long in the plane of section) contained SP- or CGRP-LI nerve fibers (see Fig 3A); due to the dark counterstaining of the glands and the section thickness it was difficult to determine whether the nerve fibers accompanied vessels within the glands or innervated the gland itself.

Substance P- or CGRP-LI nerve fibers could be seen closely apposed to the vessels at the hilus of the examined middle tracheobronchial lymph nodes; immunoreactive nerve fibers were not seen in the parenchyma (Fig 8).

Peribronchial neural ganglia were detected in 8 sampled airways (4 3B and 4 DB): nerve cell bodies were not immunoreactive, but faintly stained SP- and CGRP-LI nerve fibers seemed to contact the cell bodies (Fig 9).

To verify that any differences noted between adult and neonatal equine lungs were not due to subtle variations in technique, pulmonary tissue collected from an adult horse was processed simultaneously with that of foals #2, 5, and 6. Tissue from this adult horse (horse #14 in Table 1, Chapter 2) was fixed in the same manner as that of foals #3 to 7. In neonatal foals, the 3B, DB and the bronchi at the level of the cardiac notch (CRSB-F in Fig 1) contained numerous immunoreactive nerves associated with the respiratory epithelium and airway smooth muscle. In the corresponding airways in this adult horse, immunoreactive nerve fibers were not associated with airway smooth muscle, and sub-epithelial and intra-epithelial immunoreactive nerves were frequently detected only in the 3B and almost never in the DB or CRSB-F. The distributions of CGRP-LI nerve fibers in the DB of foal and adult lungs are compared in Fig 10. Table 3 compares the distribution of SP- and CGRP-LI nerve fibers in the lungs of foals and adults.

Discussion

Our study clearly demonstrates the presence of SP- and CGRP-like immunoreactivity within the pulmonary nerves of neonatal foals. The distributions of SP- and CGRP-LI nerve fibers were very similar in adjacent sections: most neonatal pulmonary nerves immunoreactive for SP thus may also

contain CGRP, as is frequently the case in the lungs of other species (Martling et al 1988); double staining studies would be necessary to verify whether this was indeed the case. Immunoreactive nerves were detected in airway smooth muscle, bronchial and pulmonary vessels, and the respiratory epithelium in all the neonatal pulmonary tissue examined.

Numerous nerves immunoreactive for SP and CGRP could be seen in airway smooth muscle in neonatal lungs, indicating that nerves containing these peptides may contribute to the regulation of airway tone in foals. This was in marked contrast to the adults, in which SP- or CGRP-LI nerve fibers closely associated with airway smooth muscle were only noted in 2 of 14 adults (Chapter 2). In many species including man, SP and CGRP can act directly on airway smooth muscle to cause bronchoconstriction, or indirectly to enhance cholinergically mediated bronchoconstriction (Holzer 1988; Barnes et al 1991a; Barnes et al 1991b; Solway and Leff 1991). Tachykinins do not cause a strong contraction of adult equine airways (personal communication, LeBlanc 1992; LeNinivin et al 1993). It is possible that foals differ from adults in this respect, but to date there have been no studies involving foal airways. In other species, neonatal airways generally have a less marked response to SP administration than those of older animals (Tanaka and Grunstein 1990; Tokuyama et al 1993; Haxhiu-Poskurica et al 1992).

Similarly, in the absence of physiological data regarding the effects of SP or CGRP in foals, we can only speculate about the roles of the numerous SP- and CGRP-LI nerves surrounding bronchial and pulmonary vessels. In other

species, stimulation of sensory intratracheal or pulmonary nerves, or the administration of tachykinins or CGRP cause vasodilation of tracheal and/or bronchial vessels (Matran et al 1989; Martling et al 1987a; Piedimonte et al 1992; Salonen et al 1988). Stimulation of the numerous epithelial sensory fibers in the lungs of the foal could lead, via local axon reflexes, to the release of tachykinins and CGRP in the epithelium and lamina propria, resulting in vasodilation of the small vessels of the lamina propria. This vasodilation, in turn, could decrease the airway lumen as has been documented experimentally in sheep (Mariassy et al 1991).

The actions of the nerves surrounding pulmonary vessels in foals are more difficult to determine. Sensory neurotransmitters can cause increases in pulmonary vascular resistance in the rabbit (Worthen et al 1985): if this is true in foals, SP- and CGRP-LI nerves may contribute to maintaining the high pulmonary vascular resistance typical of pre-natal life (Noden and de Lahunta 1985). An abnormally dense innervation of pulmonary arteries has been reported in children with pulmonary hypertension (Allen et al 1989), suggesting that these nerves are indeed involved in the regulation of vascular tone during development.

In rodents, SP can cause an increase in vascular permeability of tracheobronchial vessels in rodents (Rogers et al 1988; Eglezos et al 1991), a response that is reduced in immature guinea pigs (Tokuyama et al 1993). It is not known if tachykinin-mediated plasma extravasation can occur in equine

lungs; to date, this phenomenon has only been described in the lungs of rodents (Solway and Leff 1991).

In the foal, SP- and CGRP-LI nerve fibers were widely distributed in the epithelium of all cartilaginous airways, particularly near bifurcations. These sensory nerve fibers were well placed to detect inhaled irritants and changes in the intra-luminal environment. Activation of sensory nerve fibers by such stimuli can lead to local and central release of tachykinins and CGRP, followed by coughing, bronchoconstriction, and local inflammation (Holzer 1988; Barnes et al 1991b; Solway and Leff 1991; Alving 1991; Lundberg et al 1991). The activation of epithelial sensory nerve fibers could also alter airway function by acting locally on peribronchial neural ganglia, bypassing the central nervous system, since SP- and CGRP-LI nerve fibers were detected in close apposition to neuronal cell bodies in the peribronchial neural ganglia.

The distribution of SP- and CGRP-LI nerve fibers in the lungs of foals differed from that previously reported in adult horses (Sonea and Bowker 1991d; Hillman et al 1992; Chapter 2) as shown in Table 3. For instance, nerves immunoreactive for SP or CGRP were abundant in the peripheral lung of neonatal foals, whereas they were predominantly restricted to the trachea and central airways of adult lungs. Furthermore, airway smooth muscle contained numerous SP- or CGRP-LI nerve fibers in all foals, but only in 2 of 14 adult horses. These differences did not apprear to be caused by variations in the techniques employed, since these differences persisted when adult and neonatal tissues were processed simultaneously.

Some of these changes could simply reflect a "dilution" of nerves as the airways increased in length and diameter while the number of nerves remained constant. However, we believe that during the postnatal maturation of the equine lung, some of the SP- and CGRP-LI nerves either lose the ability to synthesize these peptides, or that some of their branches or the entire neurons actually die back. In either case, the end result would be a decrease in the number of nerves immunoreactive for SP and CGRP. This hypothesis is supported by two observations.

First, bronchioles frequently contained immunoreactive nerves in foals but not in adult horses. The conducting airways (trachea to bronchioles) are all present at birth (Adamson 1991; Burri 1991): the disparity between neonates and adults is therefore unlikely to be due to the addition of new airways after birth. Second, in neonatal foals, SP- or CGRP-LI nerve fibers were frequently observed within or very close to airway smooth muscle in all intrathoracic airways, in marked contrast to adult horses, in which SP- or CGRP-LI containing nerves were closely associated with airway smooth muscle in only 2 of the 14 adult horses studied (Chapter 2). A similar age-related loss of SP- or CGRP-LI nerves associated with airway smooth muscle or bronchioles is reported in human peripheral bronchi (Hislop et al 1990).

The abundance of SP- and CGRP-LI nerve fibers at all levels of the lung of foals may reflect the rapid growth occurring at this age, since a trophic role for neuropeptides has been proposed (Holzer 1988). In other species, tachykinins are trophic for skin and endothelium (Holzer 1988), and CGRP

stimulates the in vitro growth of tracheal epithelium (White et al 1993). In guinea pigs, the number of SP- and CGRP-LI nerve fibers closely associated with vessels peaked in late gestation or early post-natal life (varying with the vessel)(Dhall et al 1986), and decreased thereafter, arguing for a developmental role for these peptides in the cardiovascular system. On the other hand, in the chick (Salvi and Renda 1992) and rat (Cadieux et al 1986) lungs, sensory nerves were present before birth, but increased in number and distribution thereafter until they reached adult levels, unlike our observations in neonatal foals. It is therefore unclear whether these neuropeptides have a trophic effect in the lung.

Summary

1. Sensory nerves are abundant in both central and peripheral airways in the neonatal equine lung, in marked contrast to the predominantly central distribution of such nerves in the adult equine lung. This widespread distribution may indicate that activation of sensory nerves may have wider-ranging efferent effects on pulmonary function in the foal than in the adult horse.

2. The widespread presence of nerves immunoreactive for SP and CGRP in close apposition to airway smooth muscle in all airways examined in the neonatal lung, and the virtual absence of such nerves in the adult lung, suggests that, in the post-natal period, either these nerves lose the ability to synthetize these neurotransmitters, or that the nerves die back. In turn, this

observation suggests that these peptides may play a trophic or regulatory role in perinatal lung development.

3. The presence of nerve terminals immunoreactive for SP or CGRP in parasympathetic peribronchial ganglia provides the anatomical basis for afferent pre-synaptic modulation of chlinergic neurotransmission.

4. The age-related loss of nerves immunoreactive for SP and CGRP in the foal resembles that reported in man, suggesting that the horse may be an excellent model for the study of developmental changes in pulmonary innervation.

Table 1—Experimental animals and tissues examined

Foal	Age*	Sex#	Breed	Tissues examined†	Comments
1	7d	F	grade pony	CRL, DB, CDL	Clinically normal
2	6d	M	grade pony	3B, DB, CDSB	Clinically normal. 5.5-mm diameter abscess in cranial lung lobe
3	4d	M	grade pony	3B, CRSB-F, DB, CDSB, LN	
4	4d	F	grade	3B, CRSB-F, DB, CDSB	
5	4d	M	grade pony	3B, CRSB-F, DB, CDSB	Colostrum deprived, clinically normal
6	4d	F	grade pony	3B, CRSB-F, DB, CDSB	Small area of consolidation in ventral margin of right pulmonary lobe
7	4d	F	grade pony	3B, CRSB-F, DB, CDSB	
Adult	3y	F	Arabian cross	3B, CRSB, CRSB-F, DB, CDSB	

* Ages in years (y) or days (d).

F = female, M = male.

