NEURAL AND NEUROPEPTIDE CONTROL OF ~~GUINEA-PIG AND HUMAN~~ AIRWAY SMOOTH MUSCLE IN VITRO

by

Kerry J. Rhoden,

Department of Thoracic Medicine,

National Heart & Lung Institute,

Dovehouse Street,

London SW3 6LY

Submitted to the University of London for the degree of

Doctor of Philosophy

Abstract

Efferent neural control of airway smooth muscle is comprised of cholinergic, adrenergic, excitatory nonadrenergic noncholinergic (e-NANC) and inhibitory nonadrenergic noncholinergic (i-NANC) components. This thesis concentrates on the cholinergic and NANC innervation of guinea-pig and human airways, and aims to investigate the nature and modulation of responses produced by the stimulation of such nerves and their neurotransmitters *in vitro*.

Catecholamines inhibited cholinergic responses of human bronchi to electrical field stimulation (EFS) to a greater degree than responses to acetylcholine. Inhibition of responses to EFS was blocked by a $beta_2$-adrenoceptor antagonist. These results suggest that catecholamines may inhibit cholinergic neurotransmission, possibly via prejunctional $beta_2$-adrenoceptors.

Contractile responses of the guinea-pig trachea to tachykinins were inhibited by airway epithelium, partly due to the release of cyclooxygenase products and partly due to metabolism by neutral endopeptidase (NEP). Responses induced by e-NANC nerve stimulation in guinea-pig bronchi were modulated by NEP. Relaxant responses of guinea-pig trachea to vasoactive intestinal peptide (VIP) were inhibited by airway epithelium, due to metabolism of VIP by NEP. Responses induced by i-NANC nerve stimulation were not modulated by NEP.

Receptors mediating responses of airway smooth muscle to tachykinins and e-NANC nerve stimulation were investigated using selective agonists and antagonists. Selective agonists to NK1-, NK2- and NK4-receptors were active on guinea-pig airways, but only the NK2-receptor agonist was active on human airways. NK2-receptor antagonists were weakly active on responses of guinea-pig trachea to tachykinins and e-NANC nerve stimulation, but inhibited responses of human bronchi to tachykinins.

Relaxation of guinea-pig trachea to VIP and i-NANC nerve stimulation was potentiated by a cAMP phosphodiesterase inhibitor but not by a cGMP phosphodiesterase inhibitor. Methylene blue had no effect on relaxation to VIP or i-NANC nerve stimulation. These findings suggest that cAMP mediates relaxation to VIP and i-NANC nerve stimulation.

Acknowledgements

The work described in this thesis was supported by the Asthma Research Council. I would like to thank Professor P. J. Barnes for his supervision and guidance, and Drs. L. A. Meldrum and N. Frossard who were my co-workers for some of the studies described here. I would also like to thank the staff of the National Heart & Lung Institute library for providing endless reprints, and Ms. J. Rohde for histological expertise.

Abbreviations

Acetylcholine esterase	AChE
Adenosine cyclic 3', 5'-monophosphate	cAMP
Airway smooth muscle	ASM
Angiotensin converting enzyme	ACE
Carbachol-induced maximum contraction	$carb_{max}$
Concentration inducing 50 % inhibition	IC_{50}
Concentration-response	C-R
Dimethyl sulfoxide	DMSO
Effective concentration inducing 50 % of maximal response	EC_{50}
Effective concentration inducing 30 % of carbachol-induced maximum response	EC_{30}
Electrical field stimulation	EFS
Excitatory nonadrenergic noncholinergic	e-NANC
Frequency inducing 50 % of maximum response	EF_{50}
Frequency inducing 35 % reversal of precontraction	EF_{35}
Guanosine cyclic 3', 5'-monophosphate	cGMP
Inhibitory nonadrenergic noncholinergic	i-NANC
Krebs-Henseleit	KH
N-ethylmaleimide	NEM
Neurokinin A	NKA
Neurokinin B	NKB
Neuropeptide K	NPK
Neutral endopeptidase	NEP
Nonadrenergic noncholinergic	NANC

Abbreviations (cont.)

Not calculated	NC
Peptide histidine isoleucine	PHI
Phosphodiesterase	PDE
Sodium nitroprusside	SNP
Standard error	SE
Substance P	SP
Tetrodotoxin	TTX
Time for 50 % recovery of baseline tension	$t_{50\%}$
Vasoactive intestinal peptide	VIP

CONTENTS PAGE

LIST OF FIGURES

LIST OF TABLES

1. Introduction

1.1. Autonomic control of airway smooth muscle (ASM)

The autonomic innervation of the airways consists of afferent (sensory) and efferent (motor) pathways. The afferent innervation serves to provide sensory information to the central nervous system and spinal cord required for the control of breathing pattern and bronchomotor tone under physiological and pathological conditions. The efferent innervation controls airway smooth muscle tone, mucus secretion, transport across airway epithelium, vascular permeability and blood flow in the bronchial circulation. The efferent innervation of the airways consists of the parasympathetic (cholinergic), sympathetic (adrenergic) and nonadrenergic noncholinergic (NANC) pathways. NANC innervation may be excitatory (e-NANC) or inhibitory (i-NANC).

1.1.1. Afferent pathways

Afferent nerves arise in the airway epithelium, interalveolar spaces, smooth muscle and submucosal layer (Sant'Ambrogio, 1982; Coleridge & Coleridge, 1986). Afferent fibres pass primarily with vagus nerves to the brain stem, although some may also pass with sympathetic fibres to the spinal cord. Afferent nerve endings include (i) slowly adapting mechanoreceptors (stretch receptors)

supplied by myelinated fibres, (ii) rapidly adapting mechanoreceptors (irritant receptors) supplied by myelinated fibres and (iii) nonmyelinated C-fibre endings. Stimulation of afferent endings leads to reflex bronchoconstriction, pulmonary vasodilatation, oedema, increased airway secretion and cough (Coleridge & Coleridge, 1986).

1.1.2. Efferent pathways

1.1.2.1. Parasympathetic control

1.1.2.1.a. Innervation and function

The parasympathetic nervous system represents the major bronchoconstrictor pathway in most animals. Parasympathetic fibres arise in the vagal nuclei of the brainstem and descend via the vagus nerves to synapse at ganglia located in the airway wall. Short postganglionic fibres supply airway smooth muscle, secretory glands and pulmonary arteries (Richardson, 1979). Both preganglionic and postganglionic nerves are cholinergic and upon stimulation release acetylcholine as their neurotransmitter.

Cholinergic nerves in the airways have been localised indirectly by histochemical staining for acetylcholine esterase (AChE), the enzyme responsible for the enzymatic degradation of acetylcholine. AChE-containing fibres have

been described in airway smooth muscle of all mammalian species studied (Richardson, 1979). In human airways, AChE-containing fibres are found in all airway levels from the trachea to terminal bronchioles and their density decreases with airway diameter (Partanen *et al.*, 1982; Sheppard *et al.*,1983). The specificity of AChE staining, however, has been questioned since AChE may be localised to nerves other than cholinergic (Papka *et al.*, 1981). Ultrastructural studies have revealed the presence of nerve profiles containing predominantly small agranular vesicles and some large granular vesicles, which are considered to be cholinergic, in the smooth muscle layer of human airways (Laitinen *et al.*, 1985b).

The functional role of the parasympathetic innervation to the airways can be demonstrated in whole animals and isolated airway preparations. In animals, electrical stimulation of the distal ends of transected vagus nerves leads to a bronchoconstrictor response which can be potentiated by AChE inhibitors and blocked by the muscarinic cholinoceptor antagonist atropine (Colebatch & Halmagyi, 1963; Olsen *et al.*, 1965). Electrical field stimulation (EFS) of isolated guinea-pig and human airway smooth muscle elicits a rapid and transient contraction that is blocked by atropine and the neurotoxin tetrodotoxin (TTX), suggesting that it is mediated by the activation of cholinergic nerves and the action of

acetylcholine on muscarinic receptors on airway smooth muscle (Foster, 1964; Farmer & Coleman, 1970; Richardson & Beland, 1976).

1.1.2.1.b. Receptors

Muscarinic receptors in the airways have been identified by receptor binding studies, autoradiographic mapping and functional assays (Murlas *et al.*, 1982; Barnes *et al.*, 1983a). Muscarinic receptors are found on airway smooth muscle, submucosal glands, epithelium, airway ganglia and nerve bundles (Barnes *et al.*, 1983a; Van Koppen *et al.*, 1987).

Using selective antagonists, muscarinic receptors can be divided into three subtypes (Doods *et al.*, 1987) and all three forms may occur in the airways (Barnes, 1989). M_1 receptors are generally found in the brain and autonomic ganglia (Hammer & Giachetti, 1982) and it is possible that they may also exist in airway ganglia. In humans, the M_1 receptor antagonist pirenzepine inhibits vagally-mediated bronchoconstriction induced by SO_2, at a dose that has no effect on bronchoconstriction due to inhaled methacholine, suggesting an effect on the cholinergic reflex pathway (Lammers *et al.*, 1989). In human airways M_1 receptors are also found on submucosal glands and alveolar walls (Mak & Barnes, 1989b). M_2 receptors, originally described in the heart ("cardiac" receptors), are found on postganglionic cholinergic nerves

to the airways where they act as autoreceptors inhibiting the release of acetylcholine from nerve terminals (Faulkner *et al.*, 1986). In human airways, the M_2 receptor agonist pilocarpine inhibits cholinergic nerve-mediated contraction of isolated bronchi induced by EFS whilst having no effect on acetylcholine-induced contractions, and this response is blocked by the M_2 receptor antagonist gallamine (Minette & Barnes, 1988). Muscarinic receptors on airway smooth muscle may be of the M_3 subtype since they are sensitive to the M_3 receptor antagonists 4-DAMP and hexahydrosiladifenidol (Mak & Barnes, 1989a).

1.1.2.2. Sympathetic control

1.1.2.2.a. Innervation and function

The sympathetic innervation of the airways arises from the thoracic spinal cord, and nerve fibres synapse at extramural stellate ganglia. Postganglionic nerve fibres enter the lung and intermingle with parasympathetic fibres to form a plexus of fibres which ramifies throughout the airways as far as the bronchioles, and around blood vessels (Richardson, 1979). Preganglionic nerves are cholinergic, releasing acetylcholine as their neurotransmitter, whereas postganglionic nerves are adrenergic and release noradrenaline.

Sympathetic innervation of the airways can be demonstrated by fluorescence histochemistry. In most species catecholamine-containing fibres supply submucosal glands, bronchial arteries and parasympathetic ganglia, but a great variability in the sympathetic innervation of airway smooth muscle appears to occur between species (Richardson, 1979). Catecholamine-containing fibres innervate the smooth muscle of cat and guinea-pig airways (Silva & Ross, 1974; Doidge & Satchell, 1982) whereas in rat, rabbit, monkey and human airways sympathetic innervation is sparse or absent (Doidge & Satchell, 1982; El Bermani, 1978; Partanen *et al*, 1982).In guinea-pig airways a dense sympathetic innervation occurs in tracheal smooth muscle but is scant in bronchial smooth muscle (O'Donnell *et al.*, 1978). Ultrastructural studies on human airways have shown nerve endings containing small granular vesicles, which are considered to be adrenergic, in the proximity of smooth muscle (Laitinen *et al.*, 1985b). The number of adrenergic profiles however is low and represents only a small fraction of the fibres innervating human airway smooth muscle.

Sympathetic control of bronchomotor tone can be demonstrated in whole animals and isolated airway preparations. *In vivo,* electrical stimulation of thoracic sympathetic nerves in the presence of vagal bronchoconstriction, leads to a bronchodilatation which can be blocked by the beta-adrenoceptor antagonist propranolol (Cabezas *et al.*, 1971). This response remains

after adrenalectomy, indicating that it is not due to the release of catecholamines from the adrenal glands. EFS of guinea-pig tracheal smooth muscle causes a relaxation which can be abolished by TTX and partially blocked by propranolol, suggesting that it is partly due to the stimulation of adrenergic nerves (Farmer & Coleman, 1970; Coleman & Levy, 1974). EFS of guinea-pig bronchial smooth muscle, however only induces a contractile response (Grundstrom *et al.*, 1981b), and this agrees with the sparcity of sympathetic innervation in this tissue. EFS of human bronchial smooth muscle induces a relaxant response which is blocked by TTX but not by propranolol, suggesting that human airway smooth muscle does not possess a functional sympathetic innervation and that relaxation is mediated by other neural pathways (Richardson & Beland, 1976; Davis *et al*, 1982).

1.1.2.2.b. Circulating catecholamines

Catecholamines released by the adrenal glands into the circulation may be important in the control of airway smooth muscle tone. In humans, adrenaline is a potent bronchodilator (Warren & Dalton, 1983) but noradrenaline has no effect on airway function at physiological concentrations (Berkin *et al.*, 1985) suggesting that adrenaline, but not noradrenaline, may function as a circulating hormone. *In vitro* beta-adrenoceptor agonists potently relax human airway smooth muscle (Goldie *et al.*,

1982) despite the the sparcity of sympathetic innervation. In animals adrenalectomy and beta-adrenoceptors both potentiate bronchoconstrictor responses (Piper *et al.*, 1967; Collier & James, 1967) suggesting that circulating catecholamines may protect against bronchoconstrictor influences.

1.1.2.2.c. Receptors

Effector responses to sympathetic nerve stimulation and circulating catecholamines are mediated by alpha- and beta-adrenoceptors. Receptor binding studies show a high density of beta-receptors in lung of many species including humans (Rugg *et al.*, 1978; Barnes *et al.*, 1980b). Autoradiographic mapping studies demonstrate the presence of beta-receptors on airway smooth muscle, submucosal glands and airway epithelium, and show a decreasing density of receptors in peripheral airways (Barnes *et al.*, 1982a; Carstairs *et al.*, 1985). Beta-receptors may be subdivided into two subtypes, and functional studies on isolated airway smooth muscle suggest that receptors mediating relaxation induced by exogenous agonists are of the $beta_2$-subtype, whereas those mediating relaxation induced by sympathetic nerve stimulation are of the $beta_1$- subtype (Barnes *et al.*, 1983b). Thus $beta_1$-receptors are regulated by sympathetic

nerves ("innervated" beta-receptors) and $beta_2$-receptors are regulated by circulating adrenaline ("hormonal" beta-receptors) (Ariens & Simonis, 1983).

Alpha-adrenoceptors are also present in the lung although their density is low (Barnes *et al.*, 1979). Alpha-receptors mediate contraction of airway smooth muscle although responses can only be demonstrated under certain conditions. In canine airways alpha-receptor agonists or sympathetic nerve stimulation by EFS induce contraction only after pretreatment with histamine, 5-hydroxytryptamine or potassium chloride (Kneussl & Richardson, 1978; Barnes *et al.*, 1983c). Alpha-receptor agonists induce a contractile response in human airways that are diseased but not in normal airways (Kneussl & Richardson, 1978). Thus, alpha-receptors of animal and human airway smooth muscle appear to require prior activation or priming in order to produce a response. Alpha-receptors, like beta-receptors are subdivided into two types according to their selectivity for agonist and antagonists. Canine tracheal smooth muscle possesses a high density of $alpha_2$-receptors but a low density of $alpha_1$-receptors, and contractile responses induced by alpha-receptor agonists and sympathetic nerve stimulation are blocked by $alpha_2$-receptor antagonists (Barnes *et al.*,1982c).

1.1.2.3. Excitatory non-adrenergic non-cholinergic control

EFS of guinea-pig bronchial smooth muscle induces a cholinergic contraction followed by a slower longer-lasting contraction that is not sensitive to muscarinic or alpha-adrenoceptor antagonists (Grundstrom *et al.*, 1981b; Andersson & Grundstrom, 1983). This e-NANC component is blocked by TTX suggesting that it is neurogenic in nature. A variety of peptides have been localised to nerves supplying the airways, and of these the tachykinins (also called neurokinins) have received much attention as possible neurotransmitters for e-NANC nerves.

1.1.2.3.a. Innervation and function

The tachykinins comprise a group of bioactive peptides that share a common amino acid sequence at the C-terminal region. Substance P (SP), an 11 amino acid peptide, was discovered in equine brain and gut (Von Euler & Gaddum, 1931). Recently three other tachykinins, neurokinin A (NKA), neurokinin B (NKB) and neuropeptide K (NPK) were isolated from mammalian nervous tissue (Kimura *et al.*, 1983; Minamino *et al.*, 1984; Tatemoto *et al.*, 1985).

SP-immunoreactive nerves have been detected in the lungs of several mammalian species including guinea-pigs and humans, and are observed in close association with

airways and blood vessels (Nilsson *et al.*, 1977; Wharton *et al.*, 1979; Polak & Bloom, 1982). SP-immunoreactive nerves are found at all airway levels from the trachea to terminal bronchioles, and are seen in close contact with smooth muscle, submucosa, adventitia and epithelium.

SP-immunoreactive nerves supplying the lung are largely of vagal afferent origin. In the guinea-pig and rat SP-immunoreactivity can be detected in the vagus nerve, and SP-immunoreactive cell bodies are found in the nodose ganglia (Lundberg *et al.*, 1978; Terenghi *et al.*, 1983). SP is axonally transported down the cervical vagus nerve towards peripheral organs including the lung (Lundberg *et al.*, 1978; Gamse *et al.*, 1979a; Terenghi *et al.*, 1983). Vagal denervation and pretreatment with capsaicin both lead to a loss of SP-immunoreactivity from the trachea and bronchi of guinea-pigs (Lundberg *et al.*, 1983a). Capsaicin, which selectively degenerates sensory nonmyelinated C-fibres (Jancso *et al.*, 1977), has been shown to cause the release of SP from nonmyelinated sensory nerves (Gamse *et al.*, 1979b) and the long term depletion of SP from these nerves (Jessel *et al.*, 1978).

A small proportion of SP-immunoreactive nerves entering the lung may also be of spinal afferent origin, since stellatectomy in guinea-pigs leads to a reduction in SP-immunoreactivity in the lung (Saria *et al.*, 1985).

Capsaicin-sensitive nerves in the airways may contain other tachykinins as well as SP. Immunoreactivity to SP, NKA and NPK (but not NKB) has been detected in

capsaicin-sensitive nerves in guinea-pig and human lower airways (Hua *et al.*, 1985; Martling *et al.*, 1987). In isolated perfused guinea-pig lungs, capsaicin causes the release of SP and NKA into the effluent (Saria *et al.*, 1985).

The functional response to stimulation of e-NANC nerves can be demonstrated *in vivo* and *in vitro*, and can be shown to be mediated by capsaicin-sensitive sensory nerves. In guinea-pigs, a long-lasting and atropine-resistant bronchoconstriction can be induced by the antidromic stimulation of vagal afferent fibres, and by an intravenous infusion of SP or capsaicin (Nilsson *et al.*, 1977; Lundberg & Saria, 1982). E-NANC responses to vagal nerve stimulation, SP and capsaicin can all be reduced by a tachykinin receptor antagonist (Lundberg *et al.*, 1983c). Chronic pretreatment of animals with capsaicin, which depletes SP-immunoreactivity, reduces the bronchoconstrictor response to vagal nerve stimulation (Lundberg & Saria, 1982).

In vitro, the e-NANC response of guinea-pig bronchi to EFS is reduced by tachykinin receptor antagonists (Lundberg *et al.*, 1983c; Karlsson *et al.*, 1984; Leander *et al.*, 1984) and by pretreatment of animals with capsaicin (Lundberg & Saria, 1982). Exogenous SP causes contraction of airway smooth muscle (Nilsson *et al.*, 1977), and this response is inhibited by tachykinin-receptor antagonists (Lundberg *et al.*, 1983c). *In vitro* application of capsaicin to guinea-pig and human airways induces a

contractile response that becomes tachyphylactic, probably due to the release and subsequent depletion of SP and other tachykinins from nerve endings (Molnar *et al.*, 1969; Lundberg *et al.*, 1983b).

Capsaicin-sensitive sensory nerves mediating bronchoconstriction are thought to be part of a local axon reflex (Lundberg & Saria, 1982). Activation of chemosensitive epithelial irritant receptors by capsaicin leads to the retrograde release of sensory neuropeptides from collaterals, which mediate bronchoconstriction and increases in submucosal gland secretion and microvascular permeability. In inflammatory conditions such as asthma, this axon reflex mechanism may be exaggerated due to the exposure of sensory nerve endings following epithelial damage (Laitinen *et al.*, 1985a) and the subsequent activation of such endings by inflammatory mediators such as bradykinin (Barnes, 1986).

1.1.2.3.b. Receptors

The classification of tachykinin receptors has been based on receptor binding studies and functional assays for agonists and antagonists. Three tachykinin receptor subtypes have been identified (Lee *et al.*, 1982; Buck *et al.*, 1984; Burcher *et al.*, 1986; Regoli *et al.*, 1987):

1) the NK1 receptor, with a rank order of potency for the natural agonists of SP > NKA > NKB,

2) the NK2 receptor, with a rank order of agonist potencies of NKA > NKB > SP, and

3) the NK3 receptor, with a rank order of agonist potencies of NKB > NKA > SP.

The rank order of potency of tachykinins on airway smooth muscle corresponds to the NK2-receptor classification although studies using selective agonists suggest the presence of NK1- and NK2-receptors on guinea-pig tracheal smooth muscle (Dion *et al.*, 1987; Drapeau *et al.*, 1987). Recently, the existence of a fourth type of tachykinin receptor, the NK4-receptor, has been proposed and is suggested to be present on guinea-pig tracheal smooth muscle (McKnight *et al.*, 1987 & 1988). In human bronchial smooth muscle, responses to tachykinins appear to be mediated exclusively by NK2-receptors (Advenier *et al.*, 1987).

1.1.2.4. Inhibitory nonadrenergic noncholinergic control

1.1.2.4.a. Innervation and function

The existence of an inhibitory innervation to airway smooth muscle in addition to the adrenergic nervous system has been demonstrated in isolated airway preparations and in whole animals. In the presence of atropine and propranolol, EFS of isolated airway smooth muscle from several species, including guinea-pigs and humans, elicits a TTX-sensitive relaxation (Coburn & Tomita, 1973; Coleman

& Levy, 1974; Richardson & Beland, 1976). In the presence of propranolol, nicotine elicits a relaxation of guinea-pig trachea which can be prevented by TTX and hexamethonium suggesting that it is mediated by the activation of nicotinic receptors on postganglionic efferent nerves (Jones *et al.*, 1980). In cats and guinea-pigs, sustained electrical stimulation of the vagus nerve in the presence of atropine and propranolol induces bronchodilatation, suggesting that i-NANC responses may be mediated by vagal pathways (Chesrown *et al.*, 1980; Diamond & O'Donnell, 1980; Irvin *et al.*, 1980). However, in an isolated innervated guinea-pig tracheal preparation, electrical stimulation of the vagus nerve does not produce an i-NANC response suggesting that in this preparation vagal pathways are not involved in i-NANC responses (Blackman & McCaig, 1983).

I-NANC responses obtained *in vivo* and *in vitro* can be demonstrated at several airway levels, although species variations exist. Using tantalum bronchography, bronchodilatation in cats in response to vagal nerve stimulation occurs at all airway levels down to small bronchioles and is maximal in airways of an intermediate size (Matsumoto *et al.*, 1985). In isolated guinea-pig airways i-NANC responses can only be demonstrated in the trachea, with EFS of bronchi inducing only contractile responses (Grundstrom *et al.*, 1981b). It is possible,

however, that i-NANC responses may still be present in guinea-pig bronchi but are masked by e-NANC responses to EFS.

1.1.2.4.b. Possible neurotransmitters

The existence of an i-NANC innervation to airway smooth muscle is well established but there has been considerable controversy as to the identity of the neurotransmitter mediating i-NANC responses. Smooth muscle of the gastrointestinal tract is supplied by intramural nerves whose terminals store and release purine nucleotides such as ATP (Burnstock, 1972). Since airway smooth muscle has the same embryological origin as smooth muscle of the gut, it has been suggested that purine nucleotides may be the neurotransmitters released by i-NANC nerves. Guinea-pig tracheal smooth muscle is relaxed by adenosine, adenosine monophosphate, adenosine diphosphate and adenosine triphosphate (Coleman & Levy, 1974; Richardson & Bouchard, 1975; Satchell, 1982) and relaxant responses to purines mimic the relaxation induced by stimulation of i-NANC nerves by EFS. Dipyridamole, which blocks adenosine uptake, potentiates relaxation of guinea-pig trachea to purines and i-NANC nerve stimulation (Coleman, 1976). Furthermore, adenosine deaminase, which degrades adenosine, reduces depressor responses to adenosine and i-NANC nerve stimulation in an isolated guinea-pig tracheal tube preparation (Satchell, 1984).

Considerable evidence now exists which refutes a role for purine nucleotides as neurotransmitters for i-NANC nerves in the airways. Dipyridamole does not potentiate i-NANC relaxation in human, cat and bovine airways (Davis *et al.*, 1982; Ito & Takeda, 1982; Cameron *et al.*, 1983), and contrary to previous findings, adenosine deaminase has been shown not to reduce i-NANC responses of guinea-pig tracheal smooth muscle (Ellis & Farmer, 1989a). Furthermore, i-NANC responses persist in the presence of purine receptor antagonists (Davis *et al.*, 1982; Cameron *et al.*, 1983), after desensitisation to purines (Ito & Takeda, 1982) and in the presence of a maximal relaxation to adenosine (Karlsson & Persson, 1984).

The presence of a variety of peptides in the lung has led to the suggestion that i-NANC responses may be mediated by "peptidergic" nerves. Increasing evidence now suggests that vasoactive intestinal peptide (VIP), a 28 amino acid peptide, and/or the related peptide histidine isoleucine (PHI) may mediate i-NANC responses in the airways. This evidence can be summarised as follows:

1) VIP-immunoreactive nerves and nerve terminals are present in the lung of several species and are found in close association with tracheal and intrapulmonary airway smooth muscle (Dey *et al.*, 1981; Polak & Bloom, 1982). PHI coexists with VIP in airway nerves (Lundberg *et al.*, 1984b).

2) VIP potently relaxes isolated airway smooth muscle of several species (Said *et al.*, 1974; Wasserman *et al.*, 1982) and mimics the relaxant effect of i-NANC nerve stimulation by EFS (Matsuzaki *et al.*, 1980; Cameron *et al.*, 1983). PHI also relaxes guinea-pig tracheal smooth muscle although it is less potent than VIP (Christofides *et al.*, 1984).

3) VIP is released from airway tissues during EFS and this effect is blocked by TTX (Matsuzaki *et al.*, 1980; Cameron *et al.*, 1983).

4) Desensitisation of airway smooth muscle to VIP reduces i-NANC responses to EFS (Ito & Takeda, 1982; Venugopalan *et al.*, 1984).

5) Pretreatment of guinea-pig trachea with antisera to VIP and PHI leads to a reduction of i-NANC responses to EFS (Matsuzaki *et al.*, 1980; Ellis & Farmer, 1989a).

6) The peptidases alpha-chymotrypsin and papain reduce i-NANC responses to EFS and abolish relaxant responses to VIP and PHI in the guinea-pig trachea (Ellis & Farmer, 1989b). The persistence of a small i-NANC response in the presence of peptidases in this study may also suggest the release of a non-peptide transmitter in addition to VIP and/or PHI.

Evidence against a role for VIP in mediating i-NANC responses also exists. I-NANC relaxation of guinea-pig trachea persists in the presence of a maximally effective concentration of exogenous VIP (Karlsson & Persson, 1984). In a similar study, i-NANC responses were diminished in

the presence of a maximally effective concentration of VIP (Ellis & Farmer, 1989a), suggesting that while VIP may partly account for i-NANC responses other transmitters may be released by i-NANC nerves. In cat airways *alpha*-chymotrypsin abolishes relaxant responses to VIP but has no effect on i-NANC responses to EFS, suggesting that in this species VIP may not be the i-NANC neurotransmitter (Altiere & Diamond, 1985).

Further confirmation of a role for VIP in mediating i-NANC responses has been hindered by the unavailability of VIP-receptor antagonists. Two compounds, a growth hormone releasing factor analog and a VIP analog, have been shown to be competitive VIP receptor antagonists in intestinal and pancreatic cells (Waelbroek *et al.*, 1985; Pandol *et al.*, 1986), but neither agent is effective against VIP or PHI induced relaxation in guinea-pig trachea, suggesting that VIP receptors on airway smooth muscle may differ from those on intestinal and pancreatic cell (Ellis & Farmer, 1989a).

1.2. Modulation of airway smooth muscle responses

1.2.1. Modulation of cholinergic neurotransmission

Cholinergic neurotransmission at airway ganglia or at the neuroeffector junction of airway smooth muscle may be modulated by a variety of agents acting on preganglionic or prejunctional receptors located on cholinergic nerve terminals. Activation of such receptors leads to an increase or decrease in the output of acetylcholine from cholinergic nerve terminals and therefore serves to provide fine adjustments in neurotransmission required under varying physiological conditions. Neuromodulators may be other neurotransmitters released by nerve terminals located in the vicinity of cholinergic nerves (for example noradrenaline released by sympathetic nerves), may be released from non-neural elements in the airways (for example histamine released by mast cells) or may diffuse from the circulation (for example catecholamines released by the adrenal glands). In addition, acetylcholine may inhibit its own release by acting on preganglionic and prejunctional "autoreceptors."

The presence of catecholamine-containing nerve terminals in airway parasympathetic ganglia (Jacobowitz *et al.*, 1973; Richardson & Ferguson, 1979) has led to the suggestion that noradrenaline released by sympathetic nerves may modulate cholinergic neurotransmission at airway ganglia. In ferret paratracheal ganglia, exogenous noradrenaline decreases the amplitude of excitatory

postsynaptic potentials evoked by electrical stimulation of interganglionic nerve fibres, and this effect can be blocked by an *alpha*-receptor antagonist (Baker *et al.*, 1983). Contractions of ferret tracheal smooth muscle induced preganglionically by the electrical stimulation of the vagus are inhibited by noradrenaline and isoprenaline to a greater degree than are contractions induced postganglionically by EFS (Skoogh 1983 & 1986; Skoogh & Svedmyr, 1989). The response to noradrenaline is inhibited by an *alpha*-receptor antagonist whereas the response to isoprenaline is inhibited by a selective $beta_2$-receptor antagonist, suggesting that cholinergic neurotransmission at airway ganglia may be inhibited preganglionically by both *alpha*- and $beta_2$-receptors. Circulating adrenaline may modulate cholinergic neurotransmission at ganglia by acting preferentially on $beta_2$-receptors.

Cholinergic neurotransmission at the neuroeffector junction of airway smooth muscle may also be modulated by catecholamines. In guinea-pig and human tracheal smooth muscle, adrenergic fibres are sometimes found in close association with cholinergic fibres (Jones *et al.*, 1980; Daniel *et al.*, 1986). Indirect evidence for modulation of neurotransmission is provided by studies which compare cholinergic contractions induced by EFS to contractions induced by exogenous acetylcholine. Using this approach catecholamines have been shown to inhibit cholinergic neurotransmission in guinea-pig and human airway smooth

muscle via prejunctional *alpha*$_2$-receptors (Grundstrom *et al.*, 1981a; Grundstrom & Andersson 1985) and in canine airway smooth muscle via prejunctional *beta*-receptors (Vermeire & Vanhoutte, 1979).

Modulation of cholinergic neurotransmission can be demonstrated in intact animals. In cats & dogs sympathetic nerve stimulation inhibits the bronchoconstrictor effect of vagal nerve stimulation with little or no effect on acetylcholine-induced tone (Cabezas *et al.*, 1971; Baker & Don, 1987). A similar effect can be produced by intravenous adrenaline suggesting that circulating catecholamines may also modulate neural responses *in vivo*. (Baker & Don, 1987).

1.2.2. Modulation of airway smooth muscle responses by the epithelium

Mechanical removal of airway epithelium has been shown to alter the reactivity of airway smooth muscle to a variety of agonists *in vitro* (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Goldie *et al.*, 1986). Asthma is associated with epithelial cell damage or loss (Laitinen *et al.*, 1985a) and it has been suggested that the loss of the modulatory influence of the epithelium may contribute to bronchial hyperreactivity in asthma (Hogg & Eggleston, 1984).

Epithelium removal increases the sensitivity of isolated airway smooth muscle to contractile agents such as muscarinic agonists, histamine and 5-hydroxytryptamine, but has no effect on the sensitivity to KCl (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Goldie *et al.*, 1986). Maximal contractile responses to these agents are unchanged or increased. Epithelium removal also increases the sensitivity of airway smooth muscle to some relaxant agents such as isoprenaline, adenosine and sodium nitroprusside, but not to others such as La^{+} and papaverine (Holroyde, 1986; Farmer *et al.*, 1986). Epithelium removal may also modulate neurogenic responses of airway smooth muscle. Cholinergic contractile responses to EFS are potentiated (Flavahan *et al.*, 1986; Murlas, 1986) or unaffected (Holroyde, 1986) by epithelium removal.

The mechanism by which the epithelium modulates airway smooth muscle reactivity *in vitro* is uncertain. The epithelium has been suggested to act as a barrier to diffusion preventing access of bronchoactive molecules to the underlying smooth muscle (Holroyde, 1986). This is unlikely since the sensitivity of airway smooth muscle to several relaxant agents is decreased, and not, as expected from the diffusion barrier hypothesis, increased. Furthermore, removal of a diffusion barrier would not be expected to alter neural responses to EFS as these are produced by the local release of neurotransmitter from nerve terminals in the smooth muscle layer.

The epithelium may represent a site of uptake or metabolism of bronchoactive molecules reducing their effective concentration at the smooth muscle layer. Corticosterone, a catecholamine uptake inhibitor, abolishes the inhibitory influence of epithelium on responses of guinea-pig tracheal smooth muscle to isoprenaline, suggesting that the epithelium may contain sites of catecholamine uptake (Farmer *et al.*, 1986). Thus the effect of epithelium removal on smooth muscle responses to catecholamines at least can explained by the loss of sites of uptake. The effect of epithelium removal on responses of canine ASM to acetylcholine persists in the presence of AChE inhibitors, suggesting that metabolism does not account for the modulatory effect of epithelium on reactivity to acetylcholine (Flavahan *et al.*, 1985). Airway epithelium contains high levels of peptidases such as neutral endopeptidase (Sekizawa *et al.*, 1987b), therefore it is possible that responses of ASM to bronchoactive peptides may be modified by their metabolism at epithelial sites.

A further mechanism by which airway epithelium may modulate smooth muscle reactivity may be through the release of an inhibitory factor. Superfusion cascade experiments on guinea-pig trachea failed to demonstrate the release of a relaxant factor from airway epithelium (Holroyde, 1986). However, in a modified "sandwich" preparation consisting of an "acceptor" tracheal strip surrounded by a "donor" tracheal ring, the release of an

inhibitor factor has been demonstrated in response to ovalbumin in preparations from ovalbumin-sensitised guinea-pigs (Hay *et al.*, 1987b).

The identity of the inhibitory factor released by the epithelium is unknown, but some studies have suggested that it may be a cyclooxygenase product. Indomethacin has been reported to mimic the effect of epithelial removal on ASM responses to constrictor agents (Flavahan *et al.*, 1985; Hay *et al.*, 1986; Butler *et al.*, 1987). Cyclooxygenase has been localised to airway epithelium (Xu *et al.*, 1986; Butler *et al.*, 1987) and cyclooxygenase products, particularly PGE_2, are released by cultured airway epithelial cells (Leikauf *et al.*, 1985). Other studies however have demonstrated that the effect of epithelium removal on ASM responses is still evident in the presence of indomethacin (Barnes *et al.*, 1985; Holroyde, 1986) suggesting that whilst cyclooxygenase products may contribute to the modulatory effect of epithelium in some preparations, other inhibitory factors may also be released by the epithelium.

1.2.3. Modulation by neuropeptide metabolism

Endogenous peptides may be degraded in the lung by a variety of peptidases and recently much interest has focused on the possible modulation of airway function by such peptidases. Peptides released by nerve terminals may be inactivated or may be converted to forms with different

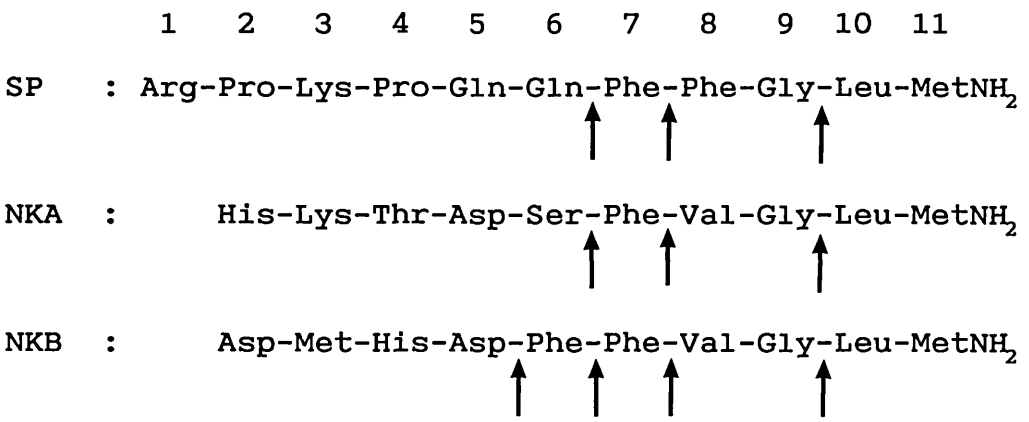
biological actions. Thus, the localisation and selectivity of peptidases in the lung may be important factors in achieving a balance between the excitatory and inhibitory influences of neuropeptides on airway function.

1.2.3.1. Neutral endopeptidase (NEP)

NEP (endopeptidase-24.11 or "enkephalinase") was first localised to the renal brush border (Kerr & Kenny, 1974) where it exists as a membrane-bound metalloendopeptidase. NEP is now known to be widely distributed in the body and high levels are found in the lung (Llorenz & Schwartz, 1981; Johnson *et al.*, 1985). In ferret airways NEP-immunofluorescence is found in the smooth muscle, interstitium and epithelium (Sekizawa *et al.*, 1987b).

NEP hydrolyses peptide bonds involving the amino groups of hydrophobic residues. In the brain NEP is the major inactivator of enkephalins, but *in vitro* studies on tissue homogenates or purified enzyme preparations reveal that NEP may hydrolyse a variety of peptides including SP (Matsas *et al.*, 1983; Skidgel *et al.*, 1984), NKA (Hooper *et al.*, 1985), NKB (Hooper & Turner, 1985), bradykinin (Gafford *et al.*, 1983), cholecystokinin (Droschodt-Lanckman & Strosberg, 1983) and others. NEP hydrolyses tachykinins at several hydrophobic bonds and the resultant peptide fragments are devoid of biological activity:

NEP hydrolysis (↑) of tachykinins:



A role for endogenous NEP in modulating the biological effects of neuropeptides is suggested by the use of selective NEP-inhibitors. *In vitro*, NEP-inhibitors potentiate responses of guinea-pig and ferret ASM to tachykinins (Stimler-Gerard, 1987; Sekizawa *et al.*, 1987a & 1987b), bradykinin (Dusser *et al.*, 1988a), cholecystokinin (Stretton & Barnes, 1989) and VIP (Liu *et al.*, 1987). *In vivo*, NEP-inhibitors potentiate the bronchoconstrictor response of guinea-pigs to SP, capsaicin and the e-NANC response to vagal nerve stimulation (Shore *et al.*, 1988; Dusser *et al.*, 1988b; Thompson & Sheppard, 1988). Inhalation exposure of guinea-pigs to toluene diisocyanate increases the bronchoconstrictor response to tachykinins and decreases tracheal NEP-activity, suggesting that inhibition of NEP may result in ASM hyperresponsiveness to tachykinins (Sheppard *et al.*, 1988).

1.2.3.2. Other peptidases

Angiotensin-converting enzyme (ACE) or peptidyl dipeptidase A is a membrane-bound metalloenzyme located primarily to vascular endothelium where it catalyses the hydrolysis of angiotensin I to angiotensin II (Erdos, 1979). ACE has also been shown to hydrolyse bradykinin (Erdos & Yang, 1967) and SP (Skidgel *et al.*, 1984), but not NKA (Hooper *et al.*, 1985) or NKB (Hooper & Turner, 1985). ACE-activity and immunoreactivity has also been detected in isolated airways (Johnson *et al.*, 1985; Dusser *et al.*, 1988a), and ACE inhibitors potentiate bradykinin-induced contractions of ferret ASM *in vitro* (Dusser *et al.*, 1988a) and SP-induced bronchoconstriction in guinea-pigs *in vivo* (Shore *et al.*, 1988), suggesting that endogenous ACE may modulate responses of ASM to bronchoactive peptides.

Mast cell activation is associated with the secretion of trypsin and chymotrypsin, and both enzymes have been implicated in the degradation of VIP (Caughey *et al.*, 1988). VIP-induced relaxation of ASM is prevented by chymotrypsin (Altiere & Diamond, 1985; Ellis & Farmer, 1989b) and reversed by mast cell supernatant containing trypsin and chymotrypsin (Franconi *et al.*, 1989). SP is also degraded by mast cell chymotrypsin but not by trypsin suggesting that a differential inactivation of excitatory

and inhibitory neuropeptides by mast cell proteases may contribute to bronchial hyperreactivity during inflammatory states (Caughey *et al.*, 1988).

In cell-free systems SP is also metabolised by acid proteases (Hanson *et al.*, 1980) and by AChE (Chubb *et al.*, 1980). SP-induced cholinergic contractions of ferret trachea, however, are not modified by leupeptin suggesting that acid proteases do not metabolise SP in the airways (Sekizawa *et al.*, 1987a). Similarly, AChE inhibitors do not potentiate tachykinin-induced contraction of ferret and guinea-pig airways *in vitro* (Stimler-Gerard, 1987; Sekizawa *et al.*, 1987b) suggesting that although AChE may be localised to the airways it may not represent an important pathway for the metabolism of tachykinins.

2. Materials and methods

2.1. Tissue preparation

2.1.1. Animal tissue

Male Dunkin-Hartley guinea-pigs (Charles Rivers Breeding Laboratories, UK) weighing 250-500 g were used throughout. Animals were housed in a temperature controlled room (21°C) with water and food freely available, and were used within 1 week of their arrival at the animal house.

Guinea-pigs were killed by cervical dislocation. The trachea and lungs were excised and placed in ice-cold oxygenated Krebs-Henseleit (KH) solution. The trachea was cleared of connective tissue and blood vessels, and was opened longitudinally by cutting through the cartilage opposite the smooth muscle layer. The trachea was cut into eight transverse segments, each comprising 3-4 opened cartilaginous rings. When necessary, alternate segments were gently rubbed with a cotton-wool probe in order to remove the epithelium. The effectiveness of this procedure was ascertained by histological examination of 5 um sections fixed in 10 % phosphate-buffered formaldehyde and stained with haematoxylin-eosin.

Guinea-pig lungs were cleared of parenchyma to expose the bronchi. Four bronchial rings were dissected, two from main bronchi and two from hilar bronchi.

2.1.2. Human tissue

Human airways were obtained from patients undergoing surgery for carcinoma of the lung. Tissue was obtained from 51 patients (43 male, age range 39-79 years; 8 female, age range 59-74 years) with a mean age of 62.0 $\pm$ 1.7 years.

Human lung was collected from surgery and placed in ice-cold oxygenated KH solution. Third order bronchi located away from the carcinoma were cleared of parenchyma and blood vessels, and were excised. Airways were either used immediately or were stored overnight in oxygenated ice-cold KH solution. Bronchi were cut into rings of 3-4 mm length. When epithelial denudation was required, a cotton wool probe was gently pulled through alternate bronchial rings.

2.2. Isometric measurement of tension

Airways were mounted in 10 ml glass organ baths containing KH solution bubbled with 95% O_2 and 5% CO_2, pH 7.4, and maintained at 37°C. Organ baths were coated with the siliconizing agent Surfasil (Pierce Chemical Co., Illinois, USA) to prevent adherence of peptides to glass. Tissues were fixed at one end to a tissue support and connected at the other end to isometric Grass FT.03 transducers (Grass Instruments, Quincy, MA, USA) via silk thread. Changes in tension were recorded on a Grass 7D

Polygraph. Tissues were placed under a tension which was found to be optimal for measurement of changes in tension. Initial tension was set at 2g for human bronchi and guinea-pig trachea, 500 ug for guinea-pig main bronchi and 300 ug for guinea-pig hilar bronchi. Tissues were allowed to equilibrate for 1 hr during which time they were washed 3-4 times with KH solution and resting tension readjusted to the optimal level.

2.3. Concentration-response (C-R) curves

C-R curves to exogenous agonists were constructed by adding increasing concentrations of agonist to the baths and allowing responses to plateau between successive additions. When no response was obtained a period of 5 min was allowed between successive additions of agonist. After the completion of each C-R curve, tissues were washed with KH solution and tension allowed to return to the baseline level. The effects of drugs on C-R curves was assessed either by (i) constructing a control C-R curve, incubating tissues with the relevant drug or solvent for a specified period of time and constructing a second C-R curve, or by (ii) incubating parallel preparations from the same animal/patient with the relevant drug or solvent and constructing only one C-R curve. Where appropriate tissues were maximally contracted with 10 uM carbachol or maximally relaxed with 0.1 mM papaverine added to organ baths at the end of each experiment.

2.4. EFS

EFS was delivered by two parallel platinum wires placed on either side of tissues, approximately 1 cm apart. Electrodes were connected to a Grass S88 stimulator and a unity voltage gain amplifier/inverter (Royal Postgraduate Medical School, UK) for the delivery of biphasic square-wave impulses. Trains of impulses were delivered for a period of time which allowed maximal responses to be developed. After each application of EFS, tension was allowed to return to the baseline level before EFS was re-applied. Optimum parameters for nerve stimulation were determined by constructing stimulus-responses curves over a range of voltages, pulse durations and frequencies. The neural nature of responses to EFS was ascertained by incubating tissues for 30 min with 3 uM TTX and repeating EFS.

2.5. Data analysis

Contractile responses to exogenous agonists and EFS were expressed as (i) absolute changes in tension measured in mg, (ii) % of maximal response obtained in each C-R curve or EFS stimulus response curve, or (iii) % of maximal response to 10 uM carbachol ($carb_{max}$) induced at the end of the experiment. Relaxant responses to exogenous agonists and EFS were expressed as (i) absolute changes in tension

measured in mg, (ii) % of maximal response to 0.1 mM papaverine induced at the end of the experiment, or (iii) % reversal of tone induced by precontracting agent.

C-R curves and EFS stimulus-response curves from each preparation (or where this was not possible from meaned results) were fitted to sigmoid curves using GraphPAD (ISI Software, Philadelphia, Mass., USA). Concentrations inducing 50 % of maximal response to each agonist (EC_{50}s for excitatory agonists or IC_{50}s for inhibitory agonists), concentrations inducing 30 % of maximal carbachol-induced contraction (EC_{30}), EFS frequencies inducing 50 % of maximal response to EFS (EF_{50}s) and EFS frequencies inducing 35 % reversal of KCl-induced tone (EF_{35}s) were determined by interpolation of the linear portion of sigmoid curves fitted to results from individual preparations or to meaned results. EC_{50}s were expressed as pD_2s (-log EC_{50}).

Results were compared by Student's t-test for paired and unpaired observations where appropriate. Observations were considered to be paired when control and test responses were obtained on the same preparation. Probability levels (p) less than 0.05 were considered to be significant. Unless otherwise indicated the number of preparations used in each group (n) was equal to the number of animals or subjects from which preparations were obtained.

2.6. Drugs and solutions

The following drugs were used: acetylcholine chloride, atropine sulphate, (-)-adrenaline, carbamyl choline (carbachol), corticosterone, hexamethonium chloride, indomethacin, (-)-isoprenaline bitartrate, methylene blue, N-ethylmaleimide, (-)-noradrenaline bitartrate, phosphoramidon (N-(alpha-rhamno-pyrano-xyloxy-hydroxy-phosphinyl)-L-leucyl-tryptophan, NH_4 salt), (±)-propranolol hydrochloride, sodium nitroprusside, SP, tetrodotoxin, tyramine hydrochloride and VIP (porcine synthetic), purchased from Sigma Chemical Co., Poole, UK; NKA and NKB, purchased from Sigma Chemical Co. and Bachem, Saffron-Waldon, UK; betaxolol, donated by Synthelabo, Paris, France; ICI 118551, donated by Imperial Chemical Industries, Macclesfield, UK; [Sar^9, $Met(O_2)^{11}$]SP, [Nle^{10}]NKA(4-10) and senktide, purchased from Cambridge Research Biochemicals, Cambridge, UK; L-659837 (cyclo(GlnTrpPhe(R)Gly[ANC-2]LeuMet)), L-659874 (Ac-LeuMet GlnTrpPhe.NH_2), L-659877 (cyclo(GlnTrpPheGlyLeuMet) and L-663109 (GlpPhePhe[ANC-2]GlyLeuMetNH_2), donated by Merk, Sharp & Dome, UK; SK&F 94120, donated by Smith, Kline & French, Welwyn, UK; zaprinast donated by May & Baker, Dagenham, UK.

Adrenaline, isoprenaline and noradrenaline were dissolved in 0.1 mg/ml ascorbic acid. Corticosterone was dissolved in ethanol. Indomethacin was dissolved in phosphate buffer, 0.02 M KH_2PO_4 and 0.12 M Na_2HPO_4 pH 7.8,

by heating to 40°C for 30 min. NKB was dissolved in 50 % sulpholane (tetrahydrothiophen-1, 1-dioxide). [Nle^{10}] NKA(4-10), L-659877, L-659874, L659837 and L-663109 were dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co.). SK&F 94120 and zaprinast were dissolved in 0.1 M NaOH. All other drugs were dissolved in distilled water. Peptides were dissolved, aliquoted into appropriate volumes and stored at -20 or -80°C until use. Other drugs were dissolved freshly on the day of use. Stock solutions of all drugs were prepared freshly and were diluted to appropriate concentrations in Krebs-Henseleit (KH) solution.

The composition of KH solution (in mM) was as follows: NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, $NaH_2PO_4.2H_2O$ 1.2, $NaHCO_3$ 25.5 and glucose 11.

3. Modulation of cholinergic neurotransmission in human airway smooth muscle by catecholamines

3.1. Aim

The present study was designed to investigate whether cholinergic neurotransmission in human airway smooth muscle may be modulated by catecholamines. Modulation by a prejunctional mechanism was investigated by comparing the effects of exogenous catecholamines on contractions of isolated human bronchi induced by exogenous and endogenous acetylcholine. The release of endogenous acetylcholine from cholinergic nerve terminals was evoked by EFS. The receptor type mediating modulation of cholinergic neurotransmission was determined with the use of selective adrenoceptor antagonists. The effect of tyramine on cholinergic responses of human bronchi was also investigated in order to determine whether noradrenaline released by any intrinsic adrenergic nerves may also modulate cholinergic neurotransmission.

3.2. Protocol

3.2.1. EFS parameters

Cholinergic nerve-mediated contractile responses were induced by EFS in human bronchi. EFS was applied for 15 s at intervals of 4 min. Optimum parameters for the stimulation of cholinergic nerves were determined by constructing stimulus-response curves over a pulse duration range of 0.01-1 ms, a voltage range of 2.5-60 V and a frequency range of 0.5-64 Hz. Tissues were incubated for 30 min with 3 uM TTX and stimulus-response curves repeated. For all subsequent experiments a pulse duration of 1 ms, voltage of 40 V and frequency range of 4-32 Hz was used.

The effect of 1 uM atropine and 1 uM hexamethonium was assessed on contractile responses to repeated applications of 32 Hz EFS.

3.2.2. Catecholamines

Preliminary experiments were carried out to determine the time course for the inhibitory effect of isoprenaline on EFS-induced contractions. Tissues were field stimulated every 4 min at 32 Hz for 10-15 min. When stable responses were obtained, 1 uM isoprenaline was added and EFS continued for 1 hr.