† See Fig 1 for the sampling sites. Third-generation bronchus = 3B; cranial small bronchi = CRSB; cranial small bronchi sampled at the cardiac notch = CRSB-F; cranial peripheral lung = CRL; dorsal bronchus = DB; caudo-dorsal small bronchi = CDSB; caudal peripheral lung = CDL; middle tracheobronchial lymph nodes = LN.

Table 2—Inner diameter of examined airways (determined after fixation)

Examined airways	Foals (mm) ± S.D.	(n)	Adult (mm)
Third-generation bronchus	5.7 ± 1.8	(6)	12
Largest "cranial small bronchus" examined in each animal	2.0 ± 0.7	(4)	6
Caudo-dorsal bronchus	4.7 ± 0.7	(7)	11
Largest "caudo-dorsal small bronchus" examined in each animal	2.9 ± 1.6	(5)	3

Table 3—Semi-quantitative comparison of SP- or CGRP-LI nerves in the lungs of foals and adult horses

	A.S.M.	P.A.	P.V.	B.V.	V.L.P.	P.E.	T.G.
Foals 3B	+ + +	+ + +	+ +	+ + +	+ + +	+ + + +	+
Adults 3B	+/-	+ + +	+	+ + +	+ + +	+ + +	+ +
Foals DB	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+
Adults DB	+/-	+ + +	+/-	+ + +	+/-	+/-	+ +
Foals SB	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+/-
Adults SB	+/-	+ + +	0	+ +	+/-	+/-	+ +
Foals Bronchioles	+ + +	+ + +	+	+ + +	N/A	+	N/A
Adults Bronchioles	+/-	+ + +	0	+ + +	N/A	0	N/A

Adult data adapted from Chapter 2.
Abbreviations: A.S.M. = airway smooth muscle; P.A. = pulmonary arteries; P.V. = pulmonary veins; B.V. = bronchial vessels; V.L.P. = vessels in lamina propria; R.E. = respiratory epithelium; T.G. = tracheobronchial glands; 3B = third-generation bronchus; DB = dorsal bronchus; SB = small bronchi (less than 5 mm diam).

Nerve densities estimated at 100X and 200X magnification, at least 20 views per sample.

0	none seen in any view
+/-	nerve fiber(s) seen in 1-5 views in some samples, or seen in less than 20% of the animals
+	nerve fiber(s) seen in 1-5 views of most samples
+ +	nerve fibers, generally more than one, seen in over 50% of views
+ + +	nerve fibers seen in nearly all views
+ + + +	numerous nerve fibers seen in all views.

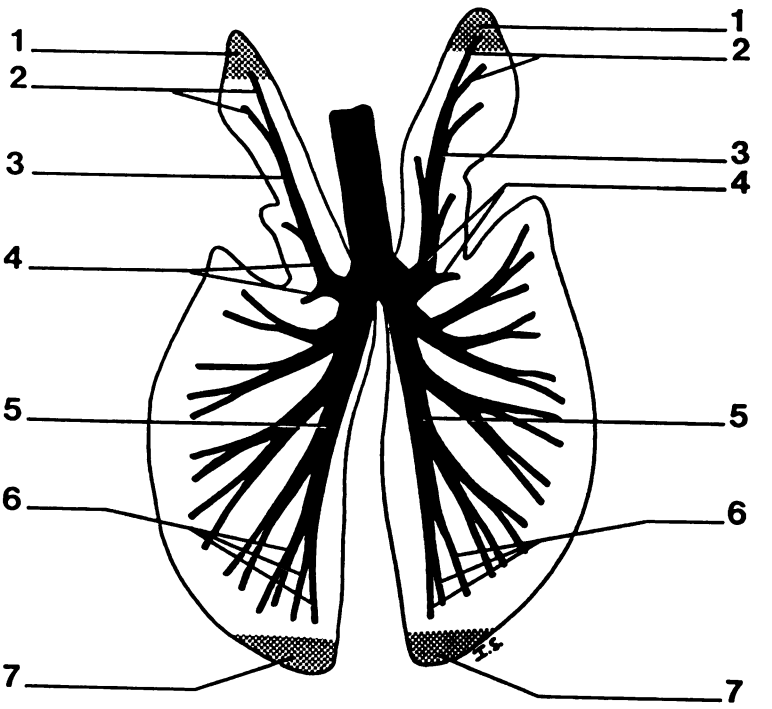


Figure 1. Schematic representation of the neonatal equine lung: sampling sites. The accessory lobe has been omitted for clarity. 1 = cranial peripheral lung; 2 = cranial small bronchi; 3 = cranial small bronchi at the level of the cardiac notch; 4 = third-generation bronchi; 5 = dorsal bronchus (sampled at the junction of the middle and caudal thirds of the lung); 6 = caudo-dorsal small bronchi; 7 = caudal peripheral lung.

Figure 2. Similar distributions of SP- and CGRP-LI nerve fibers in the neonatal equine lung. The distributions of CGRP-LI (a) and SP-LI (b) nerve fibers (arrows) were similar in adjacent, non-serial sections. Note the darker staining of CGRP-LI compared to SP-LI. Bronchial vein (V) in the peribronchial sheath of a cranial small bronchus; red blood cells are present in the lumen of the vessel. Counterstained with methylene blue, azure B, and eosin Y (MBAE). Bar = 50 μm.

Figure 3. Immunoreactive nerves in airway smooth muscle. Airway smooth muscle (M) frequently contained immunoreactive nerve fibers without accompanying vessels. Counterstained with MBAE. Bar = 50 μm. (a) CGRP-LI nerve fibers in the airway smooth muscle (M) of a third-generation bronchus. Note the immunoreactive nerve fibers (arrows) in a tracheobronchial gland (G). (b) High-magnification view of a single CGRP-LI nerve fiber apparently innervating the muscle (M), as there was no accompanying vessel in the plane of section. Note the beaded appearance of this nerve, which is typical of autonomic nerves. (c) CGRP-LI nerve fiber accompanying airway smooth muscle fibers (M) in a 1-mm diameter bronchus. Bronchial cartilage (C); respiratory epithelium (E). (d) CGRP-LI nerve fiber in the wall of a non-cartilaginous bronchiole (B). Alveoli (A).

Figures 2 and 3.

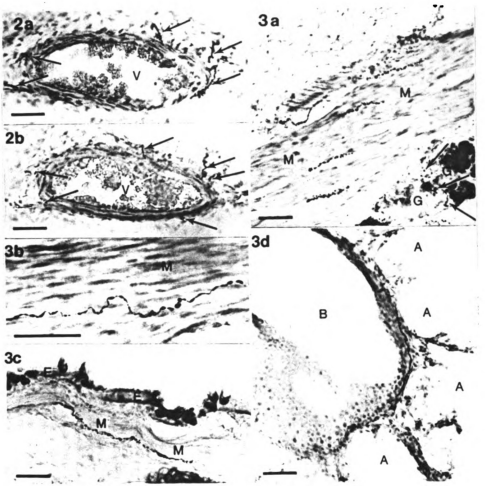


Figure 4. CGRP-LI nerve fibers in the adventitia of a large pulmonary artery. CGRP-LI nerve fibers (arrows) accompanying *vasa vasorum* (v) in the adventitia (A) of a 3-mm inner diameter pulmonary artery. Immunoreactive nerve fibers (arrows) can also be seen near but not in the muscularis (M); none are present in the intima (I) of the vessels. Counterstained with MBAE. Bar = 100 μm.

Figure 5. Immunoreactive nerves in the walls of a parenchymal pulmonary artery. The walls of small pulmonary arteries (40-μm to 2-mm diameter) in the bronchial sheath and the parenchyma contained numerous immunoreactive nerves. Oblique section through a small pulmonary artery (PA) in the cranial lung parenchyma: three CGRP-LI nerve fibers (arrows) are present in the wall of this vessel. Alveoli (A). Counterstained with MBAE. Bar = 100 μm.

Figure 6. Immunoreactive nerves in the walls of bronchial vessels. The walls of bronchial vessels contained numerous immunoreactive nerves. (a) CGRP-LI nerve fibers in the adventitia of two bronchial arteries (BA) near the large caudo-dorsal bronchus. Bronchial cartilage (C). (b) SP-LI nerve fibers in the wall of a bronchial vein (BV) below the airway smooth muscle (M) of a caudo-dorsal small bronchus. Note that some immunoreactive fibers are also present in the airway smooth muscle. Respiratory epithelium (E); lamina propria (LP). Counterstained with MBAE. Bar = 100 μm.

Figures 4, 5, and 6.

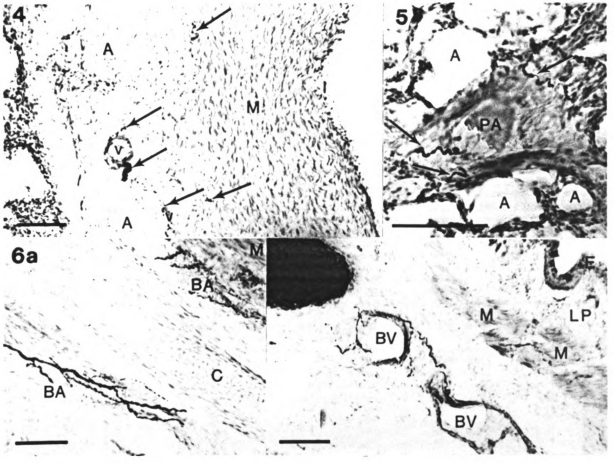


Figure 7. SP- and CGRP-LI nerve fibers in the epithelium and lamina propria. Adjacent, non-serial sections of a third-generation bronchus demonstrating SP-LI (a) and CGRP-LI (b) nerve fibers accompanying small vessels (arrows) in the lamina propria; the immunoreactive nerve fibers are particularly dense just below the epithelium. Respiratory epithelium (E); lamina propria (LP); tracheobronchial gland (G). Counterstained with MBAE. Bar = 50 μm. (c) Higher-magnification view of the same bronchus (not counterstained). CGRP-LI nerve fibers form a dense network near the basal lamina, extending thin, beaded fibers into the epithelium (E), one of which appears to reach the lumen of the airway (arrow). Lamina propria (LP). Bar = 50 μm.

Figure 8. CGRP-LI nerve fibers in a middle tracheobronchial lymph node. CGRP immunoreactive nerves accompanying a vessel (V) at the hilus of the middle tracheobronchial lymph nodes. Parenchyma of lymph node (*). Counterstained with MBAE. Bar = 50 μm.

Figure 9. Neural ganglion. Nerve fascicles (open arrow) containing CGRP-LI nerve fibers course near a peribronchial neural ganglion, and faintly immunoreactive fibers (arrows) are present within the ganglion, near the nerve cell bodies (N). Counterstained with MBAE. Bar = 50 μm.

Figures 7, 8, and 9.

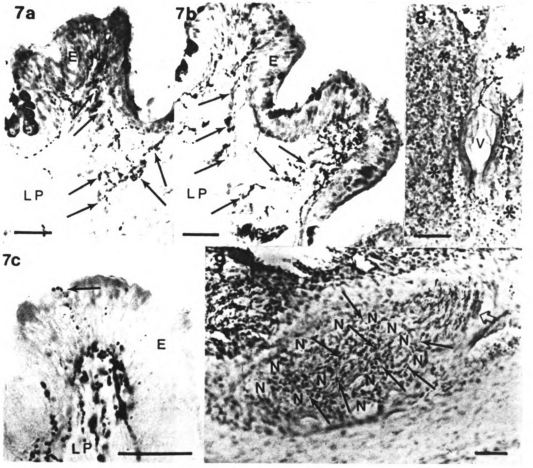
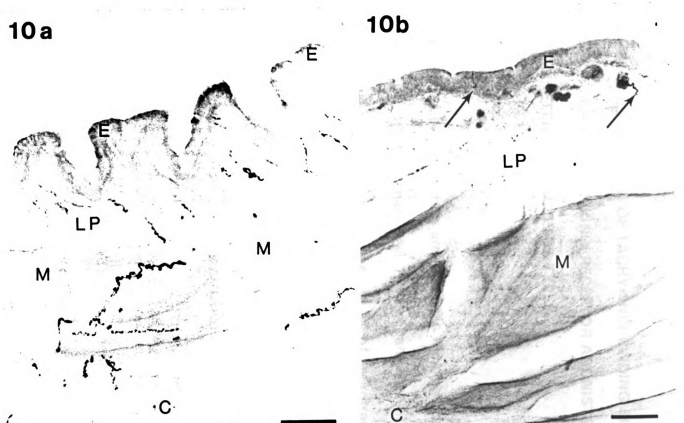


Figure 10. Comparison of adult and neonatal innervation. Immunoreactive nerve fibers were more numerous in neonatal airways. (a) CGRP-LI nerve fibers in the caudo-dorsal bronchus (5-mm inner diameter) of a foal: note the immunoreactive nerve fibers in the lamina propria (LP) and near airway smooth muscle (M). Respiratory epithelium (E); connective tissue near bronchial cartilage (C). (b) CGRP-LI nerve fibers in the corresponding bronchus (11-mm inner diameter) of an adult horse that was processed simultaneously: only two immunoreactive nerve fibers are visible (arrows). Not counterstained. Bar = 100 μm.

Figure 10.



CHAPTER 4

AUTORADIOGRAPHIC DISTRIBUTION OF TACHYKININ BINDING SITES IN EQUINE AIRWAYS: USE OF TACHYKININ RECEPTOR AGONISTS TO DETERMINE RECEPTOR TYPES

Introduction

Pulmonary afferent (sensory) nerves are present in the airways of many species (Solway and Leff 1991; Barnes et al 1991a; Barnes et al 1991b). Stimulation of these sensory nerves leads to the release of neurotransmitters from terminals in the central nervous system and, via antidromic transmission of neural impulses (local axon reflexes), from terminals on other branches of the same neurons within the lung (Solway and Leff 1991). The neurotransmitters of afferent nerves include tachykinins such as substance P (SP) and neurokinin A (NKA), which can cause increased pulmonary resistance attributed to bronchoconstriction, plasma extravasation, and increased tracheobronchial secretion (Solway and Leff 1991; Barnes et al 1991a; Barnes et al 1991b).

The intrapulmonary effects of tachykinins are mediated by specific receptors on target tissues (Regoli 1987; Quirion and Dam 1988; Regoli et al 1988). At present, tachykinin receptors are divided into three types, based on their relative affinities for different tachykinins: neurokinin-1 (NK1) receptors

bind SP) NKA) neurokinin B (NKB); neurokinin-2 (NK2) receptors bind NKA) NKB) SP and neurokinin-3 (NK3) receptors bind NKB) NKA) SP (Regoli et al 1988; Quirion and Dam 1988). All three types of tachykinin receptors modulate cholinergic neurotransmission in various tissues (Laufer et al 1985; Hall et al 1989; Watson et al 1993; Shioya et al 1993). In the airways, stimulation of NK1 receptors can cause plasma extravasation (Rogers et al 1988; Andrews et al 1989; Eglezos et al 1991) and vasodilation (Piedimonte et al 1993) while activation of NK2 receptors can cause vasodilation of systemic vessels (Hirayama et al 1993) and bronchoconstriction (Ireland et al 1991; Maggi et al 1991; Dion et al 1990; Maggi et al 1989; Black et al 1992). The presence of NK3 receptors has not been documented in the lung. Since endogenous tachykinins (SP, NKA, NKB) can bind to all three types of receptors (Regoli et al 1991), it is difficult to determine which receptor type(s) are activated in many situations, particularly in vivo.

Furthermore, in a heterogenous tissue like the lung it is particularly difficult, even in vitro, to ascribe a tachykinin-mediated effect to a specific tissue and receptor type since it is almost impossible to isolate small vessels from respiratory smooth muscle or epithelium. It would therefore be extremely useful to determine which type(s) of receptor are present in each of these tissues in the lung. Once the type(s) of receptors present has been determined, pharmacological experiments with specific receptor agonists and antagonists could be devised to focus on areas of interest.

The equine lung contains afferent nerves immunoreactive for SP and NKA (Sonea and Bowker 1991d; Hillman et al 1992; Chapter 2), and tachykinins cause a slight but significant contraction of equine airway smooth muscle (LeNinivin et al 1993). Therefore, to elucidate the contributions of tachykinins to the regulation of pulmonary function in horses, we determined in this study the distribution of specific binding sites for SP in the equine lung, using [^{125}I] receptor autoradiography. A radioligand for tachykinin receptors, [^{125}I] Bolton-Hunter substance P ([I]-BHSP), was used to detect tachykinin binding sites, which predominantly represent receptors for SP (Rothman et al 1984). Although [I]-BHSP binds preferentially to NK1 receptors, it also binds to NK2 and NK3 receptors (Quirion et al 1991). We also attempted to identify the type(s) of receptors present in equine respiratory epithelium and airway smooth muscle by comparing the binding of [I]-BHSP in the absence and presence of the following specific tachykinin receptor agonists: [Sar^{9},Met(O_2)^{11}]-substance P, an NK1 agonist, [Nle^{10}]-neurokinin $A_{(4-10)}$, an NK2 agonist, and senktide, an NK3 agonist (Quirion and Dam 1988; Regoli et al 1991). Agonists should displace [I]-BHSP from the receptors with a high affinity for that particular agonist, leading to a quantifiable decrease in total [I]-BHSP binding to tissues containing such receptors. A decrease in [I]-BHSP binding in the presence of a specific receptor agonist would thus reflect the type of tachykinin receptor present.

Materials and methods

Tissue collection and storage—Pulmonary tissues from 10 horses were studied. The animals were killed with an overdose of pentobarbital (≥100 mg/kg IV). The horses are described in Table 1; they had no clinical or macroscopic evidence of pulmonary disease at necropsy, with the exception of one horse that had a small well-encapsulated pulmonary abscess.

Pulmonary tissue was collected within 30 minutes of the animal's death. Tissue samples less than 10 mm thick were immediately frozen in crushed dry ice, then tightly wrapped in aluminium foil and stored at -40°C. Cryostat sections (20 μm) were cut at -20°C and thaw-mounted on gelatin-subbed slides. The mounted sections were dehydrated as described by Herkenham and Pert (1982), prior to storage in a sealed dessicator at -40°C.

[^{125}I]-Bolton Hunter substance P autoradiography—The incubation protocol generally followed the methods of Mantyh et al (1989). Sections were brought to room temperature and placed for 20 minutes at 22-24°C in a pre-incubation solution (50 mM Tris-HCl, 0.005% (v/v) polyethylenimine, pH = 7.4). The sections were then incubated at 22-24°C for one hour in a solution of 50 mM Tris-Hcl containing 3 mM $MnCl_2$, 200 mg/L bovine serum albumin, 2 mg/L chymostatin, 4 mg/L leupeptin, 40 mg/L bacitracin and 20 pM [I]-BHSP (pH = 7.4). Following this incubation, the sections were rinsed with four 2-minute washes at 4°C in 50 mM Tris-HCl (pH = 7.4) and dipped into 2 washes of distilled H_2O at 4°C before drying.

For each sample, one of four slides containing adjacent sections of the same tissues was placed in one of the following incubation solutions:

Solution A: contained 20 pM [I]-BHSP (Amersham).

Solution B: identical to solution A but for the addition of an excess (1 μM) of unlabelled substance P (SP) (Sigma).

Solution C: identical to solution A but for the addition of 20-120 nM of the specific NK1 receptor agonist [Sar^{9},$Met(O_2)^{11}$]-substance P (Peninsula) (NK1-Ag).

Solution D: identical to solution A but for the addition of 40-120 nM of the specific NK2 receptor agonist [Nle^{10}]-neurokinin $A_{(4-10)}$ (Peninsula) (NK2-Ag).

At a later date, we sought to verify the apparent scarcity of NK2 receptors noted in initial experiments and to determine whether NK3 receptors were present in equine pulmonary tissue. Slides with adjacent sections of the same tissues from 2 horses were simultaneously incubated in either solutions A, B, C, D or:

Solution E: identical to solution A but for the addition of 20-40 nM of the specific non-peptide NK2 antagonist MEN-10,376 (Peninsula) (NK2-Antag).

Solution F: identical to solution A but for the addition of 40 nM of the specific NK3 receptor agonist senktide (Peninsula) (NK3-Ag).

After incubation and rinsing, the slides were rapidly dried under a stream of dry, cold air before storage in a dessicator. To improve tissue preservation,

sections from horses #9 and #10 were then fixed for 3 hours with hot paraformaldehyde vapors (58°C) as described by Herkenham and Pert (1982).

The slides were then apposed to Kodak X-OMAT AR film in radiographic cassettes equiped with two Dupont Cronex Hi-Plus intensifying screens; a 20-μm thick section of [^{125}I] plastic microscales (Amersham) was also apposed to each sheet of film at the same time. Autoradiographs were developed in an automatic processor once exposure of the films was suitable for quantitative microdensitometry of airway epithelium and smooth muscle (2 to 5 days). Histological slides not selected for emulsion autoradiography were then counterstained with methylene blue, azure B, and eosin Y (MBAE) and mounted in Permount (Polysciences).