Control EFS frequency-response curves and acetylcholine C-R curves were constructed in separate preparations. Tissues were washed with KH solution and stimulus-response curves repeated following incubation with increasing concentrations (0.001-1000 uM) of isoprenaline, adrenaline or noradrenaline for 10 min. Only one catecholamine was studied on each tissue. Consecutive stimulus-response curves were constructed in adjacent bronchial rings in the absence of catecholamines to assess the reproducibility of EFS frequency-response and acetylcholine C-R curves with time.

In separate experiments tissues were field stimulated every 4 min at 32 Hz. When stable responses were obtained, tissues were incubated for 20 min with 1 uM phentolamine, 1uM propranolol, 0.1 uM betaxolol (a $beta_1$-selective antagonist) or 0.1 uM ICI 118551 (a $beta_2$-selective antagonist), and then 1 uM isoprenaline was added. Time-control experiments were carried out in parallel tissues from the same patient in the absence of any antagonist.

To investigate the effect of tyramine on EFS-induced contractions tissues were field stimulated every 4 min at 32 Hz, and after a control period, 100 uM tyramine was added.

3.3. Results

3.3.1. EFS parameters

EFS of human bronchi induced a rapid and transient contraction followed by a variable longer-lasting relaxation. The contractile response to EFS was dependent on the pulse duration, voltage and frequency of stimulation (figure 1). TTX (3uM) abolished contractile responses to EFS of pulse durations up to 0.2 ms, voltages up to 20 V and frequencies up to 16 Hz. EFS of greater pulse duration, voltage and frequency induces small TTX-resistant contractions. Optimal stimulus parameters were selected from stimulus-response curves. Hence, EFS with a pulse duration of 1 ms, voltage of 40 V and frequency of 32 Hz induces a maximal contractile response with a minimal TTX-resistant component (approximately 5%).

Atropine (1 uM) inhibited contractions induced by EFS (1 ms, 40 V, 32 Hz), with responses being reduced to 4.7 $\pm$ 3.0 % of their control level (n=6). Hexamethonium (5 uM) had no effect on similar contractions induced by EFS, with responses being 101.7 $\pm$ 6.8 % of their control level (n=6).

3.3.2. Control responses to EFS and acetylcholine

EFS (1.0 ms, 40 V, 4-32 Hz) and acetylcholine (1-300 uM) induced frequency- and concentration-dependent

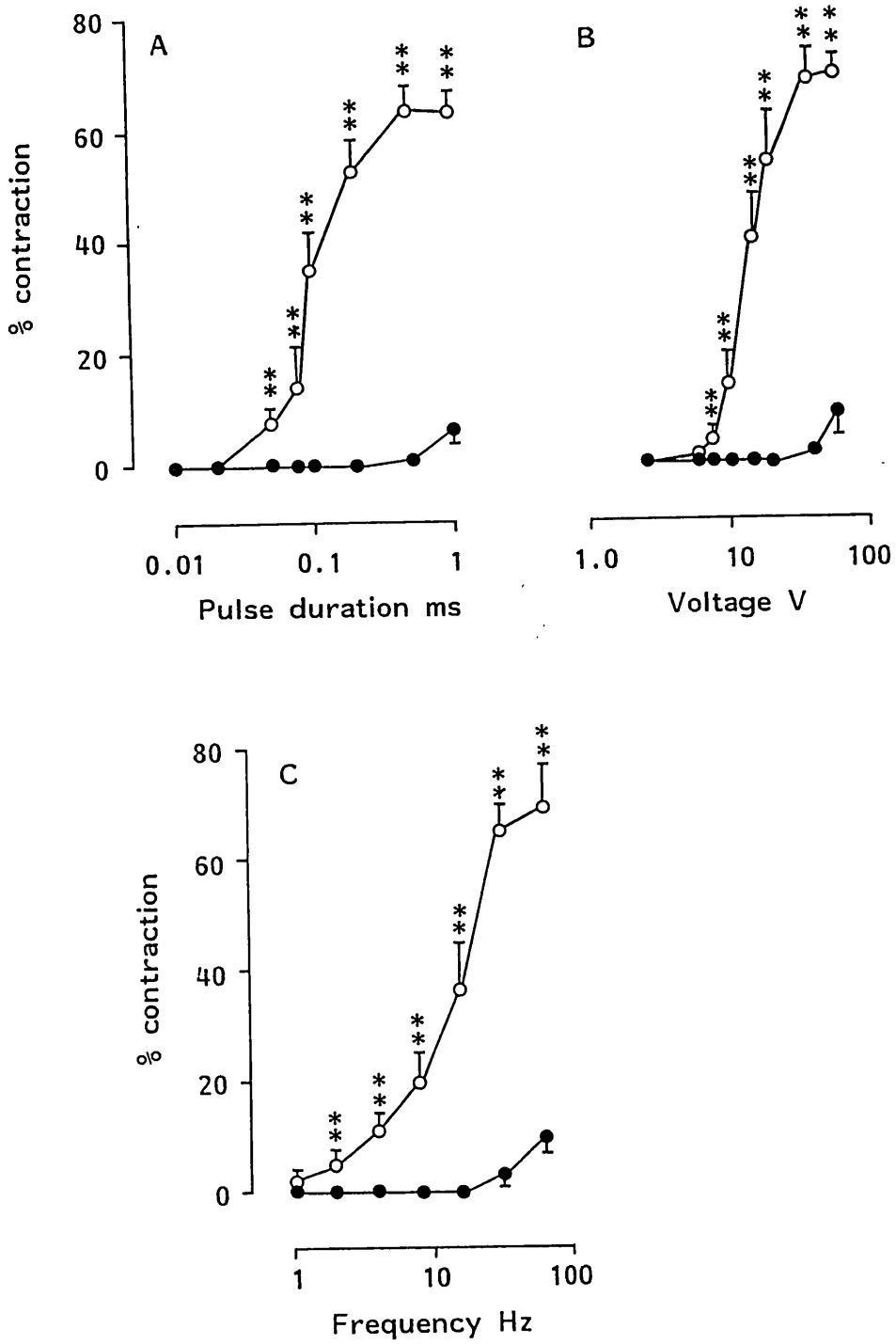


Figure 1. EFS parameters for the stimulation of cholinergic nerves in human bronchi. (a) Effect of pulse duration on contraction induced by EFS (40 V, 32Hz). (b) Effect of voltage on contraction induced by EFS (1.0 ms, 32 Hz). (c) Effect of frequency on contraction induced by EFS (40 V, 1.0 ms). Responses were obtained in the absence (O) and presence (●) of 3 uM TTX. Points represent means ± SE of n=6, ** $p<0.01$ absence vs. presence of TTX.

contractions of human bronchi, respectively (figure 2). EFS with frequencies of 4, 8, 16 and 32 Hz evoked contractions (in mg) which were comparable in magnitude to contractions evoked by 10, 30, 100 and 300 uM acetylcholine, respectively (table 1).

Consecutive EFS frequency-response curves and acetylcholine C-R curves were reproducible. Table 2 demonstrates contractile responses induced by 32 Hz EFS and 300 uM acetylcholine as part of 6 consecutive stimulus-response curves. Responses to EFS were similar for all 6 frequency-response curves. Responses to acetylcholine were similar for the first 4 C-R curves but were significantly reduced in the 5th and 6th curve. For subsequent experiments, a maximum of 6 EFS consecutive frequency-response curves and 4 consecutive acetylcholine C-R curves were used.

3.3.3. Time course

In preliminary experiments on bronchial rings from 4 patients, isoprenaline (1 uM) caused a 54.7 $\pm$ 8.7 % reduction in baseline tone (equivalent to 1144 $\pm$ 139 mg) and an 83.8 $\pm$ 6.1 % reduction in the contractile response to EFS (1.0 ms, 40 V, 32 Hz) (figure 3). Inhibition of EFS-induced contraction was maximal 6-10 min after the addition of isoprenaline and was maintained for approximately 30 min. After this time, inhibition was gradually reversed. In subsequent experiments, the effects

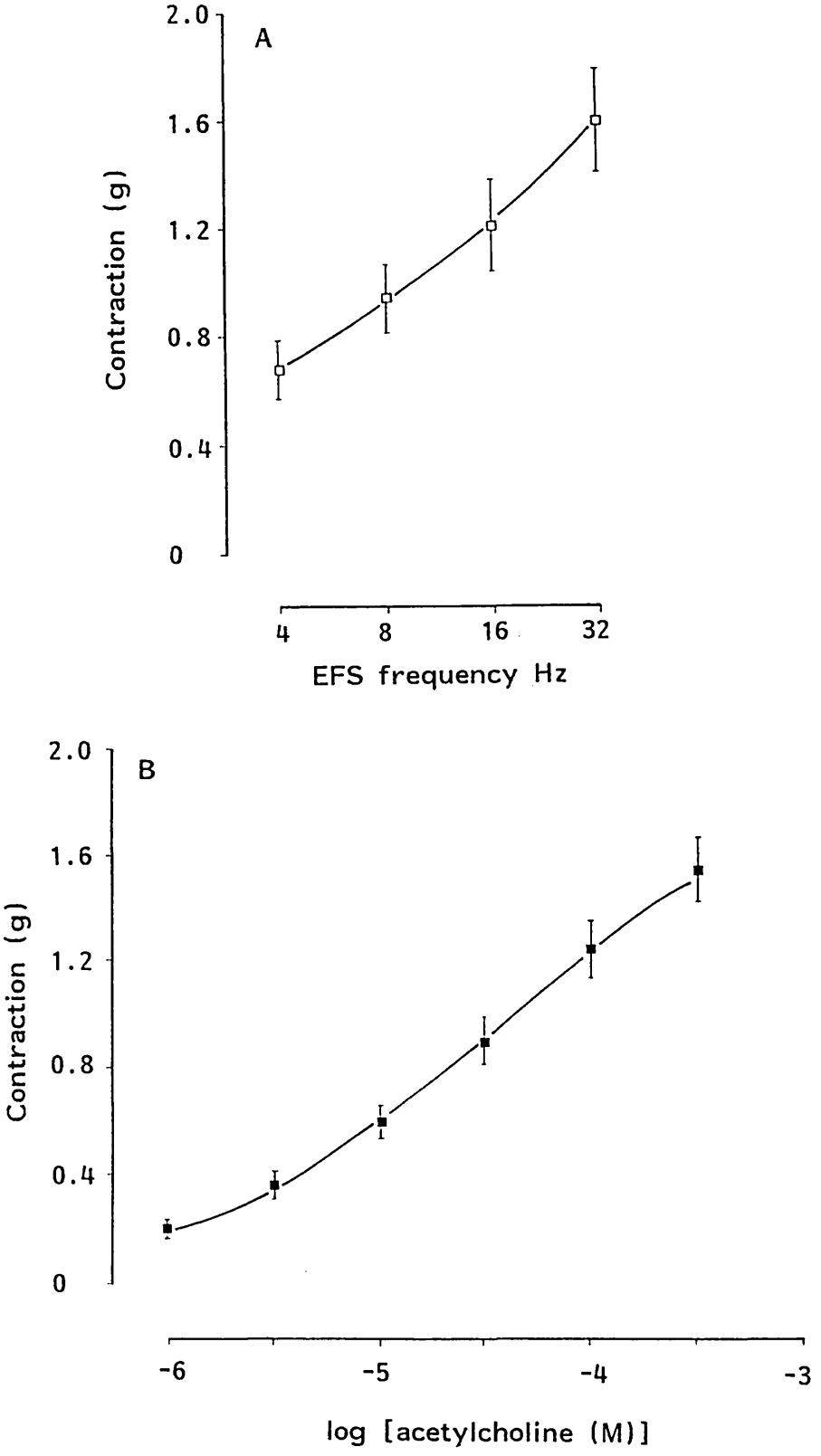


Figure 2. Control responses of human bronchi to A) EFS (1.0 ms, 40 V) (n=30 preparations from 22 patients), and B) acetylcholine (n=35 preparations from 22 patients). Points represent means $\pm$ SE.

Table 1. Matched contractile responses of human bronchi to EFS and acetylcholine

EFS (Hz)	Contraction[1] (mg)	Acetylcholine (uM)	Contraction[2] (mg)	p[3]
4	671 ± 108	10	599 ± 610	NS[4]
8	943 ± 132	30	894 ± 940	NS
16	1206 ± 171	100	1237 ± 107	NS
32	1590 ± 1879	300	1541 ± 130	NS

Figures represent means ± SE.

1. n= 35 preparations from 22 patients.

2. n= 28 preparations from 22 patients.

3. Probability level for unpaired Student's T-test comparison of matched EFS- and acetylcholine-induced contractions.

4. NS= no significance.

Table 2. Reproducibility of matched contractile responses of human bronchi to EFS and acetylcholine.

	% response[1]	
	32 Hz EFS	300 uM acetylcholine
first	100.0 ± 0.0	100.0 ± 0.0
second	100.0 ± 4.2	95.4 ± 5.2
third	103.3 ± 4.8	97.0 ± 3.7
fourth	106.4 ± 6.4	96.6 ± 5.5
fifth	103.4 ± 6.4	80.8 ± 6.3**
sixth	104.8 ± 4.2	72.3 ± 5.6**

1. Figures represent contractile responses (means ± SE of 6 preparations) induced by 32 Hz EFS or 300 uM acetylcholine as part of six consecutive stimulus-response curves, expressed as % of the response obtained in the first stimulus-response curve.

** $p<0.01$ paired Student's T-test, compared to response in first stimulus-response curve.

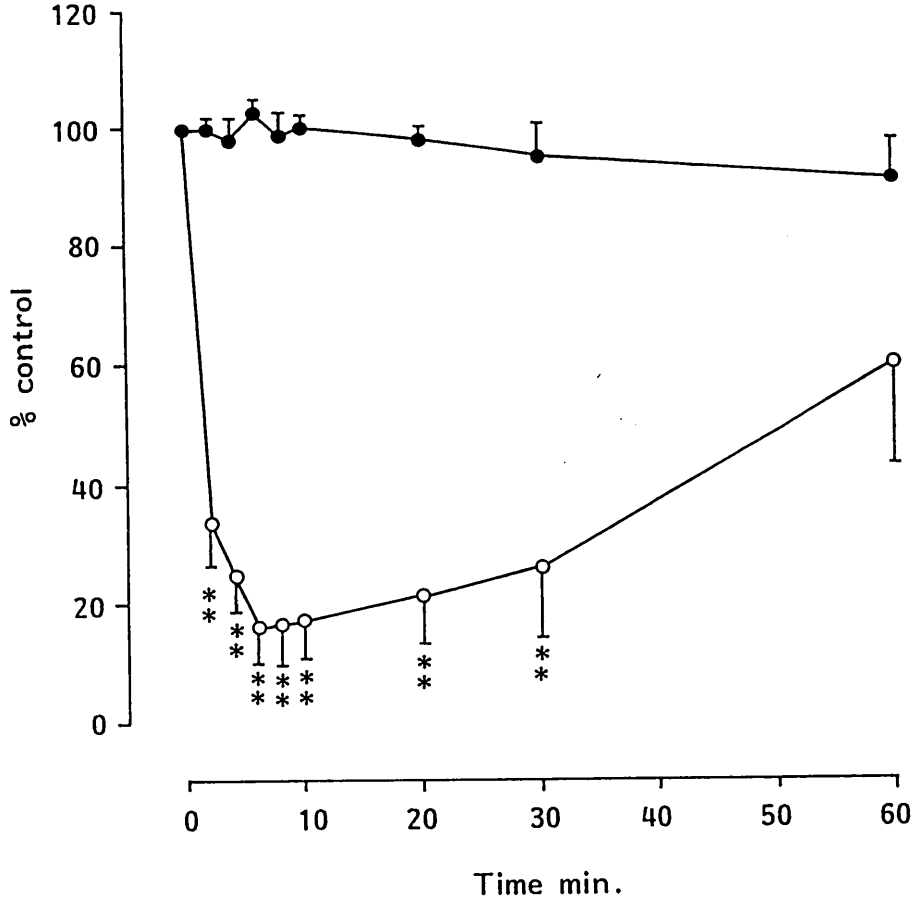


Figure 3. Time course for inhibitory effect of 1 uM isoprenaline on responses of human bronchi to EFS (1.0 ms, 40 V, 32 Hz). Cholinergic responses were measured at several time points following the addition of 1 uM isoprenaline (O). Adjacent tissues served as time controls in the absence of isoprenaline (●). Points represent means ± SE of n=4, ** $p<0.01$ compared to time control.

of catecholamines on cholinergic contractions was examined by incubating tissues with catecholamines for 10 min before the application of acetylcholine or EFS. Responses to acetylcholine and EFS were examined within 30 min of the addition of catecholamines. This period of time allowed the construction of two EFS frequency-response curves (4-32 Hz) or a limited acetylcholine C-R curve (1-300 uM).

3.3.4. Catecholamines

Isoprenaline, adrenaline and noradrenaline produced concentration-dependent inhibition of contractile responses to both EFS and acetylcholine. The rank order of potency of catecholamines for inhibition of EFS-induced responses (determined from a comparison of IC_{50}s) was isoprenaline > adrenaline > noradrenaline, and was similar at all frequencies studied (table 3). All three catecholamines were more potent at inhibiting cholinergic contractions induced by low rather than high frequencies of stimulation with IC_{50}s for inhibition of contraction induced by 32 Hz being 1 to 2 orders of magnitude greater than IC_{50}s for inhibition of contraction induced by 4 Hz. IC_{50}s for inhibition of acetylcholine-induced contraction could not always be determined as 50 % inhibition was not always achieved. However, for contractions induced by 10 uM acetylcholine , the rank order of potency for inhibition by catecholamines

was isoprenaline = adrenaline > noradrenaline. Isoprenaline and noradrenaline were more potent at inhibiting contractions induced by low concentrations of acetylcholine than high concentrations.

In order to determine whether catecholamines modulate cholinergic neurotransmission, a comparison of inhibition of matched EFS-induced and acetylcholine-induced contractions was made. Thus, inhibition of contraction induced by 4 Hz EFS was compared to inhibition of contraction induced by 10 uM acetylcholine, and so on. At all frequencies studied, isoprenaline (0.1-100 uM) caused a significantly greater inhibition of EFS-induced contractions than of acetylcholine-induced contractions (figure 4). At a stimulation frequency of 4 Hz, isoprenaline was 77 times more potent at inhibiting EFS-induced contractions than matched acetylcholine-induced contractions. This concentration-ratio (determined from comparison of IC_{50}s) increased with frequency of stimulation, such that at 32 Hz isoprenaline was >350 times more potent at inhibiting EFS-induced than acetylcholine-induced contractions. A representative tracing of the inhibitory influence of isoprenaline on EFS-induced contractions of human bronchi, at concentrations that have little effect on acetylcholine-induced contractions, is shown in figure 5.

Adrenaline (1 uM or greater) also induced a significantly greater inhibition of EFS-induced

Table 3. IC_{50}s for inhibition of cholinergic reponses by catecholamines

	EFS (Hz)	IC_{50}[1] (uM)	acetylcholine (uM)	IC_{50}[1] (uM)	ratio[2]
isoprenaline	4	0.048	10	3.7	77
	8	0.087	30	15	170
	16	0.24	100	>100[3]	>420
	32	0.29	300	>100[3]	>350
adrenaline	4	0.40	10	2.7	6.8
	8	0.68	30	NC[4]	NC
	16	1.1	100	NC[4]	NC
	32	25	300	NC[4]	NC
noradrenaline	4	56	10	300	5.4
	8	160	30	200	1.3
	16	320	100	790	2.5
	32	560	300	>1000[3]	>1.8

1. IC_{50} = concentration inducing 50 % inhibition of cholinergic contractions, interpolated from sigmoid curves fitted to mean responses.

2. Ratio of IC_{50}s for matched acetycholine- and EFS-induced contractions.

3. 50 % inhibition not achieved, therefore IC_{50} estimated to be greater than the highest concentration of catecholamine used.

4. 50 % inhibition not achieved, therefore IC_{50} not calculated (NC).

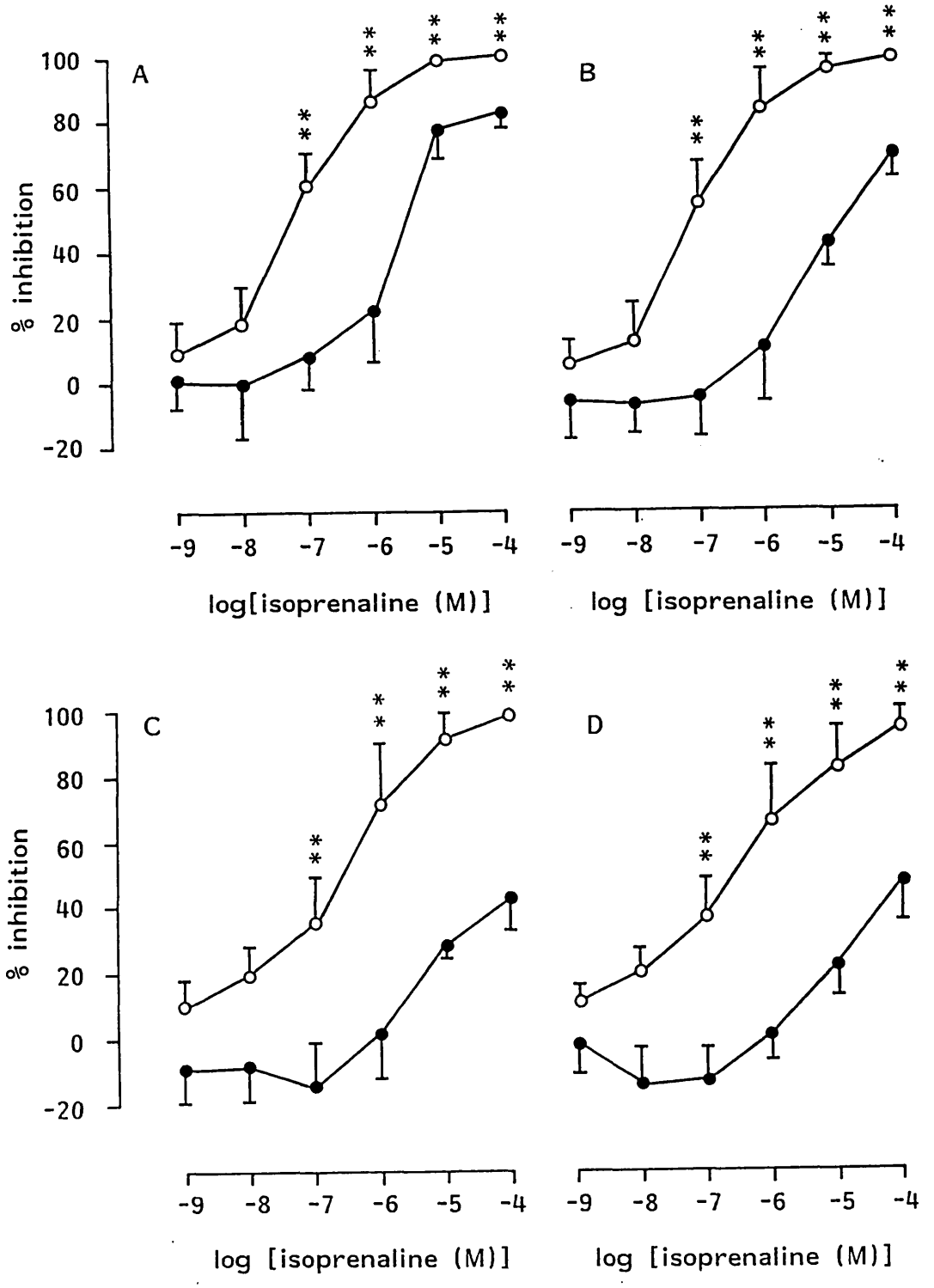


Figure 4. Effect of isoprenaline on matched responses of human bronchi to EFS (1.0 ms, 40 V) and acetylcholine. (a) 4 Hz EFS and 10 uM acetylcholine, (b) 8 Hz EFS and 30 uM acetylcholine, (c) 16 Hz EFS and 100 uM acetylcholine, and (d) 32 Hz EFS and 300 uM acetylcholine. Points represent means ± of n=7-8, ** $p<0.01$ acetylcholine-induced vs. EFS-induced responses. ○ EFS; ● acetylcholine.

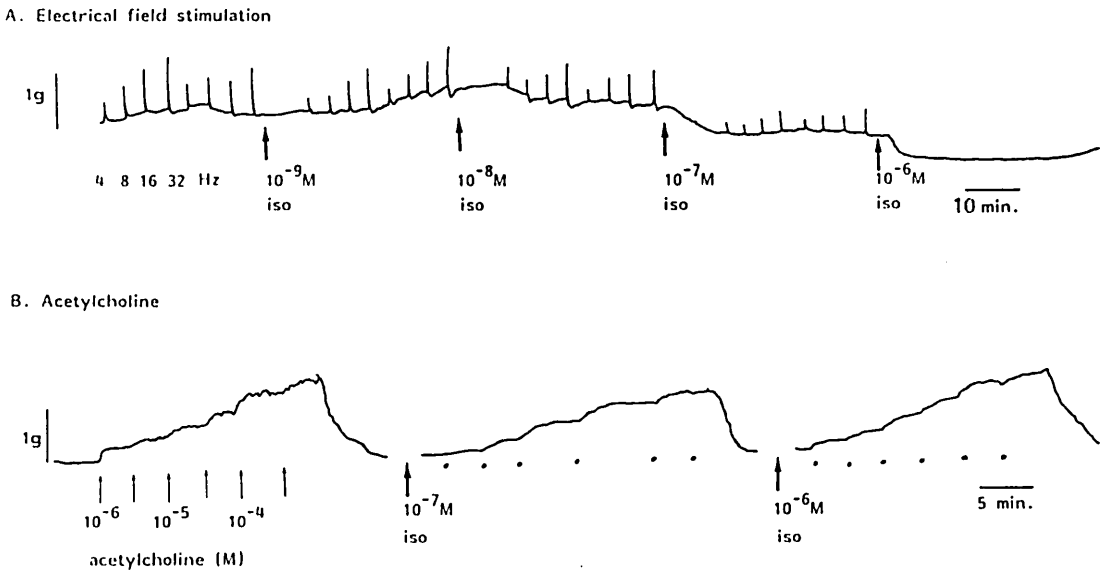


Figure 5. Representative tracing of inhibition of cholinergic responses by isoprenaline. Isoprenaline inhibits EFS-induced contractions (A), at concentrations that have little effect on acetylcholine-induced contractions (B).

contractions than of matched acetylcholine-induced contractions (figure 6), with a concentration-ratio of 6.8 at a stimulation frequency of 4 Hz.