The radioactivity of the incubation solutions remained stable during the experiments. However, for the experiment with 120 nM of NK1-Ag and NK2-Ag, [I]-BHSP radioactivity in the incubation solutions was decreased by 20%, indicating that the final concentration of [I]-BHSP was approximately 16 pM for this experiment; results were not appreciably affected.

[^{125}I]-Neurokinin A autoradiography—We also attempted to detect NK2 receptors in equine lung using (2-[^{125}I]iodohistidyl-1)-neurokinin A (Amersham) ([I]-NKA). Pulmonary tissue from 2 horses was incubated with 20 pM [I]-NKA in the presence and absence of 20-40 nM of the specific non-peptide antagonist of NK2 receptors MEN-10,376 (NK2-Antag). The incubation procedure was as described by Mantyh et al (1989), but 20 pM [I]-NKA instead

of 100 pM were used and the pre-incubation period was 20 minutes. Exposure of the autoradiography film lasted 7-10 days.

Emulsion autoradiography—Some histological slides were chosen for emulsion autoradiography to enhance the identification of the tissues labelled by [I]-BHSP or [I]-NKA. Selected slides were apposed to coverslips coated with Kodak NTB-3 emulsion (Young and Kuhar 1979). After incubation at 4°C for 8 days for [I]-BHSP or 13 days for [I]-NKA, the coverslips with their corresponding slides were developed in Dektol (diluted 1:1 with H_2O), fixed with Kodak fixer, rinsed, counterstained with MBAE, and mounted in Polymount (Polysciences). Neither negative chemography (fading of a latent image induced by exposure to tissue) nor positive chemography (creation of a latent image by non-radioactive tissue) were present on control slides, indicating that the focal increases in optic density of the autoradiographic film was entirely attributable to [^{125}I].

Microdensitometry—Each piece of autoradiographic film was identified as to the tissue, experiment, histological slide, and microscale used, and the date of development of the film. Each such piece of film was glued to a clean slide in register with the corresponding histological sections. It was then possible to examine both the histological sections and the corresponding autoradiographic image under a Leitz microscope fitted with a video camera. The final magnification of tissues or film on the video camera monitor was 83X.

Quantitative microdensitometry of the areas of interest on the autoradiographic film strips was performed using the Bioquant Meg IV (version 2.6.92) microdensitometry program installed on a computer linked to the video camera.

Features of interest (airway smooth muscle, vessels, epithelium) and landmarks (bronchial cartilage) were outlined on the video image of the histology slide, and these outlines were then superimposed upon the corresponding sites on the autoradiographic film. For each slide, the surface area and the optic density of all the visible respiratory epithelium (with the basal lamina) and airway smooth muscle (excluding the areas of intense [I]-BHSP binding to small vessels) were quantitated in 5 different fields of view at 83X (video camera monitor). The optic density in the examined film was correlated with the radioactivity in the corresponding tissues (expressed in $nCi/\mu m^2$) using the autoradiographic image of the microscales as a reference. This correlation was re-established for each sample and each measurement period. The stability of the system was verified every hour by remeasuring the same area of the microscale: if the optic density had changed by 3 or more grey values (out of a total of 250), the data collected in the previous hour was remeasured.

The binding of [I]-BHSP in the presence and absence of the specific agonists and antagonist used was expressed in $nCi/\mu m^2$ and calculated as follows:

Total binding of [I]-BHSP = binding in solution A.

Non-specific binding of [I]-BHSP = binding in the presence of an excess of unlabelled SP (solution B).

Specific binding of [I]-BHSP = binding in solution A - binding in solution B.

Total binding in the presence of NK1-Ag - binding in solution B = specific binding sites that are *not* NK1 receptors.

Total binding in the presence of NK2-Ag - binding in solution B = specific binding sites that are *not* NK2 receptors.

Total binding in the presence of NK2-Antag - binding in solution B = specific binding sites that are *not* NK2 receptors (but see below).

Total binding in the presence of NK3-Ag - binding in solution B = specific binding sites that are *not* NK3 receptors.

Results

Distribution of [I]-BHSP binding sites in equine airways—The darkest areas on the autoradiographic film, corresponding to [I]-BHSP binding sites, were present in the respiratory epithelium, the lamina propria, tracheobronchial glands, airway smooth muscle, and the small vessels within the lamina propria and the bronchial sheath (Figs 1A and 2A). The greatest density of specific [I]-BHSP binding sites was apparent over the small vessels in the lamina propria and the bronchial sheath in airways of all sizes, as well as tracheobronchial glands; the bronchial epithelium, particularly in the area of the basal lamina, also contained many specific [I]-BHSP binding sites. Specific binding was also present in airway smooth muscle and medium-sized bronchial arteries, but was always less intense than in epithelium. Specific/non-specific binding in

respiratory epithelium and smooth muscle comprised 85%/15%, and 68%/32%, respectively, of the total binding. Binding site density was low over pulmonary parenchyma.

A large amount of non-specific binding was seen in the bronchial cartilages (Figs 1B and 2B) and in the walls of large pulmonary vessels, providing useful landmarks for the exact alignment of the images of sections and autoradiographic film.

Distribution of [I]-NKA binding sites in the equine lung—The binding of [I]-NKA was slight and its distribution was similar to that of [I]-BHSP. Qualitatively, binding in the presence and absence of the NK2-Antag MEN-10,376 appeared identical, indicating that NK2-Antag did not prevent [I]-NKA binding to pulmonary tissue; specific binding therefore could not be calculated.

Specific binding of [I]-BHSP in the presence of receptor agonists and an antagonist—Qualitatively, the appearance of autoradiographic films from sections incubated with the NK1-Ag were identical to those obtained in the presence of an excess of unlabelled SP (Figs 1C and 2C), indicating that the NK1-Ag, like the excess of unlabelled SP, completely prevented specific [I]-BHSP binding. The distribution of specific [I]-BHSP binding sites in autoradiographic films of sections incubated with the NK2-Ag appeared similar to those in the absence of an agonist, but the films were slightly paler than those obtained with [I]-BHSP alone, indicating that [I]-BHSP binding to some sites had been prevented by the NK2-Ag (Figs 1D and 2D).

In preliminary studies with 2 animals, no apparent difference between the specific binding in the presence of the NK2-Antag MEN-10,376 and the specific binding of [I]-BHSP alone was noted (not quantitated). The specific binding of [I]-BHSP in the presence of 20-40 nM of the NK3-Ag senktide appeared decreased (paler autoradiographic images) when compared to the specific binding of [I]-BHSP alone, suggesting that the NK3-Ag displaced [I]-BHSP from some binding sites. Quantitative microdensitometry of one caudo-dorsal bronchus demonstrated a 35% decrease in specific [I]-BH-SP binding to equine airway smooth muscle in the presence of the NK3-Ag and a smaller (11%) decrease in epithelium, confirming our qualitative observations.

Quantitative microdensitometry of specific [I]-BHSP binding to airway epithelium and smooth muscle—Quantitative data obtained by microdensitometry were consistent with our qualitative observations. Quantitative data from experiments with 120 nM NK1-Ag and 120 nM NK2-Ag are presented in Figs 3 and 4: data was obtained from 5 large airways (mean inner diameter 8.8 mm; 3 third-generation bronchi, 2 dorsal bronchi; see Fig 1 in Chapter 2 for site of sampling) and 5 caudo-dorsal small bronchi (mean inner diameter 4.1 mm). In this particular series of experiments, the actual concentration of [I]-BHSP was approximately 16 pM (see methods).

In the respiratory epithelium and smooth muscle of large and small airways, we established that [I]-BHSP binding in the absence and the presence of NK1-Ag and NK2-Ag was significantly different (Friedman's ranked sum test, $p < 0.05$).

There was no significant difference between [I]-BHSP binding in the presence of 1 μM unlabelled SP and that in the presence of 120 nM NK1-Ag (signed ranks test, $\alpha = 0.05$), indicating that the majority of the binding sites for [I]-BHSP bound this highly specific NK1 receptor agonist. Preliminary data obtained from incubations in 20 nM NK1-Ag also demonstrate complete inhibition of specific [I]-BHSP binding.

There was a small decrease in the binding of [I]-BHSP in the presence of 120 nM NK2-Ag when compared to the specific binding of [I]-BHSP in both large and small airways. In airway smooth muscle, NK2-Ag decreased [I]-BHSP binding by 20% on average, but due to substantial variability between animals, this difference was not statistically significant (signed ranks test, $\alpha = 0.05$). The NK2-Ag-mediated decrease in [I]-BHSP binding was small (12%) but statistically significant (signed ranks test, $p < 0.05$) in respiratory epithelium. These results indicated that NK2-Ag successfully competed with [I]-BHSP for 12-20% of the specific [I]-BHSP binding sites. The results in the presence of 40 nM NK2-Ag were similar (preliminary data).

In the presence or absence of 120 nM of NK1-Ag or NK2-Ag, the specific binding of [I]-BHSP in respiratory epithelium and smooth muscle was slightly greater in the larger airways than in the smaller airways (compare Figs 3 and 4), although the differences were not statistically significant (rank sum test, $\alpha = 0.05$).

Quantitative microdensitometry of specific [I]-BHSP binding to vessels—Although the goals of the study were to characterize the binding of [I]-BHSP in airway smooth muscle with respect to receptor types, the experiments allowed us to detect dense-specific [I]-BHSP binding over many but not all vessels. Smaller pulmonary vessels in the parenchyma could not be identified with certainty, but the walls of large pulmonary vessels primarily bound [I]-BHSP non-specifically, as shown in Fig 2.

The insignificant binding of [I]-BHSP over large pulmonary vessels was in marked contrast to the intense and highly specific (over 97%) binding of [I]-BHSP over the small bronchial vessels of the lamina propria and bronchial sheath (Figs 1 and 2), as over the *vasa vasorum* of large pulmonary vessels (Fig 2). The exposure of the autoradiographic films had been adjusted to allow for quantitative microdensitometry of the much less dense [I]-BHSP binding over airway epithelium and smooth muscle. The optic density of the film above these small vessels was therefore outside the range within which a linear relationship between optic density and radioactivity exists (Davenport et al 1988). However, to give an indication of the intense binding over these areas, we estimated specific binding of [I]-BHSP to the lamina propria in two third-generation bronchi (Table 3).

Larger bronchial vessels (arteries and veins) also bound [I]-BHSP specifically, although not to the same degree as the small vessels in the lamina propria and bronchial sheath. The specific [I]-BHSP binding (70% of total binding) over a bronchial artery (external diameter approximately 800 μm) in

one third-generation bronchus was 0.3862 nCi/μm^2, slightly less than that of airway smooth muscle in the same sample.

Specific [I]-BHSP binding to tracheobronchial glands—Some of the very intense specific binding in the lamina propria (Table 3) reflected binding to tracheobronchial glands as well as small bronchial vessels; the specific [I]-BHSP binding was determined in one gland to be approximately 4.0533 nCi/μm^2.

Discussion

The specificity of [I]-BHSP binding in equine airway epithelium, small bronchial vessels, and tracheobronchial glands was high, ranging from 84% (epithelium) to over 97% (individual vessels and tracheobronchial glands in lamina propria). Since [I]-BHSP binding was completely eliminated from these tissues by an excess of unlabelled SP, the specific binding sites in these tissues almost certainly represented tachykinin receptors (Rothman et al 1984).

In airway smooth muscle and medium-sized bronchial vessels, [I]-BHSP binding was present but only constituted 60% to 70% of the total binding. These tissues may contain small numbers of tachykinin receptors or the type of tachykinin receptor present may have a low affinity for [I]-BHSP.

General observations on [I]-BHSP binding in equine lungs—Overall, the distribution of specific [I]-BHSP binding sites in equine airways resembled that described in human, guinea pig, and rat airways (Carstairs and Barnes 1986a; Sertl et al 1988), and differed most from that described in rabbit lungs (Black et al 1990). Equine airway smooth muscle contained far fewer specific

[I]-BHSP binding sites per μm^2 than the respiratory epithelium, small bronchial vessels, and tracheobronchial glands, whereas airway smooth muscle was preferentially labelled by [I]-BHSP in human, guinea pig, and rabbit (but not rat) airways (Carstairs and Barnes 1986; Black et al 1990; Sertl et al 1988). The high concentration of [I]-BHSP specific binding sites in the respiratory epithelium and small bronchial vessels of equine airways most closely resembled the reported distribution of [I]-BHSP binding sites in rat lungs (Sertl et al 1988).

In the equine lung, the intensity of [I]-BHSP binding was only slightly greater in the respiratory epithelium and airway smooth muscle of the larger bronchi compared to the smaller bronchi (compare Fig 3 and Fig 4). Since in the lung tachykinin receptor density generally correlates well with the response to tachykinins (Black et al 1990; Sertl et al 1988), marked differences in the responses of the sampled large and small equine bronchi to tachykinins are not likely.

Types of tachykinin receptors present in equine airways—The complete inhibition of specific [I]-BHSP binding in the presence of 20 to 120 nM NK1-Ag indicated that the majority of tachykinin binding sites in the equine lung probably correspond to NK1 receptors. The decreased specific binding of [I]-BHSP in the presence of NK2-Ag and NK3-Ag may indicate that a small number of NK2 and NK3 receptors were also present. Although the NK2 agonist used in these experiments ($[Nle^{10}]$-NKA_{4-10}) can bind to NK1 or NK3 receptors under certain conditions (Regoli et al 1988; Regoli et al 1991), so that the decrease

in specific [I]-BHSP observed in the presence of NK2-Ag may have been due to binding to NK1 or NK2 receptors, the NK3 agonist senktide has very little affinity for NK1 or NK2 receptors (Wormser et al 1986; Regoli et al 1988), so that the presence of NK3 receptors in equine airways is therefore quite probable.

Our attempts to confirm whether NK2 receptors were present in the equine lung using the specific NK2 antagonist MEN-10,376 (NK2-Antag) were not successful. The presence or absence of NK2-Antag did not appreciably affect [I]-BHSP binding. In a study published after our experiments were performed, MEN-10,376 was shown to have very little effect on [I]-BHSP or [I]-NKA binding to guinea pig lung membrane preparations (Geraghty et al 1992), although in functional in vivo or in vitro studies NK2-Antag has a high affinity for NK2 receptors (Patacchini et al 1991; Maggi et al 1990).

Experiments with [I]-NKA were also inconclusive. Although [I]-NKA binds specifically to NK2 receptors in the brain (Dam et al 1988) and rat intestine (Brown et al 1992), [I]-NKA appears to bind only poorly if at all to NK2 receptors in the rabbit and guinea pig lung, exhibiting some affinity for NK1 receptors instead (Black et al 1992; Xiao et al 1992; Geraghty et al 1992). In our study, [I]-NKA bound to the same sites as [I]-BHSP but with much less affinity, suggesting that this may also be the case in the equine lung.

Vasculature—Most of the numerous [I]-BHSP binding sites on small bronchial vessels appeared to represent NK1 receptors; a small number of NK2 or NK3 receptors may also be present.

Tachykinins are potent vasodilators of tracheal or bronchial vessels in other species (rat, cat, pig, and dog) (Laitinen et al 1987; Salonen et al 1988; Piedimonte et al 1992; Martling et al 1987a; Matran et al 1989). Activation of the tachykinin receptors on the numerous small vessels in the lamina propria in equine lungs is therefore most likely to result in vasodilation. In sheep, congestion of bronchial vessels in the lamina propria of 1-mm bronchioles caused a significant narrowing of the airway lumen (Mariassy et al 1991); the effect in equine airways is likely to be similar. Tachykinins could thus affect airway diameter by influencing bronchial vascular tone in horses. The specific [I]-BHSP binding sites were less dense in larger bronchial vessels, leading us to believe that tachykinins may not have as much effect on the vascular tone of these vessels as on the smaller bronchial vessels.

Neurokinin 1 (Rogers et al 1988) and, in guinea pigs, NK2 (Tousignant et al 1993) receptors can mediate plasma extravasation in the airways, but whether activation of receptors on the small vessels in the lamina propria would lead to neurogenic edema within the walls of equine bronchi is unknown. To date, tachykinin-mediated tracheal or pulmonary plasma extravasation has only been documented in rodents (Solway and Leff 1991).

The negligible specific [I]-BHSP binding to large pulmonary vessels in the equine lung probably indicates that tachykinins have few direct effects on these vessels. Consistent with this thought, SP-immunoreactive nerves are primarily associated with *vasa vasorum* in the adventitial layer of large pulmonary vessels, and are almost never seen elsewhere within them (Chapter 2). The

vasa vasorum in the adventitia of equine pulmonary vessels are bronchial in origin (Charan and Carvalho 1992).

Tracheobronchial glands—The very intense binding of [I]-BHSP to tracheobronchial glands probably reflects an important role for tachykinins in the regulation of airway secretions in the horse. For example, autoradiography of cat tracheobronchial glands revealed numerous [I]-BHSP binding sites, and tachykinins stimulate secretion by the same glands (Lundgren et al 1989; Shimura et al 1991).

Respiratory epithelium—The equine respiratory epithelium had numerous specific [I]-BHSP binding sites, most of which appeared to represent NK1 receptors. Specific [I]-BHSP binding sites in the epithelium were densest in the area of the basal lamina: some of these binding sites may have been on the numerous capillaries just below the basal lamina, while others may have been on basal cells in the epithelium. The resolution of [^{125}I]-based receptor autoradiography unfortunately does not allow us to differentiate between the sites.

The most important effect of NK1 receptor activation in equine airways may be the release of physiologically active substances such as prostanoids. In rat and guinea pig airways, tachykinin-mediated prostanoid release from the respiratory epithelium has an inhibitory effect on bronchial smooth muscle constriction (Devillier et al 1992; Frossard et al 1989). A similar mechanism is believed to account for the NK1-mediated relaxation of mouse airways (Manzini 1992). Furthermore, a tachykinin-mediated release of active

substances from the respiratory epithelium, rather than a direct effect on bronchial venules, is believed by some to cause the plasma extravasation in rat airways (McDonald 1988; McDonald et al 1988).

The numerous [I]-BHSP binding sites seen in respiratory epithelium in equine airways probably also contribute to the regulation of ion and water transport accross the epithelium, as has been demonstrated in other animals (Sestini et al 1990; Rangachari et al 1987).

Airway smooth muscle—In vitro, in the presence of the enzyme inhibitor phosphoramidon, SP caused less contraction of equine airway smooth muscle than NKA (Le Ninivin et al 1993), suggesting that the tachykinin receptors mediating smooth muscle contraction in the equine lung are NK2 or NK3 receptors. Neurokinin-2 receptors are the principal mediators of tachykinin-induced contraction of airway smooth muscle in guinea pigs (Ireland et al 1991; Maggi et al 1991) and other species (Dion et al 1990; Maggi et al 1989; Black et al 1992), although NK1 receptors are believed to contribute somewhat to the contraction of respiratory smooth muscle in guinea pigs (Maggi et al 1991; Ireland et al 1991). However, the majority of specific [I]-BHSP binding sites in equine airway smooth muscle appeared to be NK1 receptors.

It is possible that the NKA-induced bronchoconstriction in equine airways may act on NK2 receptors on airway smooth muscle: NK2-Ag inhibited [I]-BHSP binding by about 20% in airway smooth muscle. However, in equine airways atropine completely eliminates neurally induced airway smooth muscle contraction in vitro (Broadstone et al 1991; LeBlanc et al 1991), indicating that

tachykinin-mediated bronchoconstriction of equine bronchi is not likely to be due to a direct effect on the smooth muscle. It is more likely that the contractile effects of NKA result from an enhancement of cholinergic neurotransmission in the equine lung. Tachykinin receptors on parasympathetic nerves have been shown to modulate acetylcholine release in the lung in other species (Tanaka and Grunstein 1984; Tanaka and Grunstein 1986; Hall et al 1989; Martling et al 1984; Aikawa et al 1990; Shioya et al 1993). Within the airways, the nature of the tachykinin receptor(s) involved in this response has only been investigated in the guinea pig trachea, where NK1 or NK2 receptors are believed to mediate the tachykinin-dependent enhancement of acetylcholine release (Hall et al 1989; Watson et al 1993). In the guinea pig gut, which shares the same embryological origin as the lung (Noden and de Lahunta 1985), tachykinin-mediated enhancement of acetylcholine release is due to activation of NK3 receptors (Laufer et al 1985; Wormser et al 1986). It is thus reasonable to believe that in the equine lung activation of either NK2 or NK3 receptors on parasympathetic nerves might cause an enhancement of cholinergic neurotransmission.

Interestingly, epithelium removal (in the presence of phosphoramidon) did not change the response of equine airway smooth muscle to SP, but markedly increased the response to NKA (Le Ninivin et al 1993). In equine airways, removal of the epithelium and its numerous NK1 receptors (which may mediate the release of airway smooth muscle relaxing factors) may allow the contractile

effects of a small number of NK2 or NK3 receptors on airway smooth muscle to predominate.