Noradrenaline (1 mM) induced a significantly greater inhibition of EFS-induced than acetylcholine-induced contractions, but only at stimulation frequencies of 4 and 8 Hz (figure 7). At 4 Hz, noradrenaline was 5.4 times more potent at inhibiting EFS-induced than acetylcholine-induced contractions.

3.3.5. Adrenoceptor antagonists

Phentolamine (1 uM), propranolol (1 uM), betaxolol (0.1 uM) and ICI 118, 551 (0.1 uM) had no effect on contractile responses to EFS (1.0 ms, 40 V, 32 Hz), with responses being 101 $\pm$ 2 %, 103 $\pm$ 5 %, 99 $\pm$ 4 % and 100 $\pm$ 1 % respectively, of their control level. The inhibitory effect of 1 uM isoprenaline on similar contractile responses to EFS was unaffected by phentolamine and betaxolol, but was blocked by propranolol and ICI 118 551 (figure 8). The concentration of isoprenaline used for such studies was chosen as it was found to inhibit contractile responses to EFS whilst having no effect on matched acetylcholine contractions.

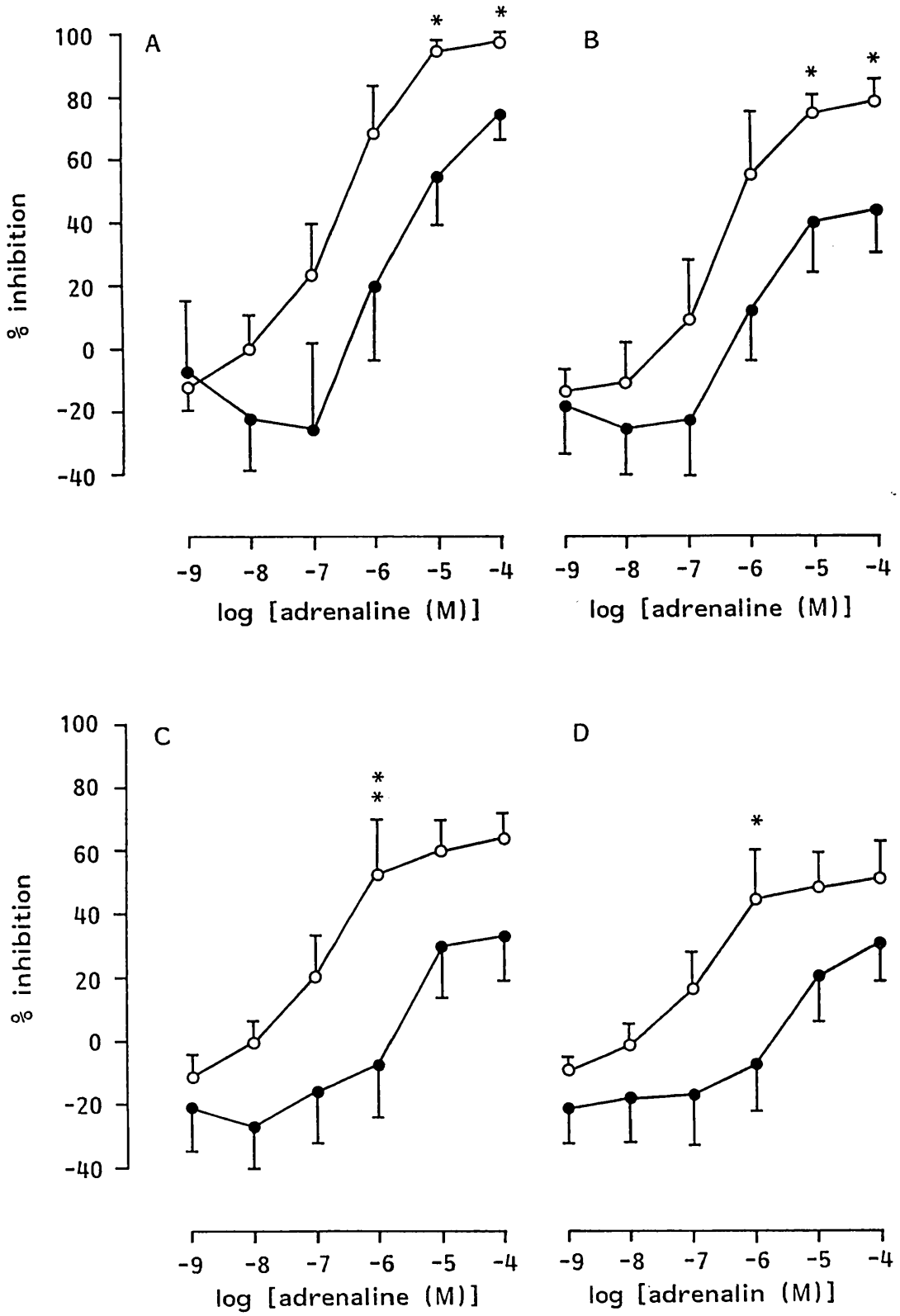


Figure 6. Effect of adrenaline on matched responses of human bronchi to EFS (1.0 ms, 40 V) and acetylcholine. (a) 4 Hz EFS and 10 uM acetylcholine, (b) 8 Hz EFS and 30 uM acetylcholine, (c) 16 Hz EFS and 100 uM acetylcholine, and (d) 32 Hz EFS and 300 uM acetylcholine. Points represent means $\pm$ SE of n=7-8, * $p<0.05$, ** $p<0.01$ acetylcholine-induced vs. EFS-induced responses.
O EFS; ● acetylcholine.

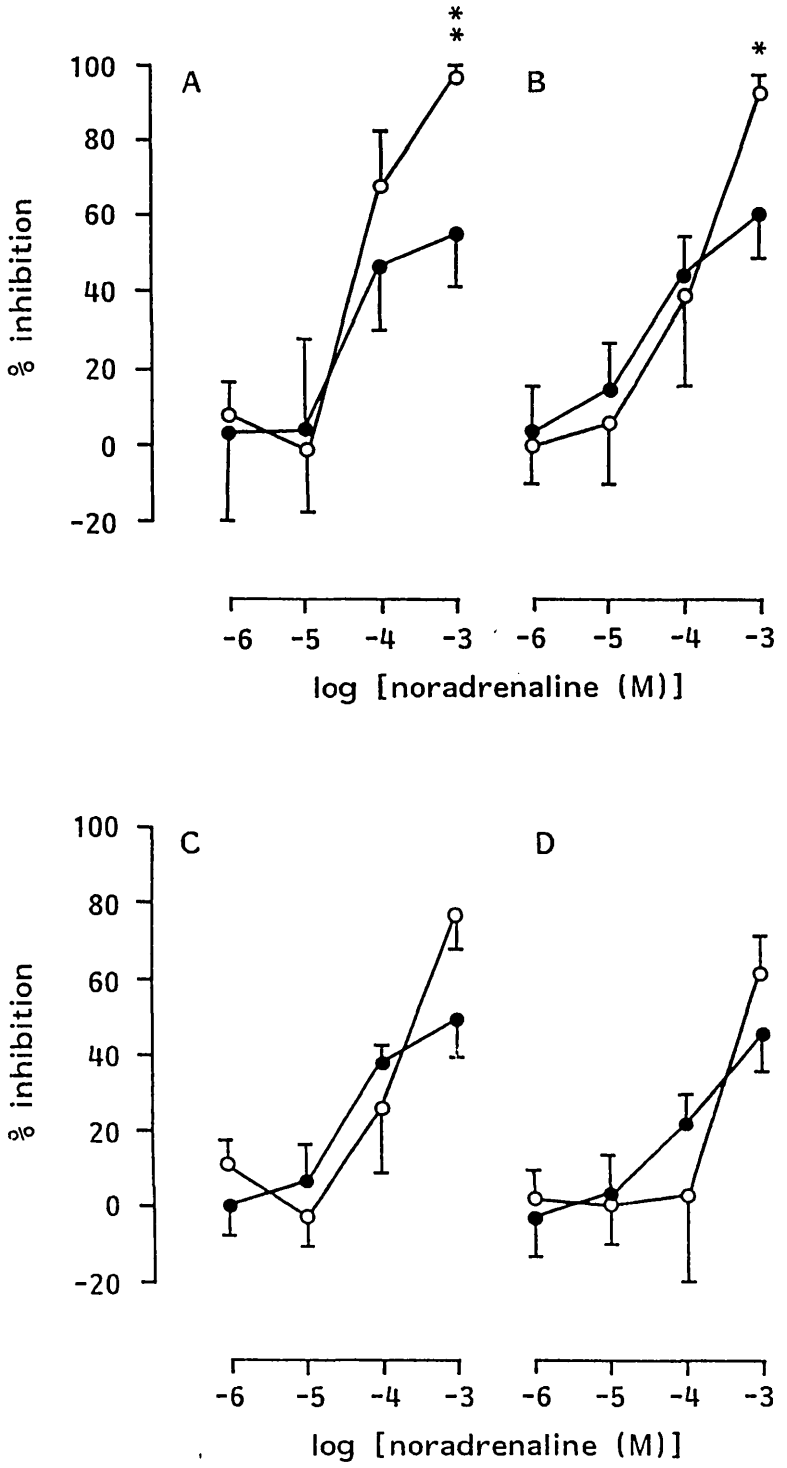


Figure 7. Effect of noradrenaline on matched responses of human bronchi to EFS (1.0 ms, 40 V) and acetylcholine. (a) 4 Hz EFS and 10 uM acetylcholine, (b) 8 Hz EFS and 30 uM acetylcholine, (c) 16 Hz EFS and 100 uM acetylcholine, and (d) 32 Hz EFS and 300 uM acetylcholine. Points represent means $\pm$ SE of n=6, * $p<0.05$, ** $p<0.01$ acetylcholine-induced vs. EFS-induced responses.

O EFS; ● acetylcholine.

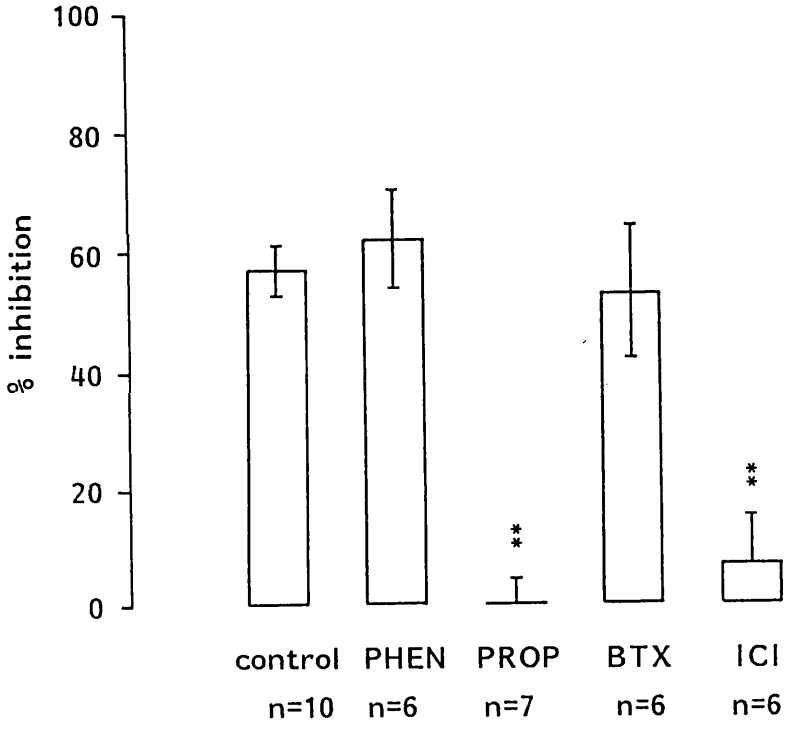


Figure 8. Effect of adrenoceptor antagonists on inhibition of responses to EFS (1.0 ms, 40 V, 32 Hz) by isoprenaline. Tissues were incubated with 1 *u*M phentolamine (PHEN), 1 *u*M propranolol (PROP), 0.1 *u*M betaxolol (BTX) or 0.1 *u*M ICI 118,551 (ICI) before 1 *u*M isoprenaline was added. Bars means $\pm$ SE of n=6-7.** $p<0.01$, compared to control.

3.3.6. Tyramine

Tyramine (100 uM) had no effect on contraction induced by EFS (1.0 ms, 40 V, 32 Hz), with responses after 10 and 30 min incubation being 105.3 $\pm$ 6.5 % and 106.0 $\pm$ 11.1 % respectively of their control level.

3.4. Discussion

The response of isolated human airway smooth muscle preparations to EFS has been well characterised by several studies (Richardson & Beland, 1976; Davies *et al.*, 1982; Taylor *et al.*, 1984). In these reports, as in the experiments, EFS of isolated bronchi elicits a contractile response that can be blocked by atropine and TTX suggesting that contraction results from the activation of intrinsic cholinergic nerves. The cholinergic contraction is followed by an inhibitory response that is blocked by TTX but is unaffected by propranolol, and is thought to be due to the activation of i-NANC nerves. A sustained non-neuronal contractile response to EFS has also been described to be present in 80 % of bronchial preparations (de Jongste *et al.*, 1987). This response may be due to a partly epithelium-dependent synthesis of excitatory arachidonic acid metabolites and is reduced by prior cooling of tissues to 4 °C. In the present experiments, a sustained contractile response to EFS was observed in preparations from only 2 out of 22 patients studied. All preparations used were maintained at 4°C prior to mounting in the organ baths, and this may explain the low incidence of non-neuronal responses to EFS in this study.

Contractile responses of human bronchi to exogenous acetylcholine were depressed by isoprenaline, adrenaline and noradrenaline, presumably by the activation of

beta-adrenoceptors located on smooth muscle membranes. Responses to cholinergic nerve stimulation by EFS were also depressed by catecholamines, and to a greater degree than were responses to exogenous acetylcholine. Several explanations may account for these findings. Exogenous acetylcholine may activate a greater number of muscarinic receptors on bronchial smooth muscle than acetylcholine released during EFS. Functional antagonism between catecholamines and acetylcholine would result in a decreased effectiveness of catecholamines to inhibit contractions induced by exogenous acetylcholine as compared to EFS-induced contractions. This explanation is unlikely since inhibition of cholinergic responses by catecholamines was determined on acetylcholine- and EFS-induced contractions of similar magnitude. Another explanation may be that the distribution of muscarinic receptors activated by endogenously released and exogenously added acetylcholine may not be the same, and this could influence the effectiveness of catecholamines to antagonise contractile responses.

In addition to postjunctional mechanisms, catecholamines may depress contractile responses to EFS by acting prejunctionally to alter neurotransmitter release. The response of smooth muscle preparations to EFS represents a composite of the effects of excitatory and inhibitory neurotransmitters. A reduction in the magnitude of cholinergic contractions induced by EFS may be due to a decrease in the release of acetylcholine from cholinergic

nerve terminals or an increase in the release of an inhibitory neurotransmitter which would antagonise contractile responses postjunctionally. The identity of the inhibitory neurotransmitter mediating relaxant responses of human bronchi to EFS is uncertain, and antagonists to this response are not available. Potentiation of the release of an inhibitory neurotransmitter can therefore not be discounted as a mechanism by which catecholamines inhibit responses to cholinergic nerve stimulation. In canine airways noradrenaline and isoprenaline suppress the amplitude of excitatory junction potentials evoked by EFS at concentrations that have no effect on membrane potential, suggesting that catecholamines may decrease the release of acetylcholine from cholinergic nerve terminals (Ito & Tajima, 1982). However, measurements of acetylcholine release from canine airways have failed to demonstrate an inhibitory effect of noradrenaline (Martin & Collier, 1986). The mechanism by which catecholamines inhibit cholinergic nerve-mediated responses of isolated airway smooth muscle therefore remains controversial, and the results of the present study cannot lead to a definitive conclusion.

Modulation of cholinergic neurotransmission by catecholamines has been demonstrated in airway ganglia (see introduction). EFS of isolated preparations excites all neuronal elements present, therefore the release of neurotransmitter from a nerve terminal results from local

changes in ionic gradients, and not from the propagation of action potentials. Cholinergic neurotransmission at the neuroeffector junction is therefore not dependent on the presence of airway ganglia, and this is confirmed by the lack of effect of hexamethonium on EFS-induced responses. Modulation of neurotransmission at any ganglia present in an isolated preparation will not necessarily be accompanied by changes in neurotransmission at the neuroeffector junction. If modulation of neurotransmitter release is the mechanism by which catecholamines inhibit responses to cholinergic nerve stimulation then modulation must occur at the neuroeffector junction of airway smooth muscle.

In guinea-pig airway smooth muscle adrenergic fibres are sometimes found in close association with cholinergic fibres (Jones *et al.*, 1980). In the rabbit small intestine complexes are formed between terminal adrenergic varicosities and cholinergic axons, and the stimulation of adrenergic and cholinergic nerves leads to the reciprocal inhibition of acetylcholine and noradrenaline release (Mamber *et al.*, 1979). Smooth muscles of the airways and the gastrointestinal tract share a common embryological origin, therefore it is possible that the stimulation of adrenergic nerves in airway smooth muscle may inhibit acetylcholine release from cholinergic nerves. In canine bronchi, propranolol augments cholinergic responses to EFS but not exogenous acetylcholine, suggesting that noradrenaline released from adrenergic nerves by EFS may

inhibit cholinergic nerve-mediated responses (Vermeire & Vanhoutte, 1979). Tyramine, which causes the pharmacological displacement of noradrenaline from adrenergic nerves, depressed cholinergic contractions evoked by EFS but not those induced by exogenous acetylcholine (Danser *et al.*, 1987). Adrenergic innervation to human airway smooth muscle is scant, although a close proximity between cholinergic nerve terminals and possible adrenergic varicosities has been reported (Davis & Kannan, 1987). EFS and tyramine both induce the release of noradrenaline from human bronchial smooth muscle (Davis & Kannan, 1987) and noradrenaline has been shown to inhibit EFS-induced contractions of human bronchi at concentrations that have little effect on acetylcholine-induced contractions (Grundstrom & Andersson, 1985). Experiments described by Grundstrom & Andersson, 1985, were carried out in the presence of the neuronal uptake inhibitor cocaine and this may explain the higher concentrations of noradrenaline needed in our experiments to inhibit contractile responses to EFS. Propranolol and tyramine had no effect on cholinergic responses induced by EFS, suggesting that in the present study noradrenaline released by any intrinsic adrenergic nerves has no significant effect on responses of human bronchi to the stimulation of cholinergic nerves.

Isoprenaline and adrenaline were both more potent than noradrenaline at inhibiting contractile responses to EFS and acetylcholine, and both yielded greater IC_{50}

concentration-ratios for inhibition of acetylcholine-induced contractions compared to inhibition of EFS-induced contractions. This raises the possibility that circulating catecholamines, rather than noradrenaline released by adrenergic nerves to smooth muscle, may be responsible for modulating cholinergic nerve-mediated responses *in vivo*. In anaesthetised cats, intravenous isoprenaline abolishes vagal tone but only reduces acetylcholine-induced tone by 50 % (Baker & Don, 1987).

The rank order of potency of catecholamines for inhibition of EFS-induced contractions was isoprenaline > adrenaline > noradrenaline, suggesting that responses to catecholamines may be mediated by *beta*-adrenoceptors. Indeed, the inhibitory influence of isoprenaline on EFS-induced contraction was prevented by a $beta_2$-adrenoceptor antagonist but not by *alpha*- or $beta_1$-adrenoceptor antagonists. Other studies have shown inhibition of cholinergic nerve-mediated responses by noradrenaline to be mediated by $alpha_2$-adrenoceptors in the guinea-pig trachea and human bronchus (Grundstom *et al.*, 1981; Grundstrom & Andersson, 1985a) and by $beta_1$-adrenoceptors in canine bronchi (Danser *et al.*, 1987). A similar study to ours has also reported a selective inhibition of EFS-induced contractions of human bronchi by isoprenaline, but the receptor type mediating such effects was not determined (Davis & Kannan, 1987). The same study however failed to show an inhibitory effect of the $alpha_2$-adrenoceptor agonist clonidine on EFS- or

acetylcholine-induced contractions. In the guinea-pig trachea the *beta*$_2$-adrenoceptor agonist procaterol suppressed end junction potentials evoked by EFS without affecting membrane potential, and this response was prevented by a *beta*$_2$-adrenoceptor antagonist (Ito, 1988). Differences in methodology, animal species and airway level used by different investigators may underlie some of the variability obtained in receptor classification, although it is difficult to reconcile the contrasting findings obtained from human bronchi regarding the role of *alpha*$_2$-adrenoceptors in mediating inhibition of EFS-induced contraction.

Inhibition of cholinergic neurotransmission in human airways may represent an important pathway by which circulating catecholamines protect against bronchoconstrictor influences. Any deficiency in this pathway would therefore have important clinical implications. Plasma adrenaline levels in resting asthmatic subjects are normal (Barnes *et al.*, 1982b) but fail to rise during an acute asthma attack (Ind *et al*, 1985). This is in contrast to the rise in catecholamine levels seen under conditions of acute stress, such as myocardial infarction (Karlsberg *et al.*, 1981). Asthmatic patients also demonstrate a blunted rise in adrenaline levels during exercise (Barnes *et al*, 1981). Since adrenaline clearance appears to be normal in stable asthmatics, the failure of plasma adrenaline levels to rise during an asthma attack or exercise may indicate an

impairment of adrenaline secretion in asthma. Nocturnal asthma is associated with a drop in circulating levels and this coincides with the time of maximum bronchoconstriction (Barnes *et al.*, 1980). Early morning bronchoconstriction may be reduced by a cholinergic receptor antagonist (Coe & Barnes, 1986), suggesting that the fall in adrenaline levels observed leads to an increase in vagal tone. *Beta*-adrenoceptor antagonists induce a bronchoconstriction in asthmatic, but not normal, subjects that is prevented and reversed by anticholinergic drugs (Grieco & Pierson, 1971; Ind *et al.*, 1989) suggesting that there is a tonic inhibition of vagal tone. Our results are in agreement with these observations since blockade of prejunctional *beta*-adrenoceptors on cholinergic nerves would remove the inhibitory influence of circulating catecholamines on cholinergic neurotransmission. The increase in acetylcholine release that would follow *beta*-adrenoceptor blockade would have a much greater effect in asthmatic subjects who are hyperreactive to cholinergic agonists. This would explain why asthmatic but not normal subjects develop bronchoconstriction in response to *beta*-adrenoceptor antagonists.

In conclusion, catecholamines inhibit cholinergic contractions mediated by the stimulation of cholinergic nerves to a greater degree than comparable contractions

evoked by exogenous acetylcholine. This may reflect prejunctional modulation of cholinergic neurotransmission in human airways by *beta*$_2$-adrenoceptors.

4. Modulation of tachykinin-induced contraction of guinea-pig and human ASM by airway epithelium: role of cyclooxygenase and NEP.

4.1. Aim

The present study was designed to investigate whether, and by what mechanism, airway epithelium modulates the response of the underlying smooth muscle to tachykinins. Contractile responses to exogenous tachykinins were induced in guinea-pig tracheal and human bronchial smooth muscle in the presence and absence of an intact epithelial layer. The effects of indomethacin and the NEP inhibitor phosphoramidon on tachykinin-induced contraction were examined in order to determine whether inhibitory cyclooxygenase products or peptide metabolism by NEP respectively may contribute to epithelial modulation of ASM responses.

4.2. Protocol

Cumulative C-R curves to tachykinins were constructed in intact and epithelium-denuded guinea-pig tracheal and human bronchial preparations. Parallel preparations from the same trachea/bronchus were pretreated with 5 uM indomethacin and/or 10 uM phosphoramidon for 30 min prior to the construction of C-R curves. When indomethacin induced relaxation, baseline tension was readjusted to the pre-existing level. Only one tachykinin C-R curve was constructed in each preparation and tachykinin-induced contractions were compared to the response induced by 10 uM carbachol at the end of each experiment.

4.3 Results

4.3.1. Guinea-pig trachea

4.3.1.1 Tachykinins

NKA, NKB and SP induced concentration-dependent contractions of guinea-pig tracheal smooth muscle. Maximal (or even 50 % of maximal) contractile responses were not achieved by any of the tachykinins studied, therefore pD_2 values could not be determined. A comparison of mean EC_{30}s suggests a rank order of potency of tachykinins of NKA > NKB > SP (-log EC_{30} 6.45 $\pm$ 0.09 NKA, 5.72 NKB and 5.30 $\pm$ 0.14 SP). NKB at the highest concentration used, produced a contraction which was less than 30 % of the maximal carbachol contraction, therefore the -log EC_{30} was extrapolated from a sigmoid curve fitted to mean NKB-induced responses.