Clinical significance of tachykinin receptors in equine lungs—The presence of specific binding sites in equine airways for [I]-BHSP in conjunction with the presence of SP-immunoreactive nerves associated with airway epithelium and small vessels (Chapter 2) lead us to believe that tachykinins could play an important role in the modulation of pulmonary function in the horse. The effects of tachykinins are most likely to become evident when airway epithelium is damaged, stimulating sensory nerves in the airways (Solway and Leff 1991).

In other species, epithelial damage due to viral infections activates sensory nerves and enhances the effects of the released tachykinins, in part at least because the endopeptidases that degrade tachykinins are bound to epithelial cells (Nadel and Borson 1991; Piedimonte et al 1990; Saban et al 1987). Consequences of similar infections in the equine lung might include airway obstruction mediated via NK1-dependent vasodilation of bronchial vessels, bronchoconstriction due to the actions of tachykinins on NK2 or NK3 receptors unopposed by NK1-mediated release of smooth muscle relaxants from the damaged epithelium, and enhancement of the actions of tachykinins due to loss of epithelium-bound peptidases.

Tachykinins may also contribute to the pathophysiology of heaves, a common equine pulmonary disease resembling asthma (Lowell 1964) with an allergic etiology (McGorum et al 1993). Inhaled allergens stimulate the release

of tachykinins in guinea pig airways (Kawano et al 1993) and cause a tachykinin-mediated vasodilation of bronchial vessels in pigs (Alving, 1991), and are likely to cause similar changes in the lungs of susceptible horses.

Our study indicates that the specific [I]-BHSP binding sites in the equine lung resemble NK1 receptors, and that NK2 and NK3 receptors may also be present: the use of radioligands that are more specific agonists of NK2 and NK3 receptors would offer final confirmation of our preliminary findings.

Summary

1. Tachykinin receptors in the equine lung are predominantly found in tracheobronchial glands, small bronchial vessels, and the respiratory epithelium. The principal effects of tachykinins in the equine lung may therefore involve changes in vascular tone and tracheobronchial secretions.

2. The majority of tachykinin binding sites appear to have the characteristics of NK1 receptors, which in other species mediate vasodilation, plasma extravasation, and tracheobronchial secretion. These receptors may mediate similar actions in the equine lung.

3. Some tachykinin binding sites may represent NK2 or NK3 receptors, which in other species mediate bronchoconstriction and enhancement of parasympathetic neurotransmission. Physiological evidence indicates that activation of NK2 receptors in the equine lung may indeed contribute to bronchoconstriction.

Figure 1. [I]-BHSP binding sites in a third-generation bronchus. Bar = 5 mm. A. Total binding in the presence of [I]-BHSP: the darker areas represent tissues with more [I]-BHSP binding sites. Note the intense binding over small vessels in the lamina propria and the bronchial sheath of the bronchus and of a non-cartilaginous bronchiole (br), whereas a bronchial artery (art) is only faintly labelled. Total binding is also intense over two large tracheobronchial glands (gland). Note that the epithelium binds more densely than the airway smooth muscle (musc), particularly at the level of the basal lamina. The [I]-BHSP binding over pulmonary parenchyma is slight.

B. Non-specific binding of [I]-BHSP in the presence of 1 μM unlabelled SP. Bronchial cartilages bind SP non-specifically, and a portion of the binding in the lamina propria of the third-generation bronchus is also non-specific.

Figure 1.

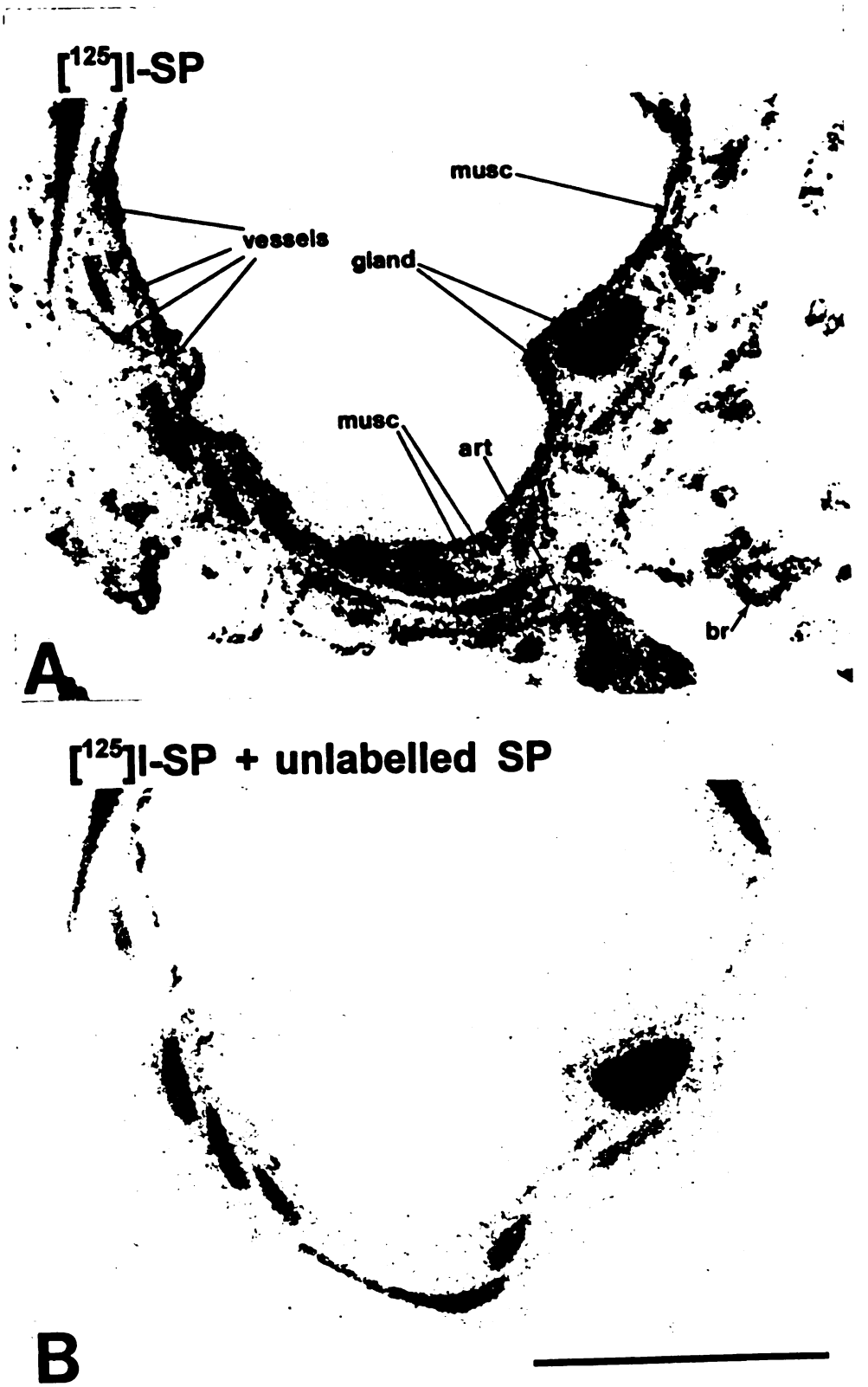

[125]I-SP
musc
vessels
gland
musc
art
br
A
[125]I-SP + unlabelled SP
B

Figure 1 (cont'd).

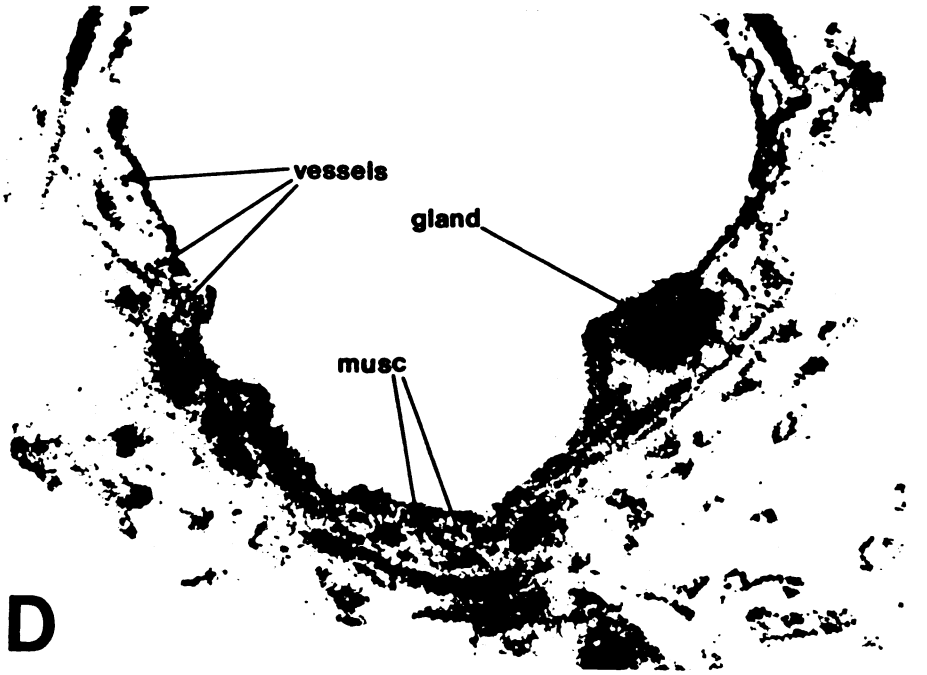
C. Binding of [I]-BHSP in the presence of a NK1-Ag cannot be distinguished from the non-specific binding in the presence of an excess of unlabelled SP, indicating that most specific binding sites in equine airways resemble NK1 receptors.

D. Binding of [I]-BHSP in the presence of a NK2-Ag resembles the binding in Figure 1A. The image is slightly paler, indicating that the NK2-Ag displaced [I]-BHSP from some binding sites: these binding sites may represent NK2 receptors.

Figure 1 (cont'd).

C

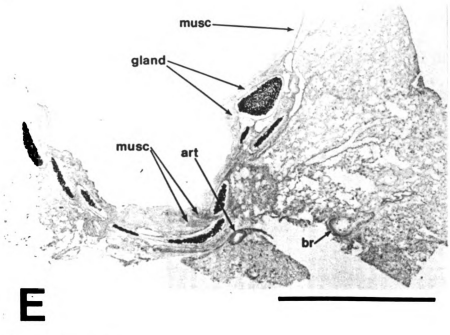
[^{125}]I-SP + NK1 agonist



D

[^{125}]I-SP + NK2 agonist

Figure 1 (cont'd).



E. Histological section from which the autoradiograph in Figure 1A was generated.

Figure 2. [I]-BHSP binding sites in a small caudo-dorsal bronchus. Bar = 5 mm. A. Total binding in the presence of [I]-BHSP. The distribution of binding sites and the intensity of the label is similar to that in the third-generation bronchus. Numerous binding sites are present in the small vessels in the lamina propria and bronchial sheath of this bronchus as well as those of non-cartilaginous bronchi (br). [I]-BHSP binding is also present in the airway epithelium (epith) and smooth muscle (musc), although it is not as dense as over small vessels. Note the intense labelling of the vasa vasorum (bronchial origin) in the adventitia of the pulmonary artery (p.a.). The pulmonary parenchyma binds very little [I]-BHSP.

B. Non-specific [I]-BHSP binding in the presence of 1 μM unlabelled SP is primarily present in bronchial cartilage and the muscularis of the pulmonary artery.

Figure 2.

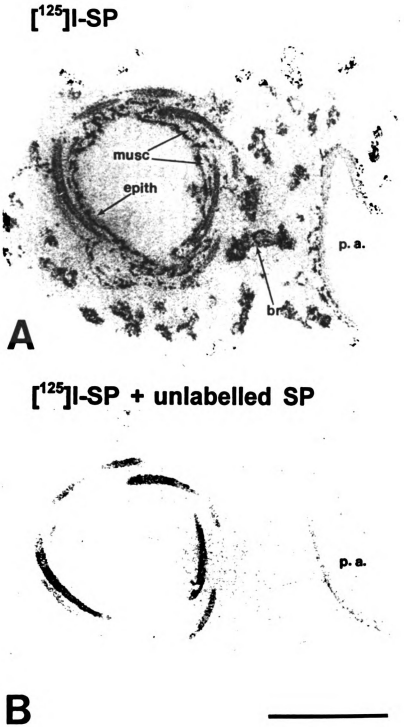
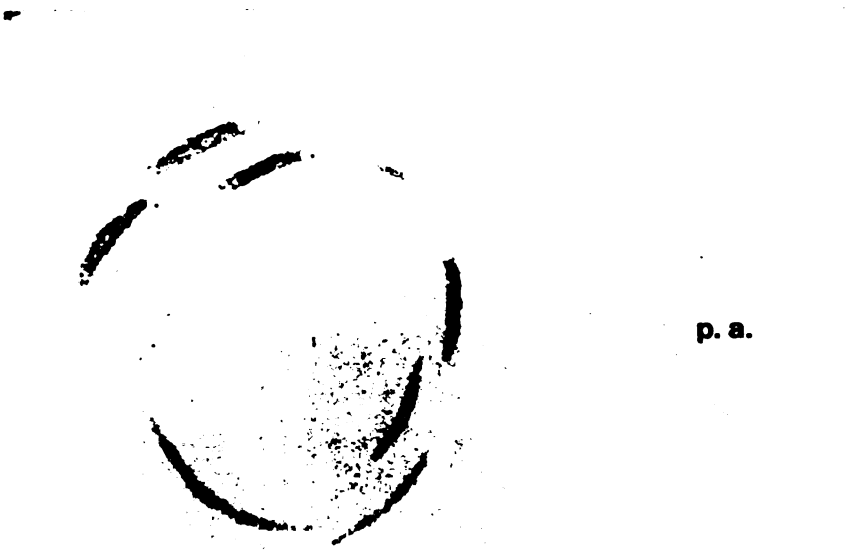


Figure 2 (cont'd).

C. Binding of [I]-BHSP in the presence of a NK1-Ag cannot be distinguished from non-specific binding in the presence of unlabelled SP, indicating that most binding sites in equine small airways resemble NK1 receptors.

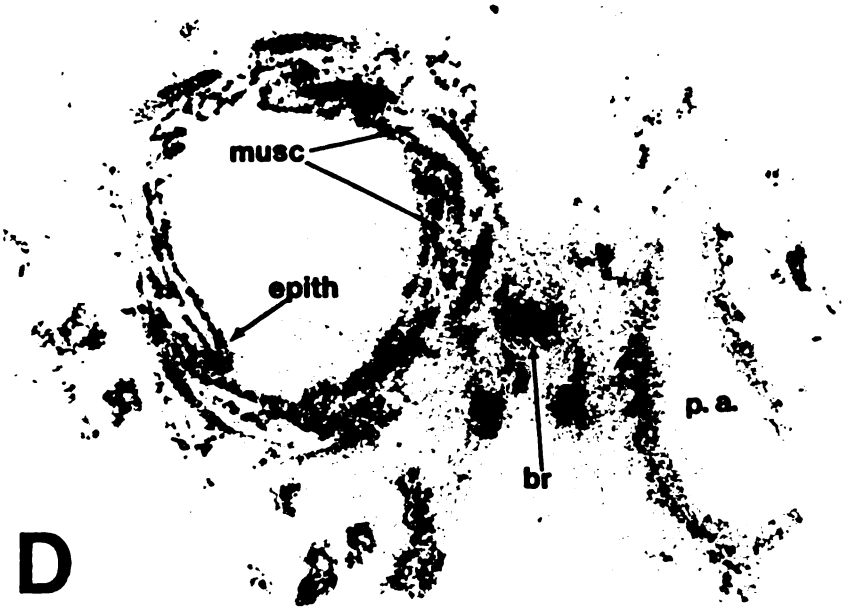
D. Binding of [I]-BHSP in the presence of a NK2-Ag is similar in distribution to that of [I]-BHSP alone, although it seems less intense.

Figure 2 (cont'd).



C

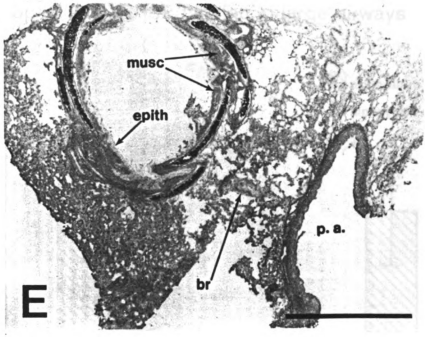
[^{125}]I-SP + NK1 agonist



D

[^{125}]I-SP + NK2 agonist

Figure 2 (cont'd).



E. Histological section from which the autoradiograph in Figure 2A was taken.

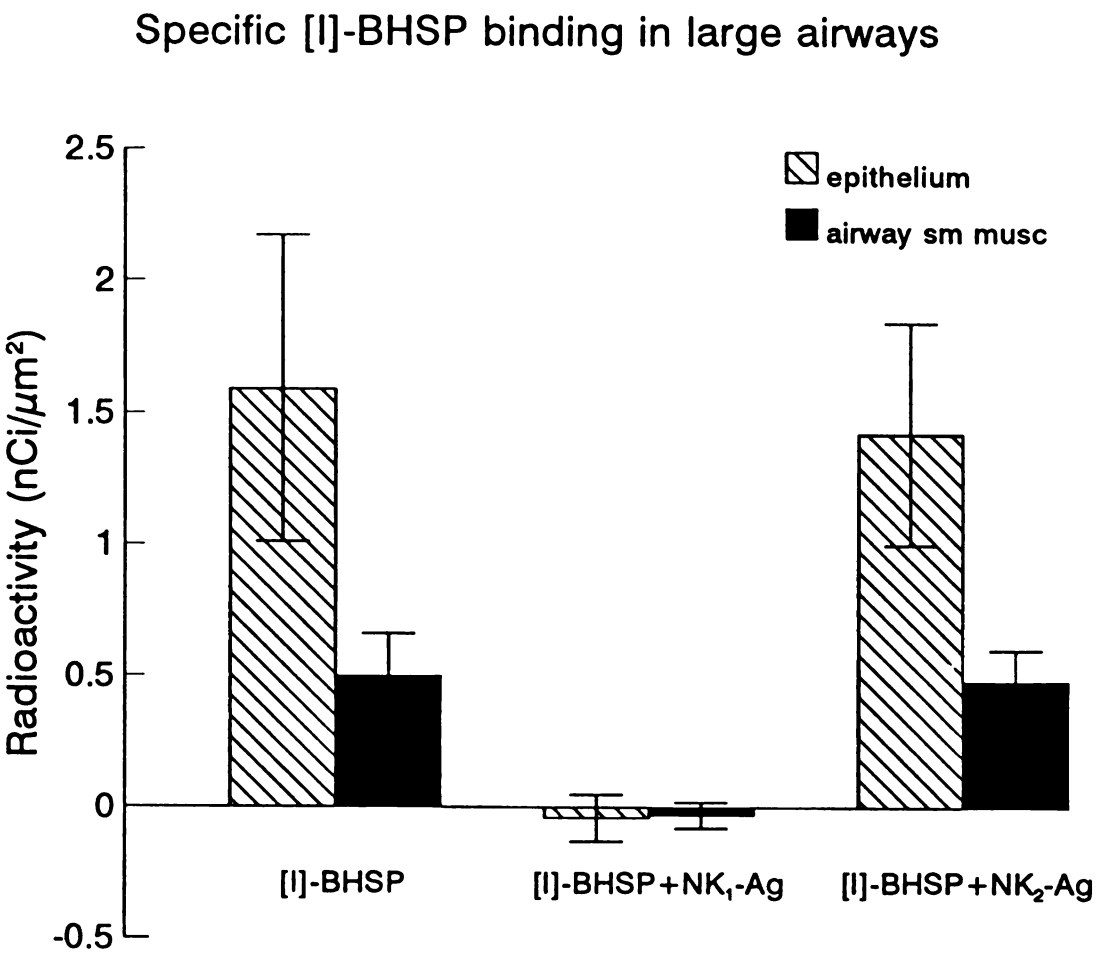


Figure 3. Specific [I]-BHSP binding (nCi/μm^2) in large airways in the absence and presence of 120 nM NK1-Ag or 120 nM NK2-Ag. Means ± S.E.. Average airway inner diameter 8.8 mm.