4.3.1.2. Effect of epithelium removal

The effectiveness of epithelium removal was assessed pharmacologically by determining the reactivity of rubbed and unrubbed preparations to acetylcholine (figure 9). Epithelium removal increased the potency of acetylcholine (pD_2 4.98 $\pm$ 0.02 unrubbed preparations, 5.91 $\pm$ 0.03 rubbed preparations, $p<0.01$, concentration-ratio 8.5, n=5) but had no effect on the maximal response to acetylcholine

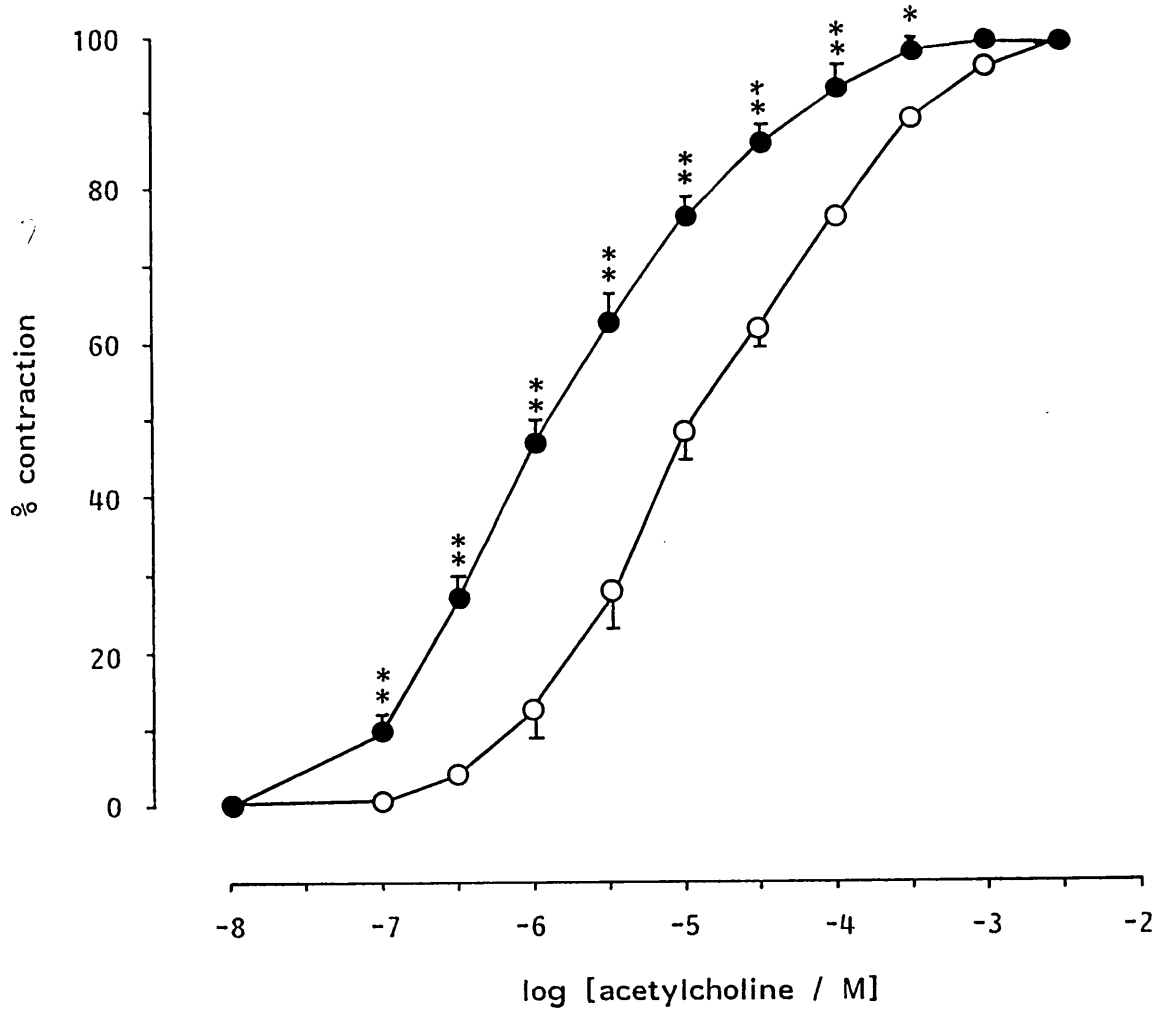


Figure 9. Effect of epithelium removal on responses of guinea-pig trachea to acetylcholine (O intact, ● epithelium-denuded preparations). Points represent means ± SE of n=5, * $p<0.05$, ** $p<0.01$ intact vs. epithelium-denuded preparations.

(2983 ± 156 mg unrubbed preparations, 2762 ± 203 mg rubbed preparations).

Epithelium removal caused a potentiation of contractile responses to all three tachykinins which was manifested in increases in potency (tables 4, 5 & 6, figures 10, 11 & 12). Mean EC_{30}s for NKA, NKB and SP were increased 12-fold, 288-fold and 138-fold, respectively. In epithelium-denuded preparations the rank order of potency of tachykinins, derived from comparisons of mean EC_{30}s and pD_2s, was NKB = NKA > SP. The effect of epithelium removal on maximal responses to tachykinins could not be determined as sufficiently high bath concentrations of tachykinins could not always be achieved.

Epithelium removal had no effect on the maximal contraction induced by 10 uM carbachol (table 7).

4.3.1.3. Effect of cyclooxygenase inhibition

Indomethacin (5 uM) induced relaxation of guinea-pig tracheal preparations (1234 ± 213 mg intact, 932 ± 154 mg epithelium-denuded preparations, n=25).

Indomethacin (5 uM) caused a potentiation of contractile responses of intact and epithelium-denuded preparations to NKA (figure 10, table 4). In intact preparations, mean -log EC_{30}s for untreated and indomethacin-treated preparations were significantly

Table 4. Effect of epithelium removal, cyclooxygenase inhibition and NEP inhibition on contractile responses of guinea-pig trachea to NKA.

	-log EC_{30}[1]	ratio[2]	pD_2[3]	ratio[3]
Untreated:				
intact	6.45 ± 0.09		-	
denuded	7.51 ± 0.07*	12	7.65 ± 0.07	-
Indomethacin:				
intact	8.10 ± 0.08		7.60 ± 0.08	
denuded	8.78 ± 0.09*	4.8	8.37 ± 0.08*	5.9
Phosphoramidon:				
intact	7.62 ± 0.10		7.73 ± 0.14	
denuded	7.82 ± 0.08	1.6	8.08 ± 0.13	2.4
Indomethacin + phosphoramidon:				
intact	9.08 ± 0.06		8.68 ± 0.08	
denuded	9.18 ± 0.07	1.3	8.77 ± 0.06	1.2

1. EC_{30}= concentration of tachykinin inducing 30 % of the maximal carbachol-induced contraction.

2. Ratio of EC_{30}s in epithelium-denuded and intact preparations.

3. pD_2= -log EC_{50}.

4. Ratio of EC_{50}s in epithelium-denuded and intact preparations.

* $p<0.05$ denuded vs. intact preparations, unpaired Student's T-test.

Figures represent means ± SE of n=8.

Table 5. Effect of epithelium removal, cyclooxygenase inhibition and NEP inhibition on contractile responses of guinea-pig trachea to NKB.

	-log EC_{30}[1]	ratio[2]	pD_2 3	ratio[3]
Untreated:				
intact	5.72		-	
denuded	8.18 ± 0.11*	288	7.77 ± 0.15	-
Indomethacin:				
intact	7.78 ± 0.13		7.48 ± 0.15	
denuded	8.45 ± 0.10*	4.7	8.15 ± 0.08*	4.7
Phosphoramidon:				
intact	7.50 ± 0.13		7.11 ± 0.07	
denuded	8.66 ± 0.15*	14	8.33 ± 0.06*	17
Indomethacin + phosphoramidon:				
intact	8.61 ± 0.09		8.14 ± 0.03	
denuded	8.79 ± 0.05	1.5	8.37 ± 0.06	1.7

1. EC_{30} = concentration of tachykinin inducing 30 % of the maximal carbachol-induced contraction.

2. Ratio of EC_{30}s in epithelium-denuded and intact preparations.

3. pD_2 = -log EC_{50}.

4. Ratio of EC_{50}s in epithelium-denuded and intact preparations.

* $p<0.05$ denuded vs. intact preparations, unpaired Student's T-test.

Figures represent means ± SE of n=7.

Table 6. Effect of epithelium removal, cyclooxygenase inhibition and NEP inhibition on contractile responses of guinea-pig trachea to SP.

	-log EC_{30}[1]	ratio[2]	pD_2[3]	ratio[4]
Untreated:				
intact	5.35 ± 0.09		-	
denuded	7.49 ± 0.10*	138	7.23 ± 0.16	-
Indomethacin:				
intact	6.78 ± 0.11		6.16 ± 0.08	
denuded	7.78 ± 0.19*	10	7.40 ± 0.12*	17
Phosphoramidon:				
intact	7.55 ± 0.07		7.14 ± 0.07	
denuded	8.18 ± 0.08*	4.3	7.71 ± 0.08*	3.7
Indomethacin + phosphoramidon:				
intact	8.27 ± 0.08		7.83 ± 0.09	
denuded	8.52 ± 0.12	1.8	8.06 ± 0.16	1.7

1. EC_{30} = concentration of tachykinin inducing 30 % of the maximal carbachol-induced contraction.

2. ratio of EC_{30}s in epithelium-denuded and intact preparations.

3. pD_2 = -log EC_{50}.

4. ratio of EC_{50}s in epithelium-denuded and intact preparations.

* $p<0.05$ denuded vs. intact preparations, unpaired Student's T-test.

Figures represent means ± SE of n=10.

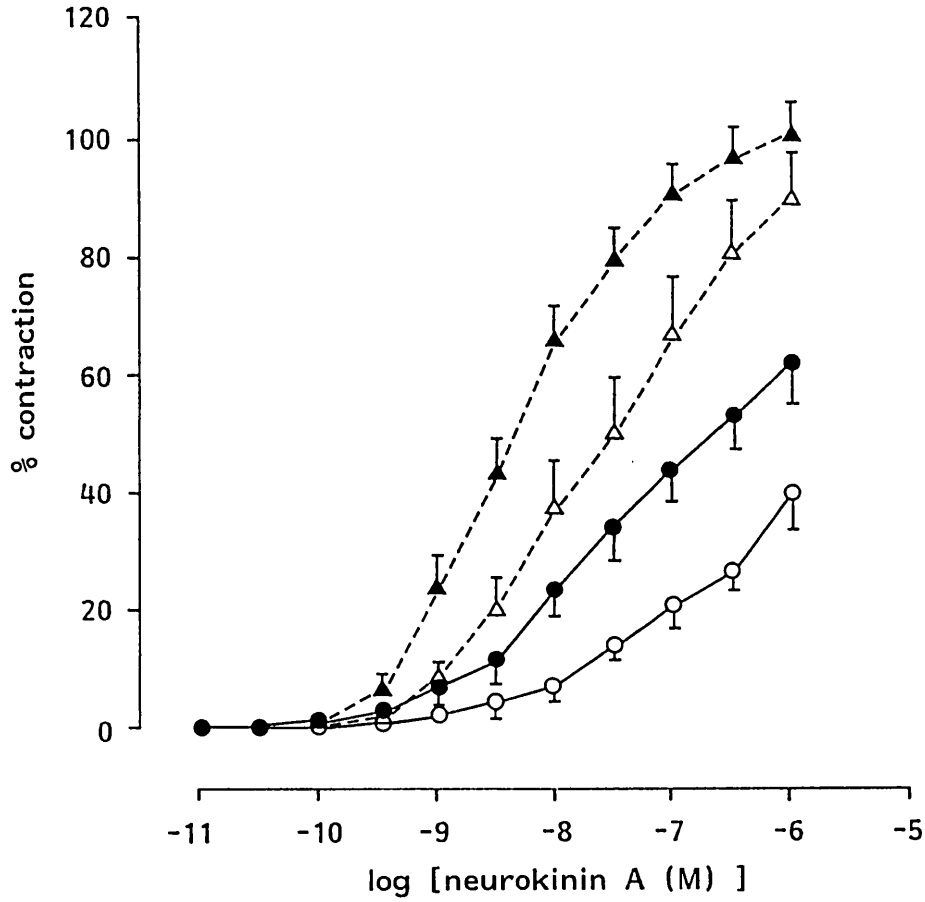


Figure 10. Effect of epithelium removal and cyclooxygenase inhibition on responses of guinea-pig trachea to NKA. Tissues were untreated (O intact, ● epithelium-denuded preparations) or pretreated with 5 uM indomethacin (Δ intact, ▲ epithelium-denuded preparations). Points represent means ± SE of n=8.

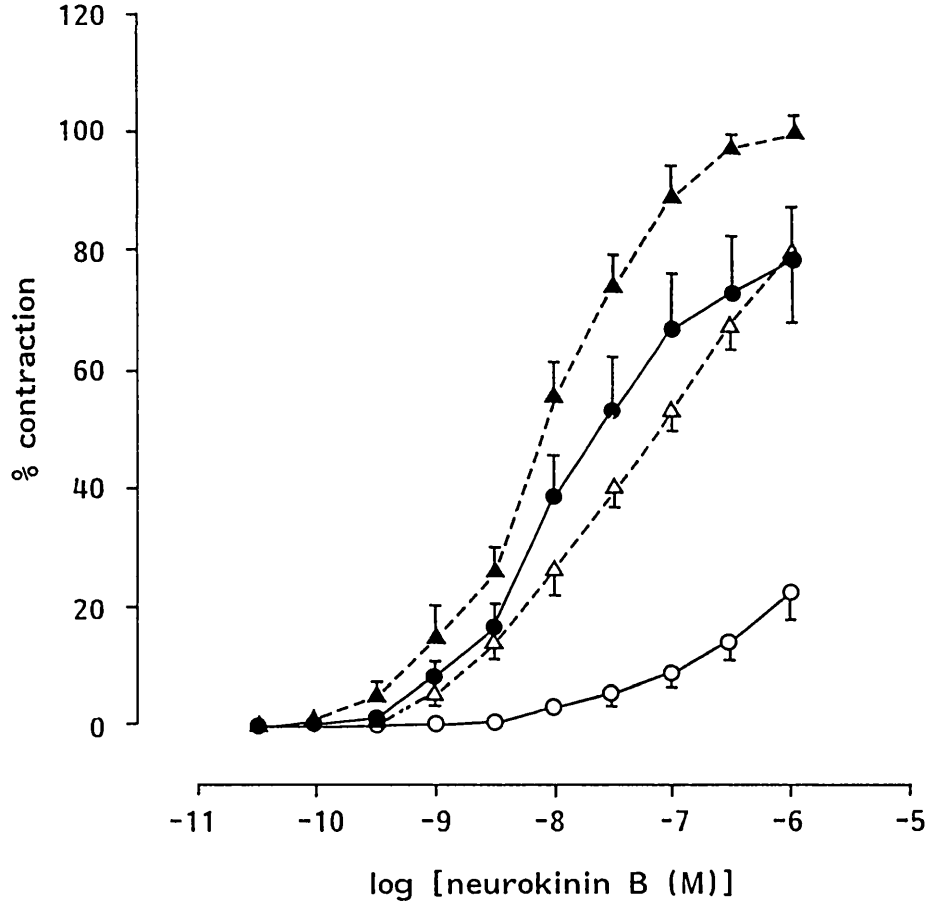


Figure 11. Effect of epithelium removal and cyclooxygenase inhibition on responses of guinea-pig trachea to NKB. Tissues were untreated (O intact, ● epithelium-denuded preparations) or pretreated with 5 uM indomethacin (Δ intact, ▲ epithelium-denuded preparations). Points represent means ± SE of n=7.

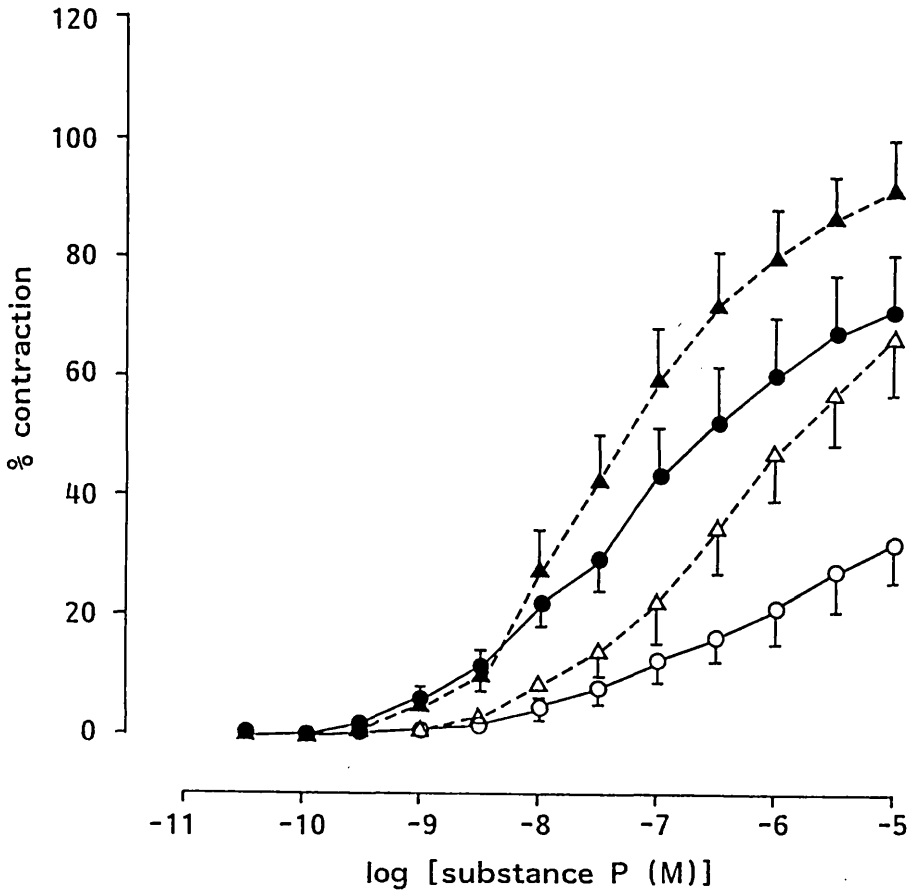


Figure 12. Effect of epithelium removal and cyclooxygenase inhibition on responses of guinea-pig trachea to SP. Tissues were untreated (○ intact, ● epithelium-denuded preparations) or pretreated with 5 uM indomethacin (△ intact, ▲ epithelium-denuded preparations). Points represent means ± SE of n=10.

Table 7. Maximal contraction induced by 10 uM carbachol

	maximal contraction (mg)[1]	
	intact preparations	epithelium-denuded preparations
NKA:		
untreated	2951 ± 162	2622 ± 309
indomethacin	2945 ± 449	2535 ± 323
phosphoramidon	2954 ± 286	2567 ± 315
indo. + phosph.	3146 ± 475	2625 ± 528
NKB:		
untreated	2278 ± 489	2597 ± 197
indomethacin	2710 ± 500	2562 ± 346
phosphoramidon	2619 ± 301	2481 ± 298
indo. + phosph.	2445 ± 350	2896 ± 268
SP:		
untreated	2452 ± 335	2130 ± 359
indomethacin	2513 ± 274	2269 ± 212
phosphoramidon	2820 ± 214	2582 ± 300
indo. + phosph.	2565 ± 257	2138 ± 211

1. maximum tension generated by 10 uM carbachol in untreated preparations or preparations pretreated with indomethacin and/or phosphoramidon after the construction of NKA, NKB or SP C-R curves.

Figures represent means ± SE of n=7-10.

different ($p<0.01$) and yielded a concentration-ratio of 45. In epithelium-denuded preparations, mean -log EC_{30} for untreated and indomethacin-treated preparations were significantly different ($p<0.01$) and yielded a concentration-ratio of 19. Potentiation of NKA-induced contractions by indomethacin was therefore greater in intact than in epithelium-denuded preparations. In the presence of indomethacin, epithelium removal still resulted in a potentiation (4.8-fold for mean EC_{30}s and 5.9-fold for mean pD_2s) of NKA-induced contractions, although this effect was smaller than in untreated preparations (12-fold for mean EC_{30}s). The effect of indomethacin on maximal responses to NKA could not be determined.

Indomethacin caused a potentiation of intact, but not epithelium-denuded, preparations to NKB (figure 11, table 5). In intact preparations, a comparison of mean -log EC_{30}s in untreated and indomethacin-treated preparations yielded a concentration-ratio of 115 (significance could not be determined). In epithelium-denuded preparations, there was no significant difference between mean -log EC_{30}s for untreated and indomethacin-treated preparations. In the presence of indomethacin, epithelium removal still resulted in a potentiation (4.7-fold for mean EC_{30}sand pD_2s) of NKB-induced contractions, and this effect was smaller than in untreated preparations (288-fold for mean EC_{30}s). The effect of indomethacin on maximal responses to NKB could not be determined in intact preparations. In

epithelium-denuded preparations, however, indomethacin potentiated the maximum contraction induced by NKB (74.2 $\pm$ 9.2 % vs. 97.1 $\pm$ 1.4 % of $carb_{max}$ untreated vss. indomethacin-treated preparations, $p<0.05$).

Indomethacin caused a potentiation of contractile responses of intact, but not epithelium-denuded, preparations to SP (figure 12, table 6). In intact preparations, mean -log EC_{30}s for untreated and indomethacin-treated preparations were significantly different ($p<0.01$), with a concentration-ratio of 27. In epithelium-denuded preparations, there was no significant difference between mean -log EC_{30}s for untreated and indomethacin-treated preparations. In the presence of indomethacin, epithelium removal still resulted in a potentiation (12-fold for mean EC_{30}s and 17-fold for mean pD_2s) of SP-induced contractions, and this effect was smaller than in untreated preparations (138-fold for mean EC_{30}s). The effect of indomethacin on maximal responses to SP could not be determined in intact preparations. In epithelium-denuded preparations, however, indomethacin had no effect on the maximum contraction induced by SP (71.8 $\pm$ 9.3 % vs. 88.1 $\pm$ 7.4 % of $carb_{max}$ untreated vs. indomethacin-treated preparations).

Indomethacin had no effect on the maximal contraction induced by 10 uM carbachol in intact and epithelium-denuded preparations (table 7).

4.3.1.4. Effect of NEP inhibition

Phosphoramidon (10 uM) induced an increase in resting tension in 4 out of 30 intact preparations (283 ± 58 mg) and 5 out of 30 epithelium-denuded preparations (333 ± 62 mg).

Phosphoramidon (10 uM) caused a potentiation of contractile responses of intact, but not epithelium-denuded, preparations to NKA (figure 13a, table 4). In intact preparations, mean -log EC_{30}s for untreated and phosphoramidon-treated preparations were significantly different ($p<0.01$) and yielded a concentration-ratio of 15. In epithelium-denuded preparations, there was no significant difference between mean -log EC_{30}s for untreated and phosphoramidon-treated preparations. In the presence of phosphoramidon, epithelium removal failed to potentiate NKA-induced contractions, with no significant difference being observed between mean -log EC_{30}s or between mean pD_2s for intact and epithelium-denuded preparations (table 4). The effect of phosphoramidon on the maximal response to NKA could not be determined.

Phosphoramidon caused a potentiation of contractile responses of intact and epithelium-denuded preparations to NKB (figure 14a, table 5). In intact preparations, mean -log EC_{30}s for untreated and phosporamidon-treated preparations yielded a concentration-ratio of 60 (significance could not be determined). In epithelium

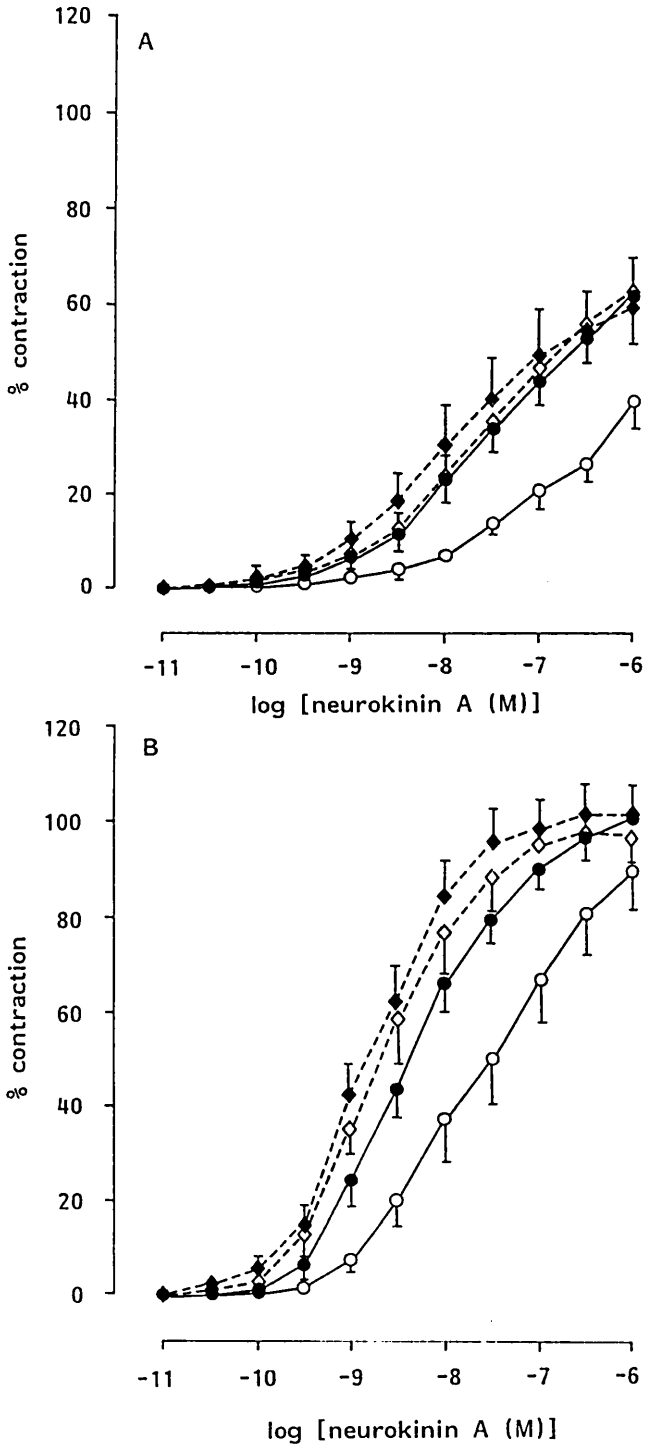


Figure 13. Effect of NEP inhibition on responses of guinea-pig trachea to NKA. In (a) tissues were untreated (Ointact, ● epithelium-denuded preparations) or pretreated with 10 uM phosphoramidon (◇ intact, ◆ epithelium-denuded preparations). In (b) tissues were pretreated with 5 uM indomethacin alone (O intact, ● epithelium-denuded preparations) or with 5 uM indomethacin plus 10 uM phosphoramidon (◇ intact, ◆ epithelium-denuded preparations). Points represent means ± SE of n=8.