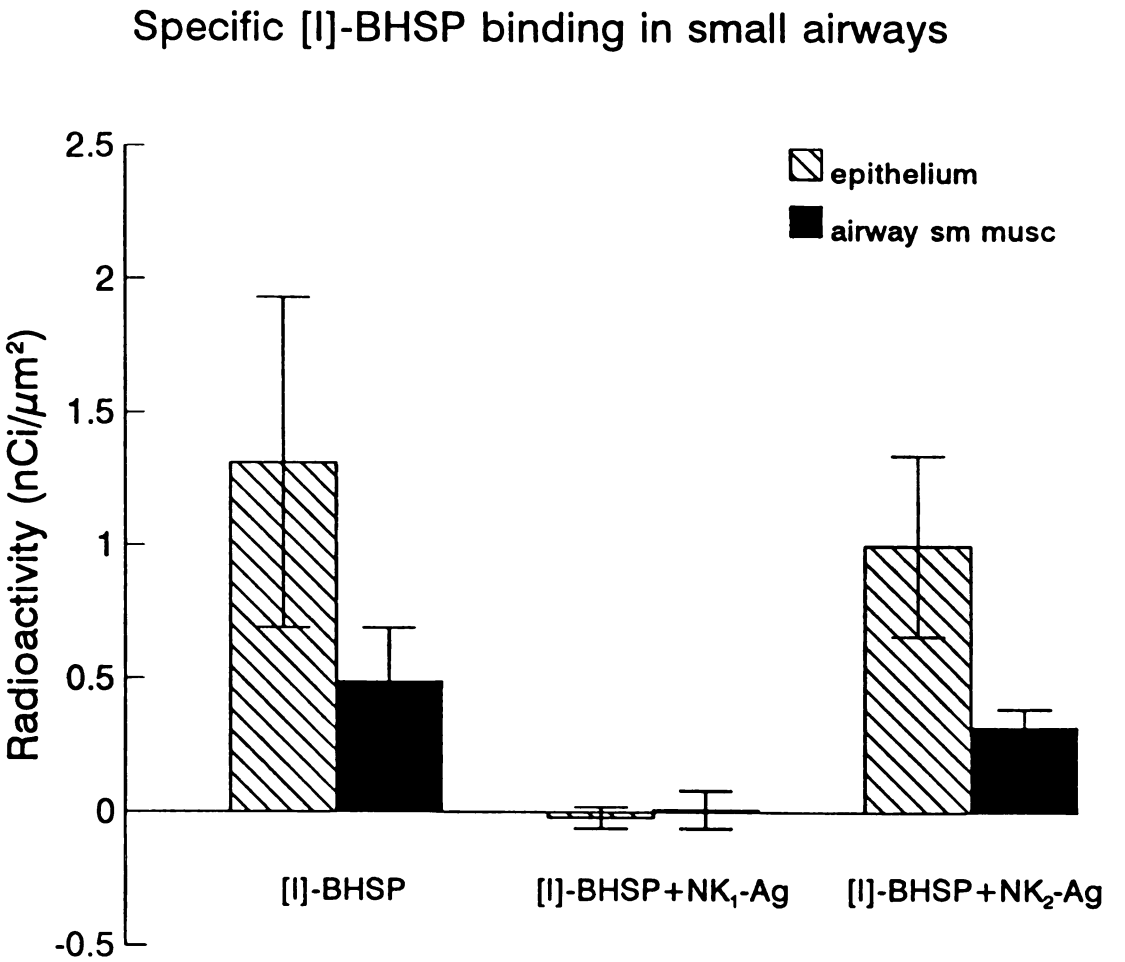


Figure 4. Specific [I]-BHSP binding (nCi/μm^2) in small airways in the absence and presence of 120 nM NK1-Ag or 120 nM NK2-Ag. Means ± S.E.. Average airway inner diameter 4.1 mm.

Table 1—Experimental animals and tissues examined

Horse	Age, sex, breed	Tissue	Comments
1	Aged, F, QTH	trachea, 3B, DB, CRSB, CDSB	Trachea used for preliminary trials
2	Aged, F, grade pony	3B, DB, CRSB, CDSB	
3	9 yr, F, Arabian	CRSB, CDSB	
4	8 yr, F, Arabian	CRSB, CDSB	
5	24 yr, F, grade pony	3B, DB, CRSB, CDSB	
6	5 yr, F, Arabian	3B, DB, CRSB, CDSB	
7	4 yr, G, Arabian	3B, DB, CRSB, CDSB	
8	15 yr, F, TB	3B, DB, CRSB, CDSB	Ataxic.
9	Aged, F, TB	DB, CDSB	Tissues also incubated with MEN-10,376, senktide and [I]-NKA. Ataxic.
10	3 yr, M, grade pony	3B, DB, CRSB	Tissues also incubated with MEN-10,376, senktide and [I]-NKA. 5-cm diam walled-off abscess in caudo-dorsal lung: CDSB consequently not collected.

Abbreviations: F = female; G = gelding (castrated male); M = intact male; 3B = third-generation bronchus; DB = large caudo-dorsal bronchus at junction of middle and caudal thirds of lung; CRSB = cranial small bronchi; CDSB = caudal small bronchi (see Fig 1 in Chapter 2 for site sampling). Aged: over 15 years old but exact age undetermined.

Table 2—Specific binding of [I]-BHSP in vessels and tracheobronchial glands (nCi/μm^2)

	[I]-BHSP (A)	[I]-BHSP and NK1-Ag	[I]-BHSP and NK2-Ag (B)	A - B
Lamina propria*	2.4871	0.1383	2.1201	0.3670

*Individual vessels and many glands within the lamina propria were impossible to outline accurately. The optic density of the entire surface of 2 views at a final magnification of 83X of the lamina propria (including small vessels and glands) of 2 third-generation bronchi was quantitated and the mean is given above. Within the lamina propria, the areas with the densest [I]-BHSP binding corresponded to ≈ 4.112 nCi/μm^2; the densest binding of [I]-BHSP in the presence of the NK2-Ag corresponded to ≈ 4.03 nCi/μm^2 (measured in one third-generation bronchus). These areas of dense binding were located over small vessels and tracheobronchial glands.

CHAPTER 5

CONCLUSIONS

1. Nerves immunoreactive for SP and CGRP were predominantly present in the central airways of adult horses, where they were closely associated with bronchial and pulmonary vessels and the respiratory epithelium. These nerves are well placed to detect inhaled irritants and to release their neurotransmitters near vessels. The release of neurotransmitters from afferent nerves is most likely to predominantly affect the nearby epithelium, tracheobronchial glands, and vessels.

2. In foals, nerves immunoreactive for SP and CGRP were present in central and peripheral airways in the lung, as well as in airway smooth muscle. This much wider distribution of nerves containing tachykinins and CGRP may indicate that these nerves and peptides play a role during development or during the adjustment to extra-uterine life.

3. In the adult equine lung, specific binding sites for [I]-BHSP were abundant over small bronchial vessels, tracheobronchial, glands and the respiratory epithelium. These binding sites appeared to predominantly reflect NK1

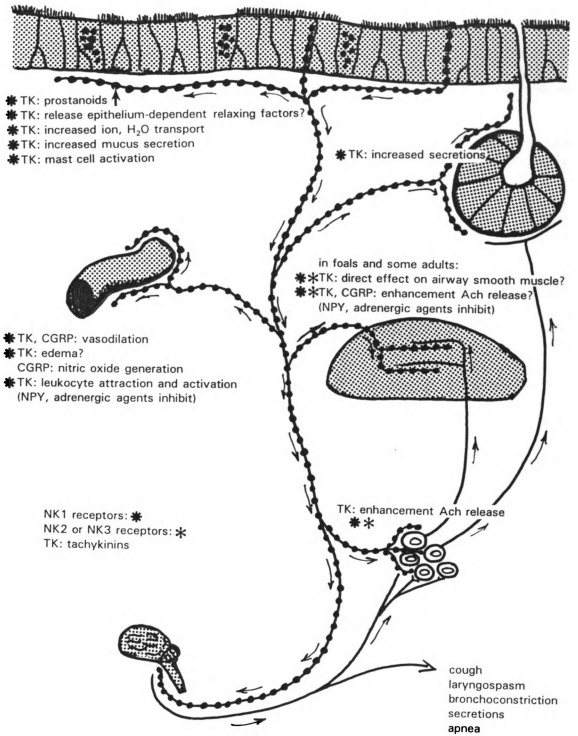
receptors, although NK2 and NK3 receptors may also have been present in small numbers. The binding sites of [I]-BHSP on airway smooth muscle, which appear to be NK1 receptors, are not very numerous, an observation that correlates well with the physiological data indicating that tachykinins have little direct effect on smooth muscle in the equine lung.

4. The equine lung offers an ideal model for the study of the efferent actions of sensory nerves that may underlie the inflammation and the bronchial hyperreactivity typical of asthma.

Figure 1 is a schematic representation of how activation of afferent nerves might affect pulmonary function in the adult equine lung.

Figure 1. Schematic representation of the possible effects of activation of afferent nerves in a central bronchus in the adult equine lung. Stimulation of afferent nerves in the epithelium leads to generation of neural impulses that travel orthodromically to generate centrally mediated impulses, and to modulate parasympathetic neurotransmission in peribronchial ganglia. Neural impulses also travel antidromically to other branches of the same nerve fibers to release neurotransmitters within the lung. Activation of NK1 receptors on epithelial cells may mediate the release of prostanoids and other vasoactive products as well as alterations in ion and mucus secretion from the respiratory epithelium. Tachykinins may also stimulate secretion and extrusion of glandular secretions from tracheobronchial glands. Activation of NK1 receptors on small bronchial vessels may cause congestion and plasma extravasation within the lamina propria, contributing to airway obstruction, as well as the release of endothelium-depedent relaxing factors. Although a direct effect of tachykinins on airway smooth muscle is not very likely, stimulation of intrapulmonary sensory nerves may cause activation of NK2 or NK3 receptors on prejunctional parasympathetic nerves, thereby enhancing cholinergic neurotransmission and, in turn, airway obstruction. Airway smooth muscle tone may also be modified by the tachykinin-mediated release of prostanoids and other factors from the respiratory epithelium or the vessels within the lamina propria. A tachykinin-mediated influx of leukocytes and activation of mast cells may significantly contribute to the inflammatory response of the lung.

Figure 1.


✱TK: prostanoids
✱TK: release epithelium-dependent relaxing factors?
✱TK: increased ion, H_2O transport
✱TK: increased mucus secretion
✱TK: mast cell activation
✱TK: increased secretions
in foals and some adults:
✱✱TK: direct effect on airway smooth muscle?
✱✱TK, CGRP: enhancement Ach release?
(NPY, adrenergic agents inhibit)
✱TK, CGRP: vasodilation
✱TK: edema?
CGRP: nitric oxide generation
✱TK: leukocyte attraction and activation
(NPY, adrenergic agents inhibit)
NK1 receptors: ✱
NK2 or NK3 receptors: ✱
TK: tachykinins
TK: enhancement Ach release
✱✱
cough
laryngospasm
bronchoconstriction
secretions
apnea

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