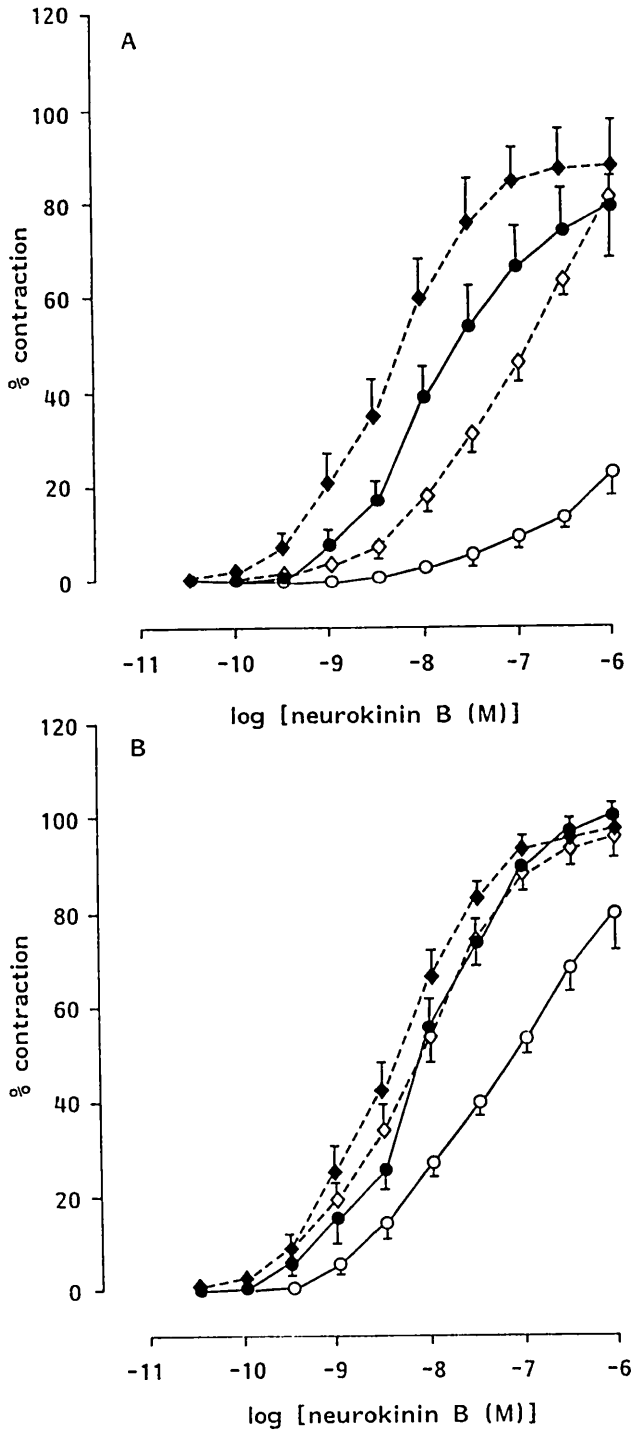


Figure 14. Effect of NEP inhibition on responses of guinea-pig trachea to NKB. In (a) tissues were untreated (○intact, ● epithelium-denuded preparations) or pretreated with 10 uM phosphoramidon (◇ intact, ◆ epithelium-denuded preparations). In (b) tissues were pretreated with 5 uM indomethacin alone (○ intact, ● epithelium-denuded preparations) or with 5 uM indomethacin plus 10 uM phosphoramidon (◇ intact, ◆ epithelium-denuded preparations). Points represent means ± SE of n=7.

denuded preparations, mean -log EC_{30}s for untreated and phosphoramidon-treated preparations were significantly different ($p<0.05$) and yielded concentration-ratios of 3.4. Potentiation of NKB-induced contractions by phosphoramidon was therefore greater in intact than in epithelium-denuded preparations. In the presence of phosphoramidon, epithelium removal still resulted in a potentiation (14-fold for mean -log EC_{30}s and 17-fold for mean pD_2s) of NKB-induced contractions, and this effect was smaller than in untreated preparations (257-fold for mean -log EC_{30}s). The effect of phosphoramidon on maximal responses to NKB could not be determined in intact preparations. In epithelium-denuded preparations phosphoramidon had no effect on the maximum contraction induced by NKB (74.2 $\pm$ 9.2 % vs. 86.7 $\pm$ 9.0 % untreated vs. phosphoramidon-treated preparations.)

Phosphoramidon caused a potentiation of contractile responses of intact and epithelium-denuded preparations to SP (figure 15a, table 6). In intact preparations, mean -log EC_{30}s for untreated and phosporamidon-treated preparations were significantly different and yielded a concentration-ratio of 158. In epithelium denuded preparations, mean -log EC_{30}s for untreated and phosphoramidon-treated preparations were significantly different ($p<0.05$) and yielded concentration-ratios of 5.8 for mean EC_{30}s and 3.0 for mean pD_2s. Potentiation of SP-induced contractions by phosphoramidon was therefore greater in intact than in epithelium-denuded preparations.

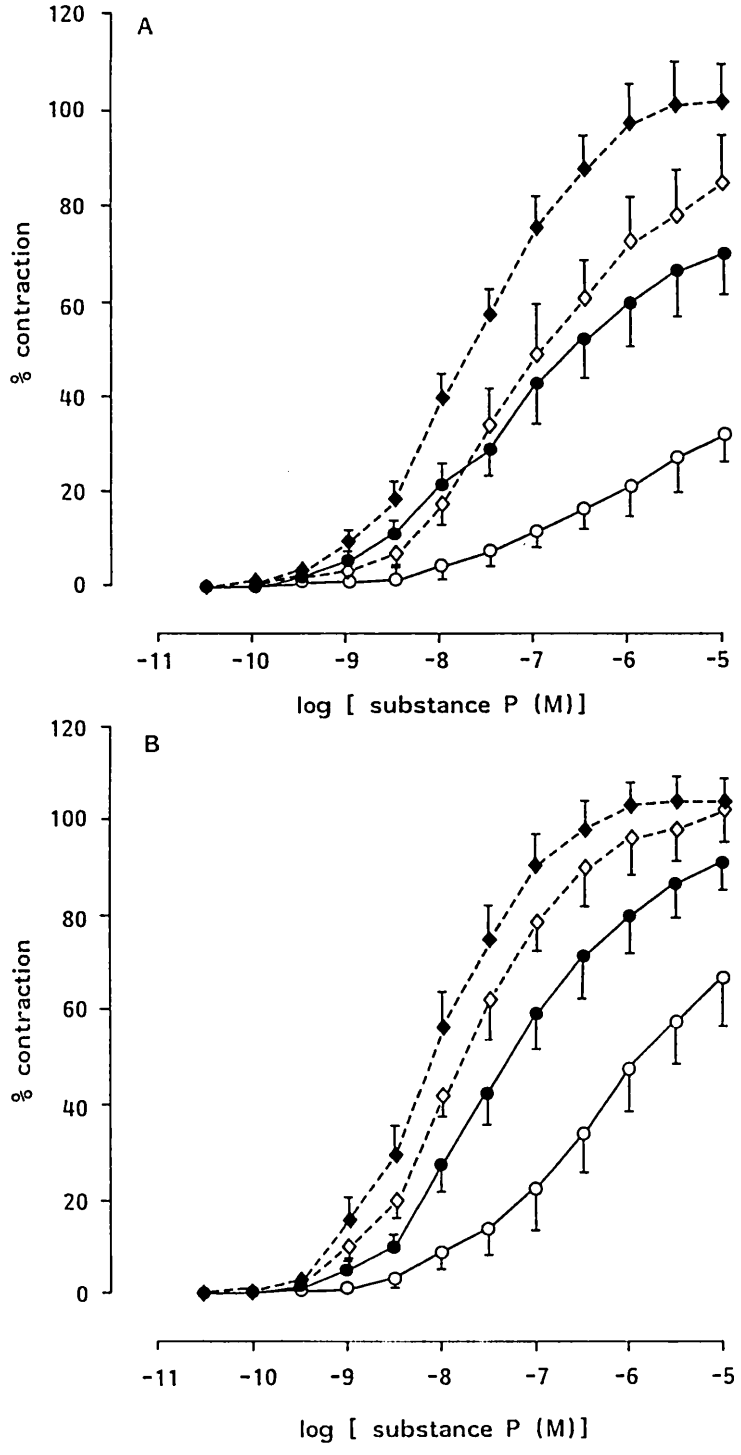


Figure 15. Effect of NEP inhibition on responses of guinea-pig trachea to SP. In (a) tissues were untreated (○intact, ● epithelium-denuded preparations) or pretreated with 10 uM phosphoramidon (◇ intact, ◆ epithelium -denuded preparations). In (b) tissues were pretreated with 5 uM indomethacin alone (○ intact, ● epithelium-denuded preparations) or with 10 uM indomethacin and 10 uM phosphoramidon (◇ intact, ◆ epithelium-denuded preparations). Points represent means ± SE of n=10.

In the presence of phosphoramidon, epithelium removal still resulted in a potentiation (4.3-fold for mean -log EC_{30}s and 3.7-fold for mean pD_2s) of SP-induced contractions, and this effect was smaller than in untreated preparations (138-fold for mean -log EC_{30}s). The effect of phosphoramidon on maximal responses to SP could not be determined in intact preparations. In epithelium-denuded preparations phosphoramidon potentiated the maximal contraction induced by SP (71.8 $\pm$ 9.3 % vs. 106.5 $\pm$ 8.1 % of $carb_{max}$ untreated vs. phosphoramidon-treated preparations, $p<0.05$).

Phosphoramidon had no effect on maximal contraction induced by 10 uM carbachol in intact and epithelium-denuded preparations (table 7).

Phosphoramidon had no effect on contractile responses of intact preparations to acetylcholine (pD_2 5.05 $\pm$ 0.25 untreated, 4.77 $\pm$0.26 treated preparations, n=5).

3.2.1.5. Effect of combined cyclooxygenase and NEP inhibition

In the presence of indomethacin, phosphoramidon caused a further potentiation of NKA-induced contractions in intact, but not epithelium-denuded, preparations (figure 13b, table 4). In intact preparations, mean -log EC_{30}s and pD_2s for indomethacin-treated and indomethacin plus phosphoramidon-treated preparations were significantly different ($p<0.01$) and yielded

concentration-ratios of 9.5 for mean -log EC_{30}s and 12 for mean pD_2s. In epithelium-denuded preparations there was no significant difference between mean -log EC_{30}s or between mean pD_2s for indomethacin-treated and indomethacin plus phosphoramidon-treated preparations. In the presence of indomethacin and phosphoramidon, epithelium removal failed to potentiate NKA-induced contractions, with no significant differences in mean -log EC_{30}s or pD_2s being observed between intact and epithelium-denuded preparations (table 4).

In the presence of indomethacin, phosphoramidon caused a further potentiation of NKB-induced contractions in intact, but not epithelium-denuded, preparations (figure 14b, table 5). In intact preparations, mean -log EC_{30}s and pD_2s for indomethacin-treated and indomethacin plus phosphoramidon-treated preparations were significantly different ($p<0.05$) and yielded concentration-ratios of 6.8 for mean -log EC_{30}s and 4.6 for mean pD_2s. In epithelium-denuded preparations there was no significant difference between mean -log EC_{30}s or between mean pD_2s for indomethacin-treated and indomethacin plus phosphoramidon-treated preparations. In the presence of indomethacin and phosphoramidon, epithelium removal failed to potentiate NKB-induced contractions, with no significant differences in mean -log EC_{30}s and pD_2s being observed between intact and epithelium-denuded preparations (table 5).

In the presence of indomethacin, phosphoramidon

caused a further potentiation of SP-induced contractions in intact and epithelium-denuded preparations (figure 15b, table 6). In intact preparations, mean -log EC_{30}s and pD_2s for indomethacin-treated and indomethacin plus phosphoramidon-treated preparations were significantly different ($p<0.01$) and yielded concentration-ratios of 31 for mean -log EC_{30}s and 47 for mean pD_2s. In epithelium-denuded preparations, mean -log EC_{30}s and pD_2s for indomethacin-treated and indomethacin plus phosphoramidon-treated preparations were significantly different ($p<0.05$) and yielded concentration-ratios of 4.5 for mean -log EC_{30}s and 4.6 for mean pD_2s. In the presence of indomethacin and phosphoramidon, epithelium removal failed to potentiate SP-induced contractions, with no significant differences in mean -log EC_{30}s or pD_2s being observed between intact and epithelium-denuded preparations (table 6).

Indomethacin and phosphoramidon had no effect on maximal contraction induced by 10 uM carbachol in intact and epithelium-denuded preparations (table 7).

4.3.2. Human bronchi

NKA caused concentration-dependent contractions of human bronchi. Maximal or even 50% of maximal contraction was not achieved, therefore a pD_2 could not be determined.

Epithelium removal produced a 2.3-fold increase in the potency of acetylcholine (pD_2 4.42 $\pm$ 0.11 intact

preparations, 4.78 ± 0.08 epithelium-denuded preparations, $p<0.05$, n=6), with no effect on maximal contraction (1976 ± 237 intact preparations, 1765 ± 222 mg epithelium-denuded preparations), (figure 16). Epithelium removal had no effect on NKA-induced contraction (figure 17), with no significant difference being observed between mean -log EC_{30}s for intact (6.91 ± 0.23) and epithelium-denuded (7.19 ± 0.08) preparations (n=8).

Phosphoramidon had no effect on resting tension but caused a potentiation of NKA-induced contraction of intact and epithelium-denuded preparations (figure 17). In intact preparations, mean -log EC_{30}s for untreated (6.91 ± 0.23) and phosphoramidon-treated (8.05 ± 0.31) preparations were significantly different ($p<0.01$, n=8) and yielded a concentration-ratio of 13. In epithelium-denuded preparations mean -log EC_{30}s for untreated (7.19 ± 0.08) and phosphoramidon-treated (8.42 ± 0.16) preparations were significantly different ($p<0.01$, n=8) and yielded a concentration-ratio of 17. In the presence of phosphoramidon there was no significant difference between mean -log EC_{30}s for intact and epithelium-denuded preparations. However, mean pD_2s for intact (7.77 ± 0.08) and epithelium-denuded (8.18 ± 0.14) preparations in the presence of phosphoramidon were significantly different ($p<0.05$) and yielded a concentration-ratio of 2.6. The effect of phosphoramidon on maximal responses to NKA could not be determined. Phosphoramidon had no effect on the maximum contraction induced by 10 uM carbachol at the end

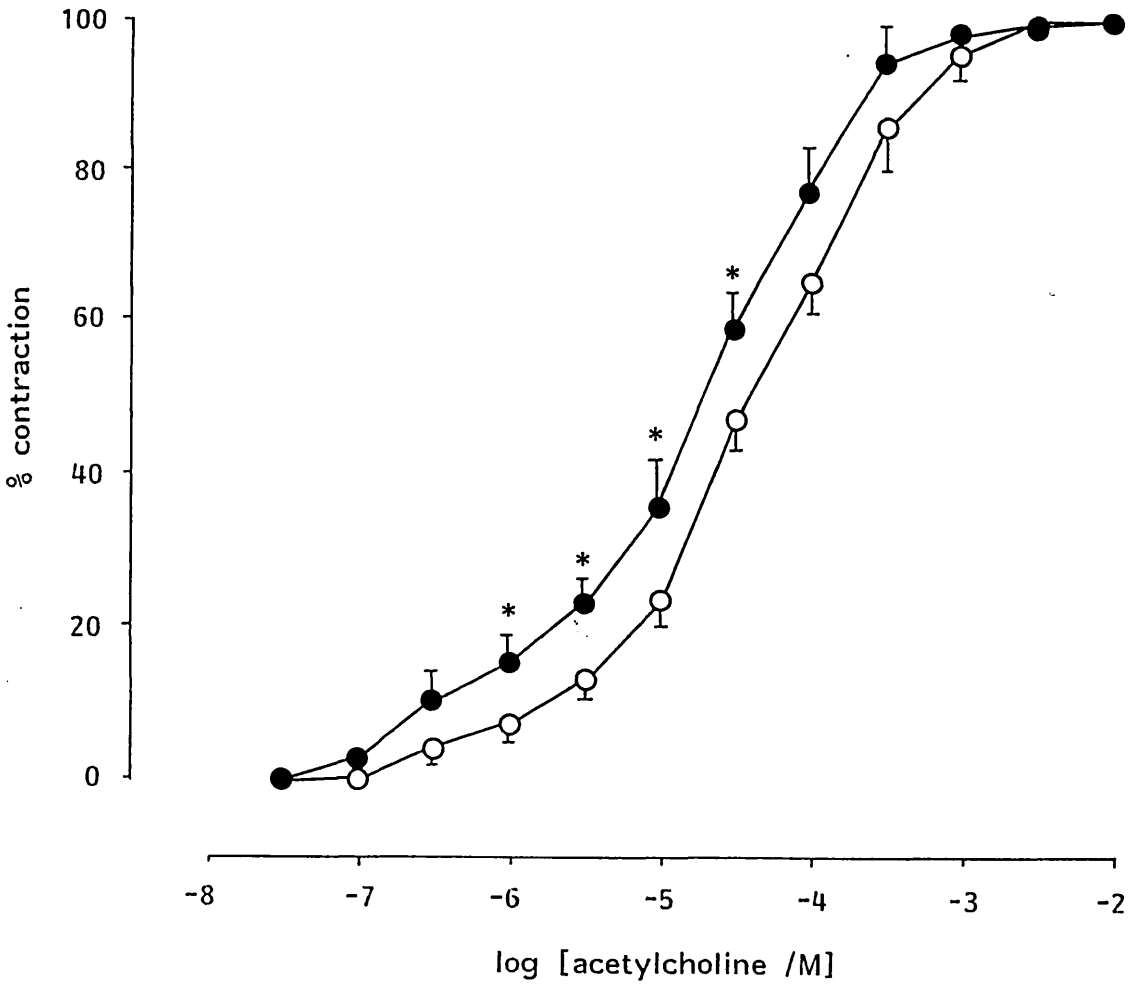


Figure 16. Effect of epithelium removal on responses of human bronchi to acetylcholine (○intact, ● epithelium-denuded preparations). Points represent means ± SE of n=6, * $p<0.05$ intact vs. epithelium-denuded preparations.

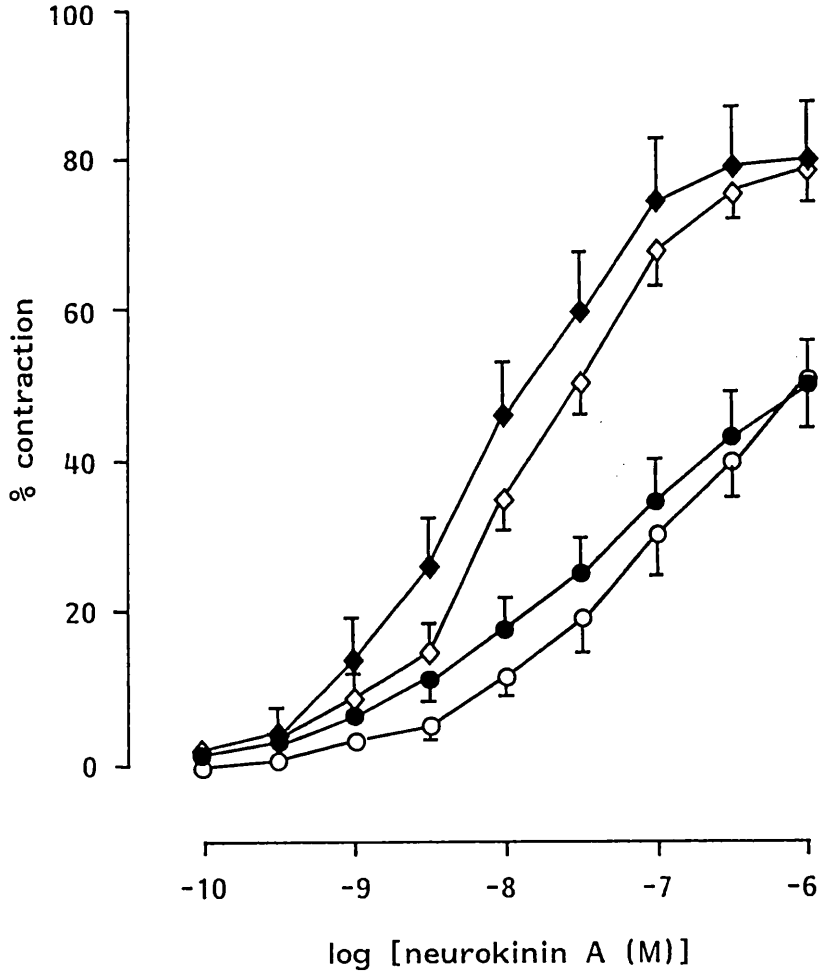


Figure 17. Effect of epithelium removal and NEP inhibiton on responses of human bronchi to NKA. Tissues were untreated (○ intact, ● epithelium-denuded preparations) or pretreated with 10 *u*M phosphoramidon (◇ intact, ◆ epithelium-denuded preparations). Points represent means ± SE of n=8.

of each experiment in intact (2471 ± 273 mg untreated , 2493 ± 405 mg phosphoramidon-treated) or epithelium-denuded (2506 ± 360 mg untreated, 2275 ± 333 mg phosphoramidon-treated) preparations.

Indomethacin had no effect on resting tension or on NKA-induced contraction of human bronchi (figure 18), with no significant difference being observed between mean -log EC_{30}s for untreated (6.81 ± 0.13) and indomethacin-treated (6.65 ± 0.13) preparations (n=8). Indomethacin had no effect on maximal contraction induced by 10 uM carbachol (2471 ± 273 mg untreated, 1983 ± 341 mg indomethacin-treated preparations).

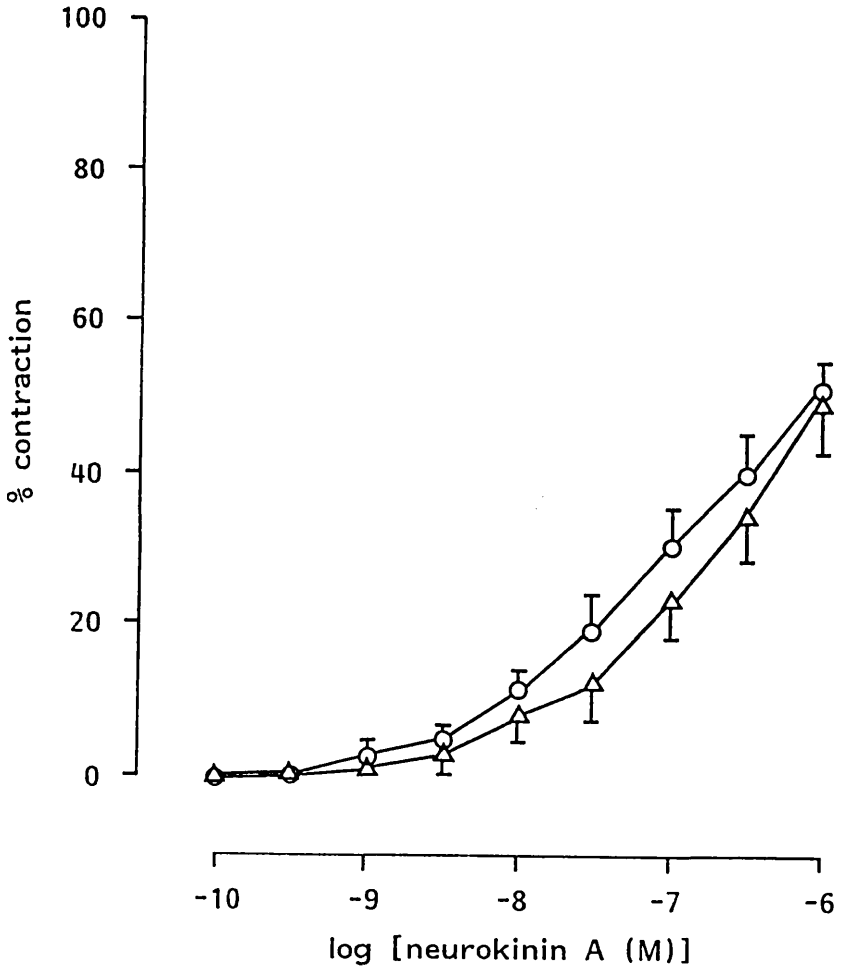


Figure 18. Effect of cyclooxygenase inhibition on responses of human bronchi to NKA. Tissues were untreated (O) or pretreated with 5 uM indomethacin (Δ). Points represent means ± SE of n=8.

4.4. Discussion

Airway epithelium is known to modulate the reactivity of the underlying smooth muscle to contractile and relaxant agonists (see introduction). The mechanism responsible for this effect is unclear and indeed a variety of pathways may be involved. Our results suggest that the modulation of responses of the guinea-pig trachea to tachykinins may be mediated in part by the release of cyclooxygenase metabolites of arachidonic acid and in part by the enzymatic degradation of peptides by NEP located in the epithelial layer.

Epithelium removal caused a potentiation of contractile responses of the guinea-pig trachea to NKA, NKB and SP which was reflected in an increase in the potency of tachykinins. Potentiation was greater than that generally reported for other agonists (Murlas, 1986; Goldie *et al.*, 1986; Holroyd, 1986; Hay *et al.*, 1986) esting that additional factors may be involved in the epithelial modulation of responses to tachykinins. Potentiation was similar to that reported by others (Tschirhart & Landry, 1986; Devillier *et al.*, 1988) although a recent report has found that epithelium removal has no effect on contraction of guinea-pig trachea to SP (Lunblad & Persson, 1988). The effect of epithelium removal on the maximal response of guinea-pig trachea to tachykinins could not be determined in our experiments, however, responsiveness to other agonists is generally

unchanged (Murlas, 1986; Goldie *et al.*, 1986; Holroyde, 1986).

Cyclooxygenase products are involved in the maintenance of tone in the guinea-pig trachea and may modulate its sensitivity and/or responsiveness to contractile agonists (Orehek *et al.*, 1975). Indomethacin caused a potentiation of contractile responses to NKA, NKB and SP suggesting that responses to tachykinins may be modulated by cyclooxygenase products. Potentiation was greater in the presence than absence of epithelium, suggesting that cyclooxygenase products may originate at least in part from the epithelium. Cyclooxygenase has been localised to airway epithelium (Xu *et al.*, 1986; Butler *et al.*, 1987) and cyclooxygenase products are released by cultured epithelial cells (Leikauf *et al.*, 1985). Qualitative and quantitative differences in the potentiation of contraction by indomethacin were observed between the three tachykinins. Indomethacin increased the potency of all three tachykinins in intact preparations, whereas only that of NKA was increased in epithelium-denuded preparations. SP-induced contractions were only potentiated in intact preparations. Cyclooxygenase products released by the epithelial layer may therefore be more important in modulating responses of SP and NKB than to NKA, with cyclooxygenase products from other sources within the airways also contributing to the modulation of NKA-induced contractions. In the presence of indomethacin, modulation of responses to tachykinins by

the epithelium was diminished but was still evident, suggesting that whereas cyclooxygenase products released by the epithelium may contribute to its modulatory influence, other mechanisms may also be involved.

Phosphoramidon caused a potentiation of contractile responses of the guinea-pig trachea to NKA, NKB and SP suggesting that tachykinins may be degraded by endogenous NEP. Phosphoramidon had no effect on responses to acetylcholine thus eliminating the possibility of a non-specific effect of phosphoramidon. Potentiation was greatest for SP suggesting that this tachykinin may be more susceptible to NEP activity than NKA or NKB. This is in contrast with findings in the ferret trachea which suggest NKB to be the most potently affected by NEP (Sekizawa *et al.*, 1987b) and in the guinea-pig trachea which suggest NKA to be the most potently affected by NEP (Devillier *et al.*, 1988). Potentiation of responses of guinea-pig trachea to NKA by phosphoramidon mimicked the effect of epithelium removal and was absent from epithelium-denuded preparations. NKB- and SP-induced responses were potentiated in intact and epithelium-denuded preparations, but potentiation was greatest in the presence of epithelium. These results suggest that NEP may be primarily located in the epithelial layer and that degradation of tachykinins by NEP may contribute to (or account for in the case of NKA) the modulatory effect of epithelium on smooth muscle reactivity. Studies on the ferret and guinea-pig trachea

have demonstrated that the modulatory effect of epithelium on responses to all three tachykinins is abolished by thiorphan (Sekizawa *et al.*, 1987b; Devillier *et al.*, 1988). In the ferret trachea NEP is localised to the interstitium, smooth muscle and epithelium (Sekizawa *et al.*, 1987b). Hence, epithelium removal would reduce NEP levels and increase the concentration of tachykinins in the smooth muscle layer. This would be particularly important to exogenously applied tachykinins since agonist molecules must pass the epithelial layer in order to reach the smooth muscle. The small potentiation of SP- and NKB-induced contractions in epithelium-denuded preparations would result from degradation of tachykinins by NEP in the interstitium and smooth muscle. Modulation of SP- and NKB-induced responses by the epithelium could still be observed in the presence of phosphoramidon, but was abolished by the combination of phosphoramidon and indomethacin. Epithelial modulation of responses to SP and NKB therefore results from the combined effects of degradation by NEP and the release of inhibitory cyclooxygenase products from the epithelium. A recent report has demonstrated a residual effect of epithelium removal on SP-induced contraction in the presence of phosphoramidon and indomethacin, suggesting that additional factors may be involved (Fine *et al.*, 1989).

Epithelium removal has also been shown to potentiate the sensitivity of human tracheal and bronchial smooth

muscle to contractile agonists (Raeburn *et al.*, 1986; Aizawa *et al.*, 1988) and indeed we were able to show a small potentiation of acetylcholine-induced responses. Epithelium removal, however, tended to but failed to significantly potentiate contractile responses of human bronchi to NKA. Histological examination of transverse sections revealed that the epithelium was often damaged in "normal" airways, and therefore the modulatory effect of the epithelium may already be absent or reduced. A recent study has demonstrated a 4-5 fold potentiation of responses to NKA in human bronchi by epithelium removal (Naline *et al.*, 1988).

Indomethacin had no effect on resting tone of human bronchi suggesting that cyclooxygenase products do not contribute to muscle tone in human airways (Brink *et al.*, 1980; Davis *et al.*, 1982). Human lung is able to generate large quantities of cyclooxygenase products (Piper & Walker, 1973; Haye-Legrand *et al.*, 1986) but they do not appear to modulate contractile responses *in vitro* (Brink *et al.*, 1980; Haye-Legrand *et al.*, 1986). In agreement with this, indomethacin had no effect on NKA-induced contraction of human bronchi in our experiments.

Phosphoramidon potentiates NKA-induced contraction of human bronchi (Black *et al.*, 1988; Naline *et al.*, 1988). Although an effect of epithelium removal could not be demonstrated in untreated preparations, potentiation of NKA-induced contraction by phosphoramidon was consistently observed in rubbed and unrubbed preparations suggesting

that NKA is degraded by NEP located in non-epithelial sites. This contrasts with our findings on the guinea-pig trachea which suggest the epithelium to be the primary site of degradation of NKA by NEP. In the presence of phosphoramidon, epithelium removal did in fact increase the sensitivity of human bronchi to NKA suggesting that modulation of smooth muscle responses in human airways by airway epithelium must involve mechanisms other than enzymatic degradation of neuropeptides by NEP.

In conclusion, contractile responses of guinea-pig trachea to tachykinins are modulated by airway epithelium. Modulation may be due to the combined effects of degradation of tachykinins by NEP located in the epithelial layer and the release of inhibitory cyclooxygeanse products from the epithelium. Epithelial modulation of smooth muscle responsiveness may also occur in human airways although the mechanism is unclear. Degradation of tachykinins by NEP in non-epithelial sites may also occur in human and guinea-pig airways.

5. Effect of NEP inhibition on contraction of guinea-pig bronchi induced by e-NANC nerve stimulation and by tachykinins

5.1. Aim

The observation that responses of isolated ASM to exogenous tachykinins are modulated by NEP raises the possibility that tachykinins released endogenously by peptidergic nerves in the airways may also be susceptible to degradation by NEP. Guinea-pig bronchi possess an e-NANC innervation, for which tachykinins are putative neurotransmitters (Lundberg *et al.*, 1983c). The aim of the present study was therefore to investigate the effect of the NEP inhibitor phosphoramidon on e-NANC nerve-induced responses of guinea-pig bronchi evoked by EFS and on responses induced by exogenous tachykinins.

5.2. Protocol

All experiments were carried out in the presence of 5 uM indomethacin in order to prevent the generation of cyclooxygenase metabolites of arachidonic acid. Indomethacin was included in the KH solution at the start of the equilibration period and was present throughout.

5.2.1. EFS parameters

Contractile responses to the stimulation of e-NANC nerves by EFS were examined in guinea-pig bronchi. EFS was applied for 30 s at intervals of 30 min. Optimum parameters for the stimulation of e-NANC nerves were determined by constructing stimulus-response curves over a pulse duration range of 0.01-1 ms, voltage range of 1-60 V and frequency range of 0.25-32 Hz. E-NANC responses tended to decrease with time, therefore a maximum of 6 stimulations were applied on each preparation. For each stimulus parameter, three control responses were obtained, tissues were incubated for 30 min with 3 uM TTX, and EFS repeated three times.

For all subsequent experiments a pulse duration of 0.5 ms, voltage of 40 V and frequency of 8 Hz were chosen as eliciting a maximal response with a minimal TTX-resistant component.

5.2.2. NEP inhibition

The effect of NEP inhibition on e-NANC responses of guinea-pig main and hilar bronchi was examined by field stimulating tissues three times to obtain control responses, incubating with 10 uM phosphoramidon for 30 min and repeating three stimulations. Contractile responses to EFS were compared to a maximal carbachol-induced contraction obtained at the end of each experiment.

Cumulative C-R curves to NKA, NKB and SP were constructed in guinea-pig main and hilar bronchi. Adjacent preparations were pretreated for 30 min with and without 10 uM phosphoramidon. Only one tachykinin C-R curve was constructed in each preparation and responses were compared to the contraction induced by 10 uM carbachol at the end of each experiment.

5.3. Results

5.3.1. EFS parameters

EFS of guinea-pig hilar bronchi induced a rapid contraction followed by a slow-developing longer-lasting contraction. The magnitude of the second component was dependent on the pulse duration, voltage and frequency of stimulation (figure 19). TTX (3uM) abolished contractile responses to EFS of pulse durations up to 0.2 ms, voltages up to 15 V and frequencies up to 4 Hz. EFS of greater pulse duration, voltage and frequency induced small TTX-resistant contractions. Optimal stimulus parameters were selected from stimulus-response curves. Hence, EFS with a pulse duration of 0.5 ms, voltage of 40 V and frequency of 8 Hz induced a near-maximal contractile response with a minimal TTX-resistant component, and was used in all subsequent experiments.

The second contractile component of the response to EFS (0.5 ms, 40 V, 8 Hz) was unaffected by 1 *u*M atropine or 1 *u*M phentolamine, with responses being 104 $\pm$ 3 % and 100 $\pm$ 4 % respectively of their control level. The response is therefore neither cholinergic nor adrenergic in nature, and is assumed to be due to the stimulation of e-NANC nerves.

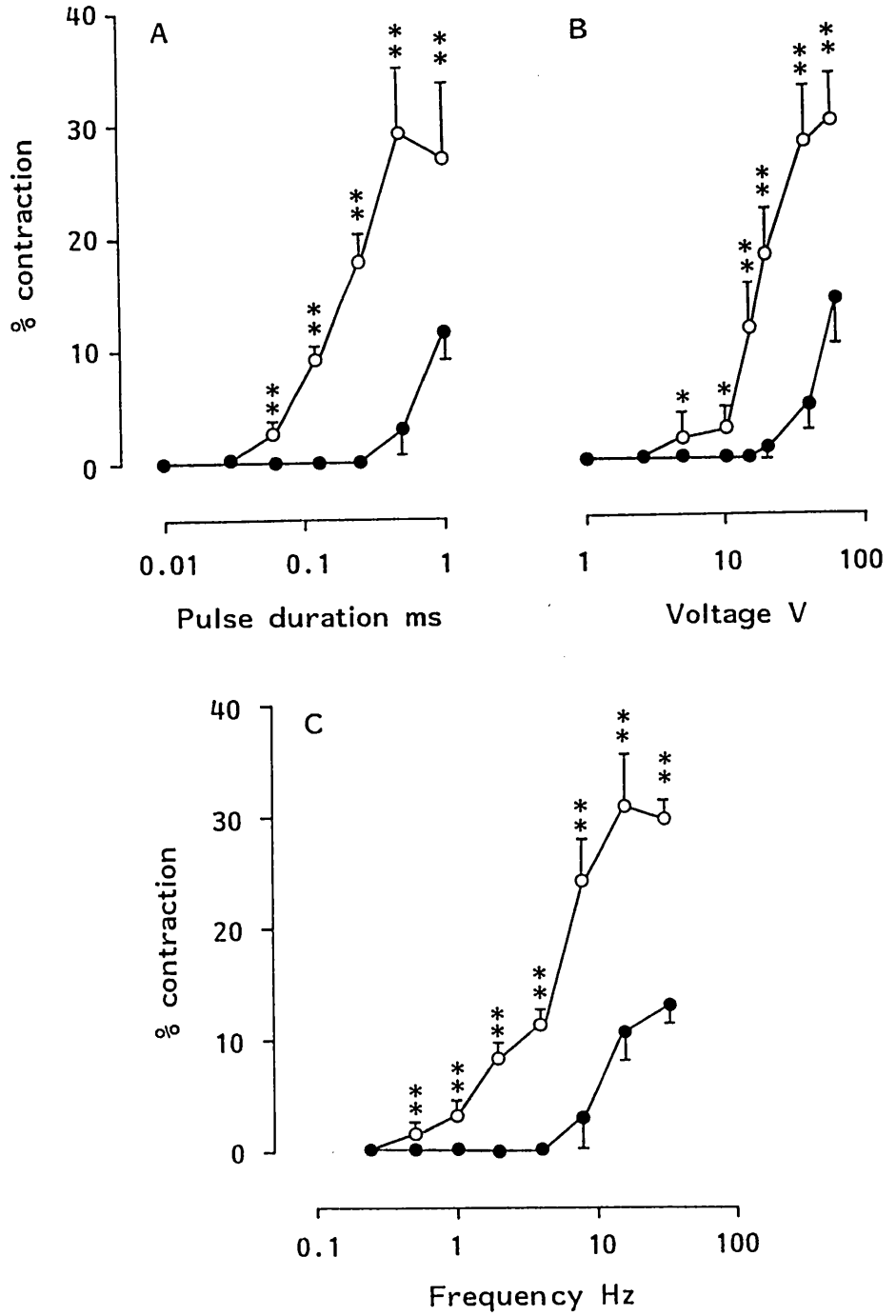


Figure 19. EFS parameters for the stimulation of e-NANC nerves in guinea-pig hilar bronchi. (a) Effect of pulse duration on e-NANC responses induced by EFS (40 V, 8 Hz). (b) Effect of voltage on e-NANC responses induced by EFS (0.5 ms, 8 Hz). (c) Effect of frequency on e-NANC responses to EFS (40 V, 0.5 ms). Responses were obtained in the absence (O) or presence of 3 uM TTX (●). Indomethacin (5 uM) was present throughout. Points represent means ± SE of n=4-7, * $p<0.05$, ** $p<0.01$ absence vs. presence of TTX.

3.2.2.2. Effect of NEP inhibition

Phosphoramidon caused a potentiation of e-NANC responses induced by EFS (0.5 ms, 40 V, 8 Hz) in guinea-pig bronchi (figures 20 and 21). Phosphoramidon increased the magnitude of e-NANC contractions by 45.6 $\pm$ 9.3 % in main bronchi and by 52.8 $\pm$ 9.6 % in hilar bronchi. Phosphoramidon also increased the duration of e-NANC responses induced by EFS. In main bronchi, the mean time taken for 50 % recovery of baseline tension ($t_{50\%}$) in control (8.1 $\pm$ 1.6 min) and phosphoramidon-treated (17.0 $\pm$ 4.6 min) preparations were significantly different ($p<0.01$). Similarly, in hilar bronchi mean $t_{50\%}$s in control (9.4 $\pm$ 3.1 min) and phosphoramidon-treated (32.3 $\pm$ 4.4 min) preparations were significantly different ($p<0.01$). In the presence of phosphoramidon (but not in its absence), e-NANC responses induced by EFS in hilar bronchi were longer-lasting than those induced in main bronchi, with a significant difference in mean $t_{50\%}$s ($p<0.01$).

Contractile responses of guinea-pig bronchi to tachykinins were also potentiated by 10 uM phosphoramidon (figures 22 and 23; table 8). In main bronchi, phosphoramidon caused a 93-fold, 148-fold and 141-fold increase in mean EC_{30}s for NKA, NKB and SP respectively. In hilar bronchi, phosphoramidon caused a 115-fold, 115-fold and 245-fold increase in mean EC_{30}s for NKA, NKB and SP respectively.

Phosphoramidon potentiated contractile responses of

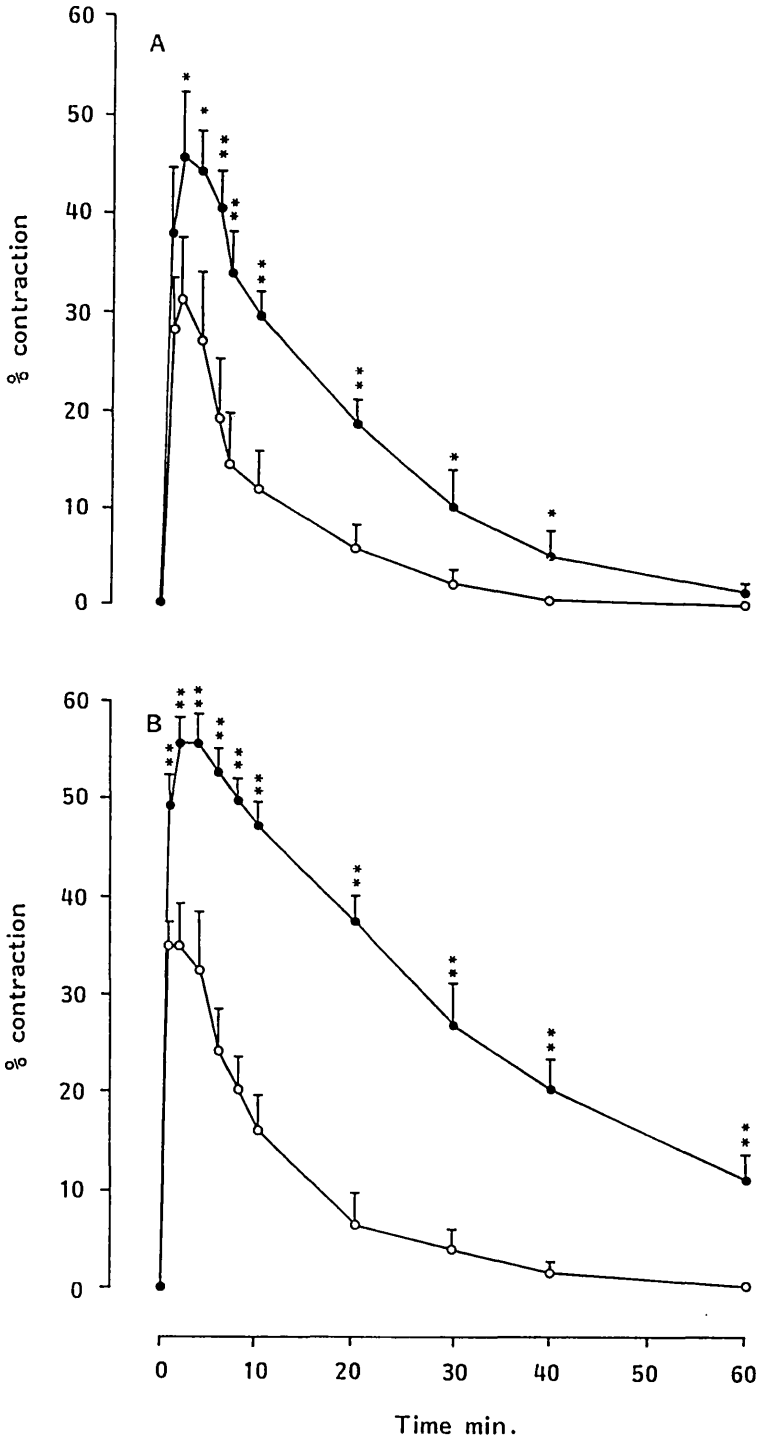


Figure 20. Effect of NEP inhibition on e-NANC responses induced by EFS in guinea-pig bronchi. EFS (0.5 ms, 40 V, 8 Hz) was applied to (a) main bronchi and (b) hilar bronchi in the absence (O) and presence (●) of 10 uM phosphoramidon. Indomethacin (5 uM) was present throughout. Points represent mean ± SE of n=5, * $p<0.05$, ** $p<0.01$ absence vs. presence of phosphoramidon.

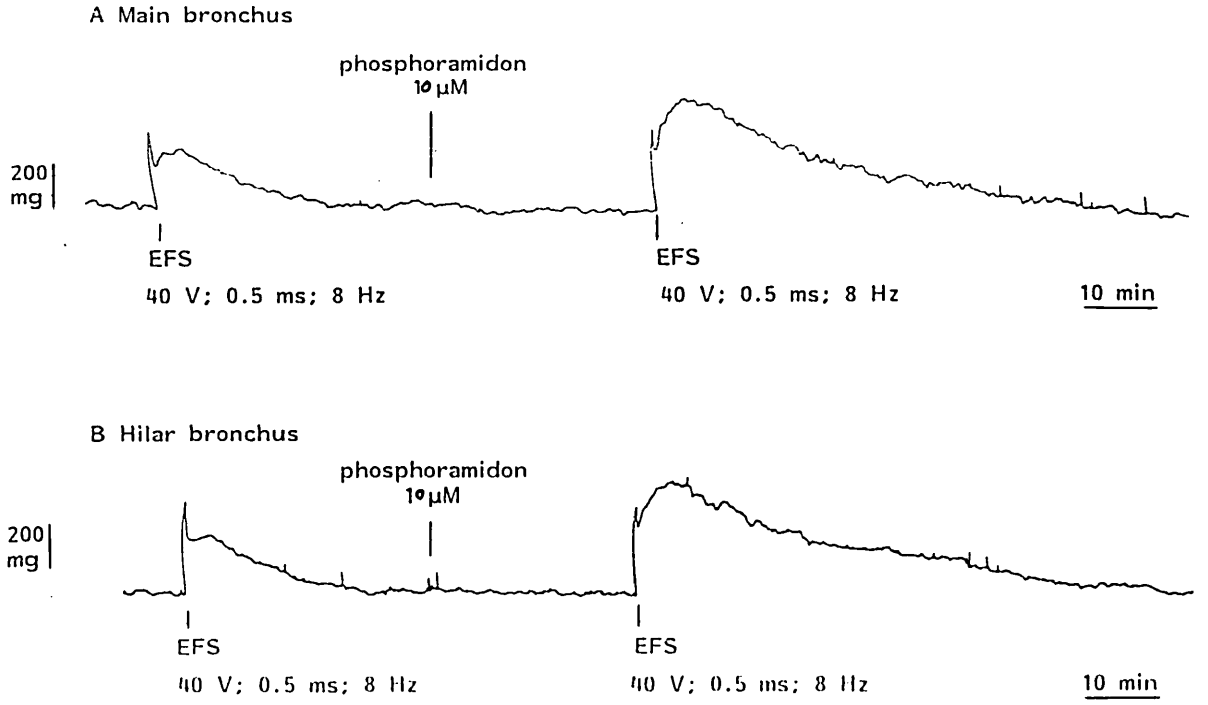


Figure 21. Representative tracing showing effect of phosphoramidon on responses of (A) main bronchi and (B) hilar bronchi to stimulation of e-NANC nerves. Responses were induced by EFS (0.5 ms, 40 V, 8 Hz). Indomethacin (5 uM) was present throughout.
N.B. Phosphoramidon-treated tissues did not always return to baseline tension following EFS.

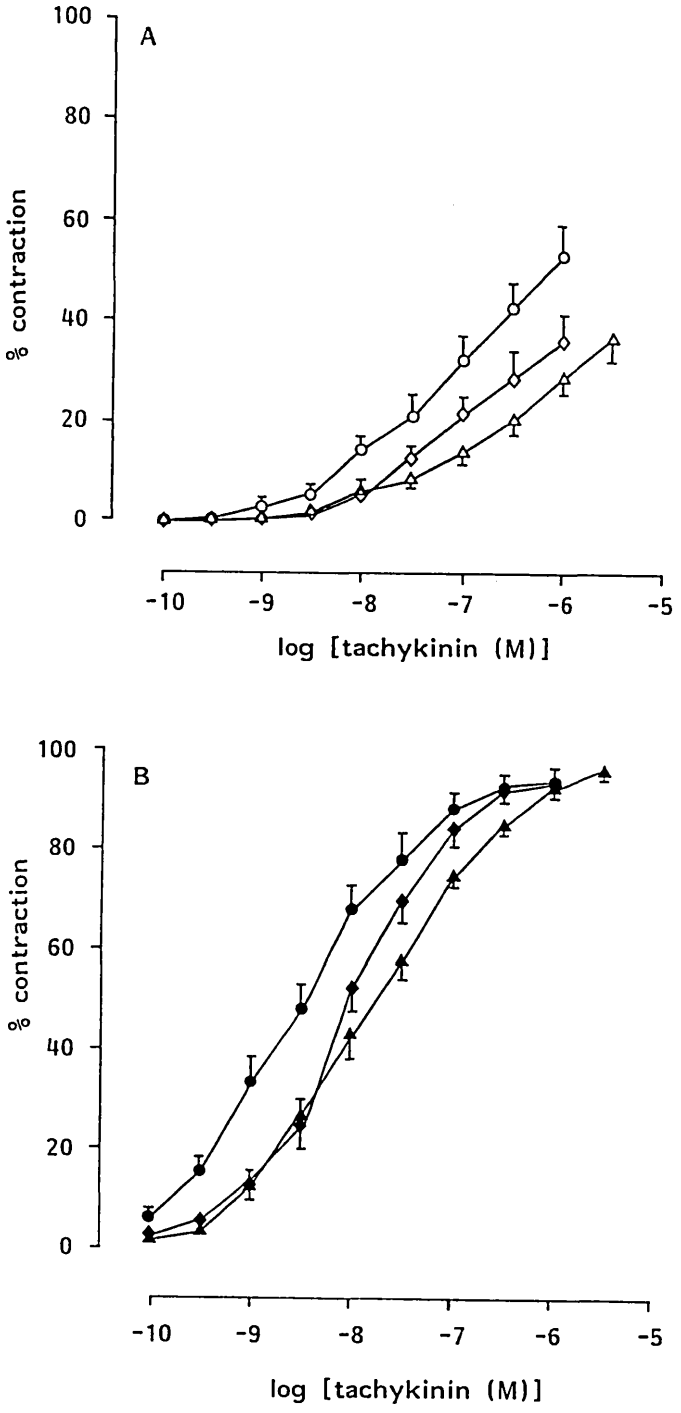


Figure 22. Effect of NEP inhibition on responses of guinea-pig main bronchi to tachykinins. Responses were obtained in the absence (O NKA, ◇ NKB, Δ SP) or presence of 10 uM phosphoramidon (● NKA, ◆ NKB, ▲ SP). Indomethacin (5 uM) was present throughout. Points represent means ± SE of n=6-8.

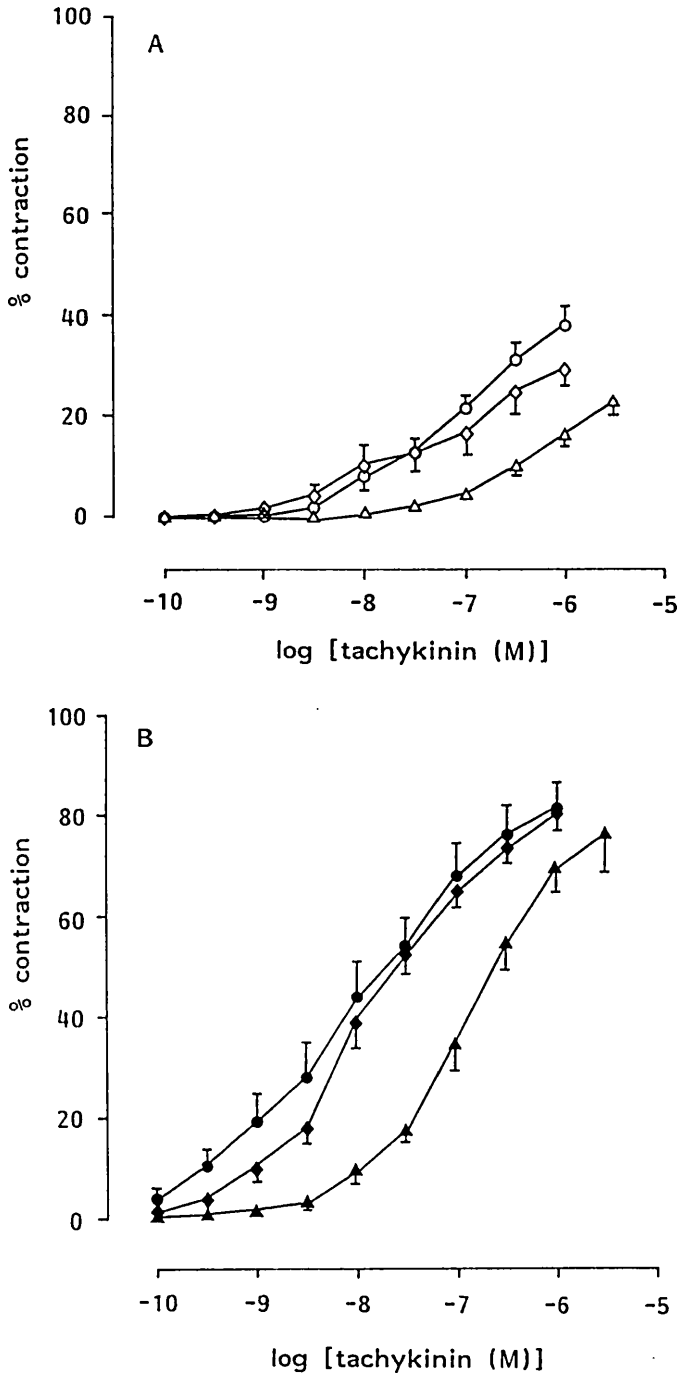


Figure 23. Effect of NEP inhibition on responses of guinea-pig hilar bronchi to tachykinins. Responses were obtained in the absence (○ NKA, ◇ NKB, △ SP) or presence of 10 uM phosphoramidon (● NKA, ◆ NKB, ▲ SP). Indomethacin (5 uM) was present throughout. Points represent means ± SE of n=6.

Table 8. Effect of NEP inhibition on responses of guinea-pig bronchi to NKA, NKB and SP.

	Main bronchus		
	-log EC_{30} [1]	pD_2 [2]	n
Indomethacin:			
NKA	7.01 ± 0.05	-	8
NKB	6.37 ± 0.10	-	6
SP	5.92 ± 0.09	-	7
Indomethacin + phosphoramidon:			
NKA	9.07 ± 0.06*	8.55 ± 0.03	8
NKB	8.43 ± 0.05*	8.06 ± 0.03	6
SP	8.31 ± 0.08*	7.79 ± 0.05	7

	Hilar bronchus		
	-log EC_{30} [1]	pD_2 [2]	n
Indomethacin:			
NKA	6.45 ± 0.21	-	6
NKB	5.98 ± 0.18	-	6
SP	4.95	-	6
Indomethacin + phosphoramidon:			
NKA	8.42 ± 0.21*	8.06 ± 0.22	6
NKB	8.15 ± 0.22*	7.81 ± 0.12	6
SP	7.10 ± 0.16*	6.80 ± 0.08	6

1. EC_{30} = tachykinin concentration inducing 30 % of maximal carbachol-induced contraction.
2. pD_2 = -log EC_{50}.
Figures represent means ± SE of n preparations.
* $p<0.05$ unpaired Student's T-test indomethacin-treated vs. indomethacin plus phosphoramidon-treated.

guinea-pig bronchi to exogenous tachykinins to a greater degree than comparable e-NANC responses induced by EFS. In the absence of phosphoramidon 0.98 nM NKA, 0.43 *u*M NKB and 1.20 *u*M SP induced contractions (30 % of $carb_{max}$) that were similar in magnitude to contractions induced by EFS (0.5 ms, 40 V, 8 Hz). Phosphoramidon (10 *u*M) potentiated contractions induced by 0.98 nM NKA, 0.43 *u*M NKB and 1.20 *u*M SP by 191 %, 206 % and 211 % respectively. This contrasts with the 45.6 % potentiation of EFS-induced e-NANC responses by phosphoramidon.

5.4. Discussion

EFS of guinea-pig bronchi induced a contractile response which was neurogenic in nature but was not due to the activation of adrenegic or cholinergic nerves. E-NANC responses of guinea-pig bronchi are antagonised by tachykinin-receptor antagonists (Lundberg *et al.*, 1983c; Leander *et al.*, 1984; Karlsson *et al.*, 1984) suggesting that they are mediated by the release of tachykinins from peptidergic nerves in airway smooth muscle.

Phosphoramidon caused a potentiation of contractile responses evoked by the stimulation of e-NANC nerves in guinea-pig bronchi suggesting that endogenously released tachykinins may be degraded by NEP. A similar finding has been reported recently for guinea-pig bronchi (Djokic *et al.*, 1989). In the ferret trachea, SP enhances the release of acetylcholine from cholinergic nerve terminals and this effect is potentiated by phosphoramidon (Sekizawa *et al.*, 1987a). Phosphoramidon potentiated not only the magnitude of e-NANC responses but also their duration. The action of neurotransmitters on postsynaptic membranes is terminated by neuronal or extraneuronal uptake of neurotransmitter molecules, by enzymatic degradation and by diffusion away from the synaptic cleft. If NEP has a functional role in the termination of responses induced by endogenously released tachykinins, then NEP inhibition would prolong their duration of action. In the absence of phosphoramidon e-NANC responses of main and hilar bronchi were of similar

magnitude and duration. In the presence of phosphoramidon the magnitude of e-NANC responses was similar in main and hilar bronchi but were of a longer duration in hilar bronchi. This may reflect a greater importance of NEP in the inactivation of tachykinins in hilar bronchi as opposed to main bronchi.

Phosphoramidon also potentiated contractile responses of guinea-pig bronchi to exogenous tachykinins. Potentiation was greater than that observed in the guinea-pig trachea suggesting that NEP activity may be greater in small airways. Potentiation of responses to exogenous tachykinins was greater than the potentiation of comparable e-NANC responses evoked by EFS. This could reflect the importance of NEP located in the epithelial layer and the interstitium in the degradation of exogenous but not endogenous tachykinins. E-NANC responses evoked by EFS result from the local release of neurotransmitter from nerve terminals in smooth muscle and tachykinins released endogenously would therefore not be expected to be degraded by NEP in the epithelium and interstitium. Exogenously applied tachykinins on the other hand would have to pass through the epithelial layer and interstitium before reaching the smooth muscle and would be more susceptible to degradation by NEP.

In conclusion, contractile responses of guinea-pig bronchi induced by endogenous and exogenous tachykinins may be modulated by NEP in the airways.

6. Characterisation of tachykinin receptors on guinea-pig and human ASM

6.1. Aim

Agonist potency ratios suggest the existence of three tachykinin receptor subtypes (see introduction), although a fourth type has been proposed to exist on guinea-pig tracheal smooth muscle (McKnight *et al.*, 1987 & 1988). We have used the natural tachykinin-receptor agonists NKA, NKB and SP, selective agonists to NK1-, NK2- NK3- and the putative NK4-receptor, and selective NK2-receptor antagonists, to characterise receptors mediating tachykinin-induced contraction of guinea-pig and human ASM. Since tachykinins may mediate responses induced by the stimulation of e-NANC nerves (Lundberg *et al.*, 1983c) we have also examined the effect of a selective NK2-receptor antagonist on e-NANC contractions of guinea-pig bronchi induced by EFS. Tachykinins may be degraded in the airways by NEP (Sekizawa *et al.*, 1987a & 1987b) therefore we have examined the effect of NEP inhibition by phosphoramidon on the classification of tachykinin-receptors.

6.2. Protocol

All experiments on guinea-pig airways were carried out in the presence of 5 uM indomethacin in order to prevent the generation of cyclooxygenase metabolites of arachidonic acid. Indomethacin was included in the KH solution at the begining of the equilibration period.

Cumulative C-R curves to tachykinins and tachykinin-related peptides were constructed in guinea-pig tracheal and bronchial preparations, and in human bronchial preparations. For agonist studies, adjacent preparations were pretreated for 30 min with and without 10 uM phosphoramidon. For antagonist studies, all preparations were pretreated for 30 min with 10 uM phosphoramidon, and adjacent preparations were pretreated for 15 min with the appropriate antagonist or solvent (0.1 % DMSO). Only one tachykinin C-R curve was constructed in each preparation and responses to tachykinins were compared to the response to 10 uM carbachol determined at the end of each experiment.

The effect of tachykinin-receptor antagonists on contractile responses induced by the stimulation of e-NANC nerves was examined in guinea-pig bronchial preparations. E-NANC responses were induced by EFS (0.5 ms; 40 V; 8 Hz for 30 s). Tissues were field stimulated three times to obtain control responses, incubated with antagonist or solvent (0.1 % DMSO) for 15 min and EFS repeated. In order to allow a comparison with the effect of antagonists on

NKA-induced contractions, experiments were also carried out in the presence of 10 uM phosphoramidon. However, we have previously shown that in the presence of phosphoramidon, tension did not always return to baseline level, therefore repeated electrical stimulations could not be made. Instead, control e-NANC responses to EFS were obtained, and adjacent preparations were incubated with 10 uM phosphoramidon for 30 min and with antagonist or solvent (0.1 % DMSO) for 15 min before repeating EFS once. Contractile responses to EFS were compared to a maximal carbachol-induced contraction obtained at the end of each experiment.

The effect of tachykinin-receptor antagonists on carbachol-induced contractions of guinea-pig and human airways was examined by constructing a control carbachol C-R curve, incubating with antagonist or solvent (0.1 % DMSO) for 15 min and reconstructing a second C-R curve.

6.3. Results

6.3.1. Guinea-pig airways

6.3.1.1. Rank order of potency of tachykinins

The contractile effects of NKA, NKB and SP on guinea-pig airways have already been described in sections 4 and 5, and are summarised in table 9. In indomethacin-treated tracheal preparations, the rank order of potency of tachykinins was NKA > NKB > SP, with NKA being 21 times more potent than SP (as determined from EC_{30} comparisons). In the presence of 10 uM phosphoramidon, the order of potency was maintained although the potency ratio was reduced to 6. The potency of tachykinins decreased from the trachea to hilar bronchi although the rank order of potencies was similar at all airway levels studied (trachea, main bronchus and hilar bronchus). In the absence of phosphoramidon, NKA, NKB and SP were 45, 63 and 67 times more potent in the trachea than hilar bronchi (as determined from EC_{30} comparisons). Potentiation of tachykinin-induced contractions by phosphoramidon was greater in bronchi than in the trachea. For example, phosphoramidon induced a 9-, 115- and 100-fold potentiation of NKA-induced contraction in the trachea, main bronchus and hilar bronchus respectively (as determined from EC_{30} comparisons).

Table 9. Responses of guinea-pig airways to tachykinins

	-log EC_{30}		
	NKA	NKB	SP
Indomethacin:			
trachea	8.10 ± 0.20	7.78 ± 0.13	6.78 ± 0.11
main bronchus	7.01 ± 0.05	6.37 ± 0.10	5.92 ± 0.09
hilar bronchus	6.45 ± 0.21	5.98 ± 0.18	4.95
Indomethacin + phosphoramidon:			
trachea	9.08 ± 0.16	8.61 ± 0.19	8.27 ± 0.14
main bronchus	9.07 ± 0.06	8.43 ± 0.05	8.31 ± 0.08
hilar bronchus	8.42 ± 0.30	8.15 ± 0.22	7.10 ± 0.16

	pD_2		
	NKA	NKB	SP
Indomethacin:			
trachea	7.60 ± 0.16	7.48 ± 0.15	6.16 ± 0.17
main bronchus	-	-	-
hilar bronchus	-	-	-
Indomethacin + phosphoramidon:			
trachea	8.68 ± 0.08	8.14 ± 0.13	7.83 ± 0.12
main bronchus	8.55 ± 0.03	8.06 ± 0.03	7.79 ± 0.05
hilar bronchus	8.06 ± 0.22	7.81 ± 0.12	6.80 ± 0.08

EC_{30} = -log of tachykinin concentration inducing 30 % of maximal carbachol-induced contraction.
pD_2 = -log EC_{50}.
Figures represent means ± SE of 6-8 preparation.

6.3.1.2. Selective tachykinin-receptor agonists

The effects of selective NK1-, NK2- and NK3-receptor agonists on guinea-pig airways is shown in figures 24, 25 and 26.

The NK1-receptor agonist [Sar^9,$Met(O_2)^{11}$]SP induced concentration-dependent contractions of guinea-pig airways, with mean pD_2s of 7.39 $\pm$ 0.24 (trachea, n=6), 7.29 $\pm$ 0.08 (main bronchus, n=6) and 6.55 $\pm$ 0.06 (hilar bronchus, n=6). Maximal responses were not achieved at the highest concentrations of agonist used. Mean pD_2s obtained in the trachea and hilar bronchus were significantly different ($p<0.01$) indicating a decrease in potency with decreasing airway diameter. Phosphoramidon (10 uM) potentiated contraction induced by [Sar^9,$Met(O_2)^{11}$]SP in the trachea (pD_2 7.99 $\pm$ 0.23, $p<0.05$, 4.0-fold potentiation), main bronchus (pD_2 7.79 $\pm$ 0.06, $p<0.05$, 3.2-fold potentiation) and hilar bronchus (pD_2 7.73 $\pm$ 0.05 hilar bronchi, $p<0.05$, 15-fold potentiation 15). In the presence of phosphoramidon, mean pD_2s obtained in the trachea and hilar bronchus were not significantly different.

The NK2-receptor agonist [Nle^{10}]NKA(4-10) induced concentration-dependent contractions of guinea-pig trachea (pD_2 7.77 $\pm$ 0.13, n=6), main bronchus (pD_2 7.43 $\pm$ 0.09, n=6) and hilar bronchus (pD_2 7.18 $\pm$ 0.04, n=6). Mean pD_2s obtained in the trachea and hilar bronchus were significantly different ($p<0.01$) indicating a decrease in

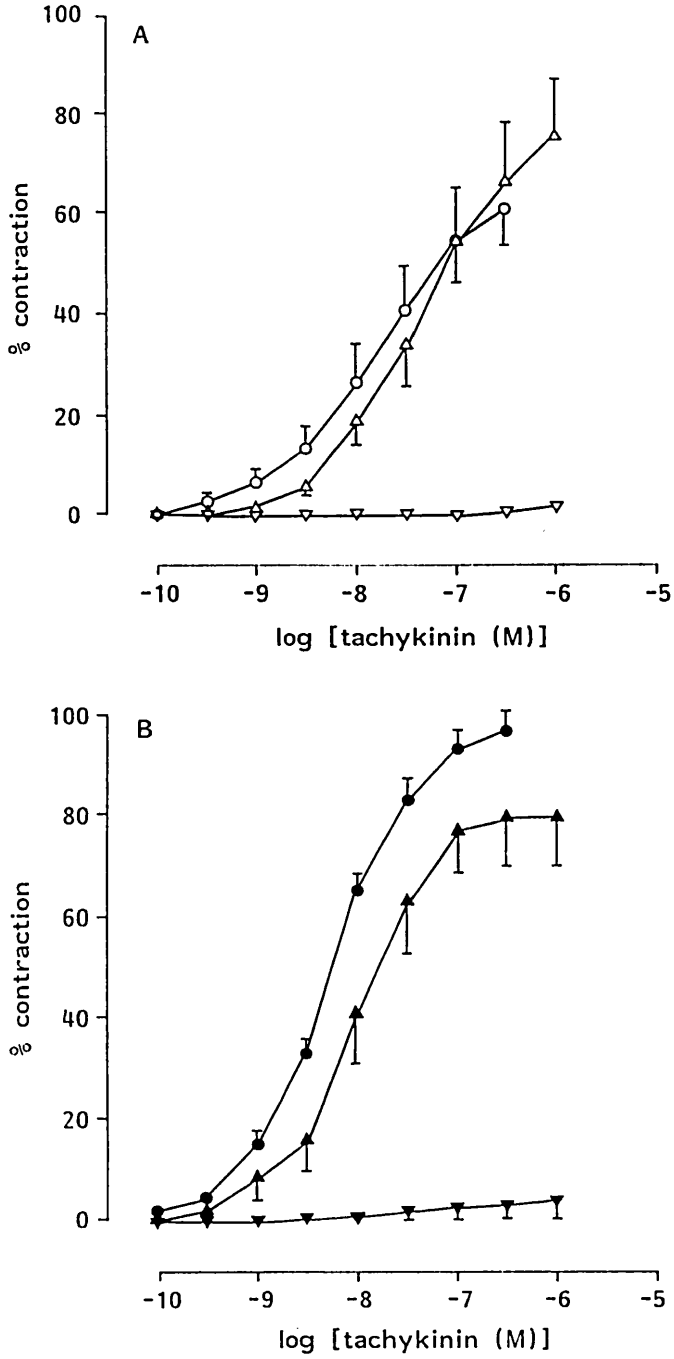


Figure 24. Responses of guinea-pig trachea to selective tachykinin-receptor agonists. Contraction was induced in the absence (○ [Sar^9,$Met(O_2)^{11}$SP; △ [Nle^{10}]NKA (4-10); ▽ senktide) or presence (● [Sar^9,$Met(O_2)^{11}$SP; ▲ [Nle^{10}]NKA(4-10); ▼ senktide) of 10 uM phosphoramidon. Indomethacin (5 uM) was present throughout. Points represent means ± SE of n=6.

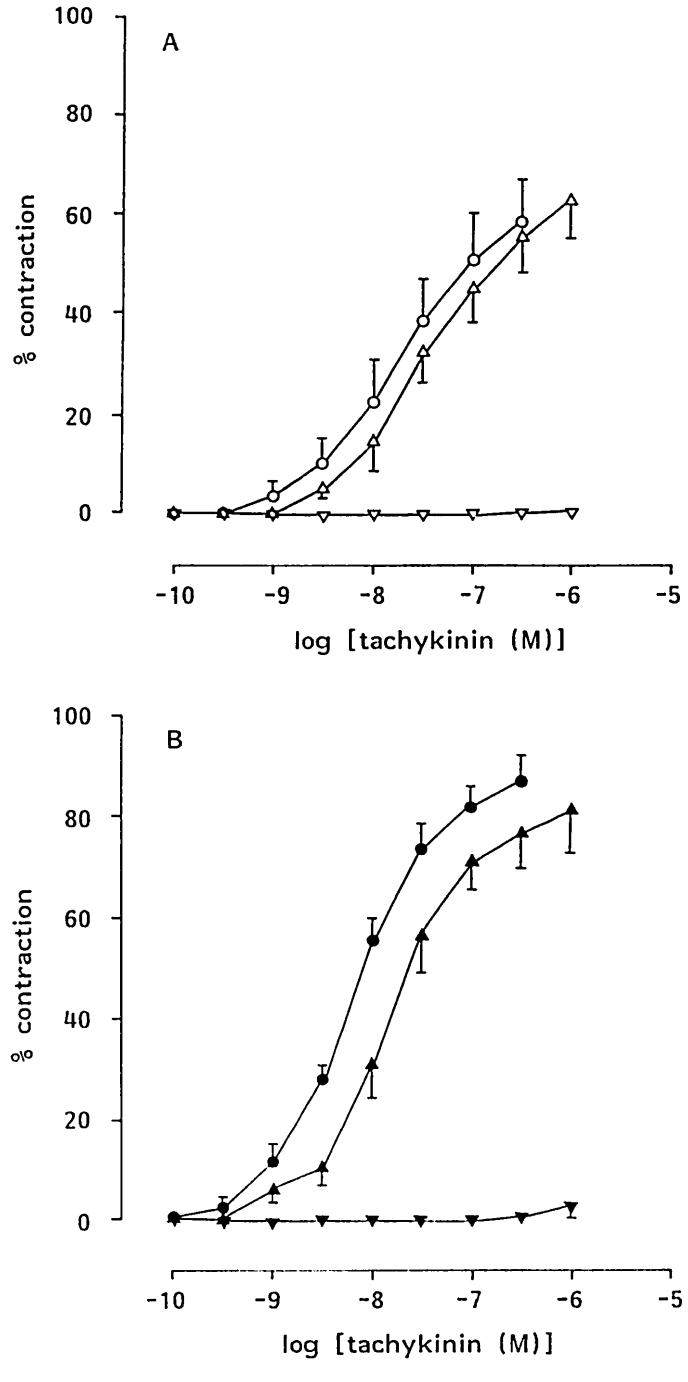


Figure 25. Responses of guinea-pig main bronchi to selective tachykinin-receptor agonists. Contraction was induced in the absence (O [Sar^9,$Met(O_2)^{11}$SP; Δ [Nle^{10}]NKA(4-10); ∇ senktide) or presence (● [Sar^9, $Met(O_2)^{11}$SP; ▲ [Nle^{10}]NKA(4-10); ▼ senktide) of 10 *u*M phosphoramidon. Indomethacin (5 *u*M) was present throughout. Points represent means ± SE of n=6.

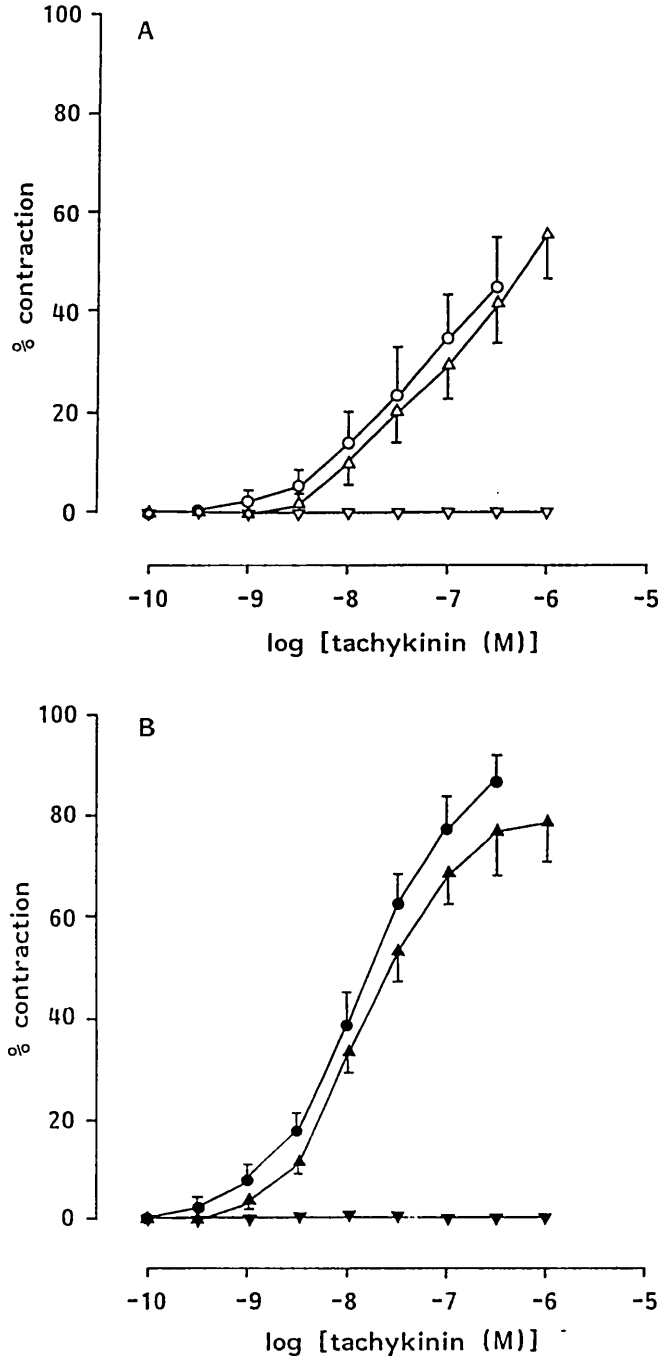


Figure 26. Responses of guinea-pig hilar bronchi to selective tachykinin-receptor agonists. Contraction was induced in the absence (O [Sar^9,$Met(O_2)^{11}$SP; Δ [Nle^{10}]NKA(4-10); ∇ senktide) or presence (● [Sar^9, $Met(O_2)^{11}$SP; [Nle^{10}]NKA(4-10); ▼ senktide) of 10 uM phosphoramidon. Indomethacin (5 uM) was present throughout. Points represent means ± SE of n=6.

potency with decreasing airway diameter. Phosphoramidon (10 uM) potentiated contraction induced by [Nle^{10}]NKA(4-10) in the trachea (pD_2 8.26 $\pm$ 0.22, $p<0.05$, 3.1-fold potentiation), main bronchus (pD_2 8.14 $\pm$ 0.09, $p<0.05$, 5.1-fold potentiation) and hilar bronchus (pD_2 7.78 $\pm$ 0.07, $p<0.05$, 4.0-fold potentiation).

The NK3-receptor agonist senktide induced only small contractions of guinea-pig ASM(<5% of $carb_{max}$) at concentrations greater than 0.1 uM (n=4). Contractions were not potentiated by 10 uM phosphoramidon. Senktide had no effect on main and hilar bronchi, even in the presence of 10 uM phosphoramidon (n=4).

6.3.1.3. L663109

L663109, the putative NK4-receptor agonist, induced concentration-dependent contractions of guinea-pig trachea (figure 27). Contractions were not influenced by epithelium removal (pD_2 6.59 $\pm$ 0.19 intact, 6.88 $\pm$ 0.29 epithelium-denuded preparations, n=8), nor by 10 uM phosphoramidon (6.50 $\pm$ 0.25 intact, 6.48 $\pm$ 0.30 epithelium-denuded preparations, n=8).

L663109 also induced contractions of guinea-pig bronchi (figure 28). Phosphoramidon (10 uM) had no effect on L663109-induced contractions of main bronchi (pD_2 6.89 $\pm$ 0.26 control, 6.87 $\pm$ 0.33 phosphoramidon-treated, n=7) or hilar bronchi (6.48 $\pm$ 0.22 control, 6.60 $\pm$ 0.22 phosphoramidon-treated, n=5).

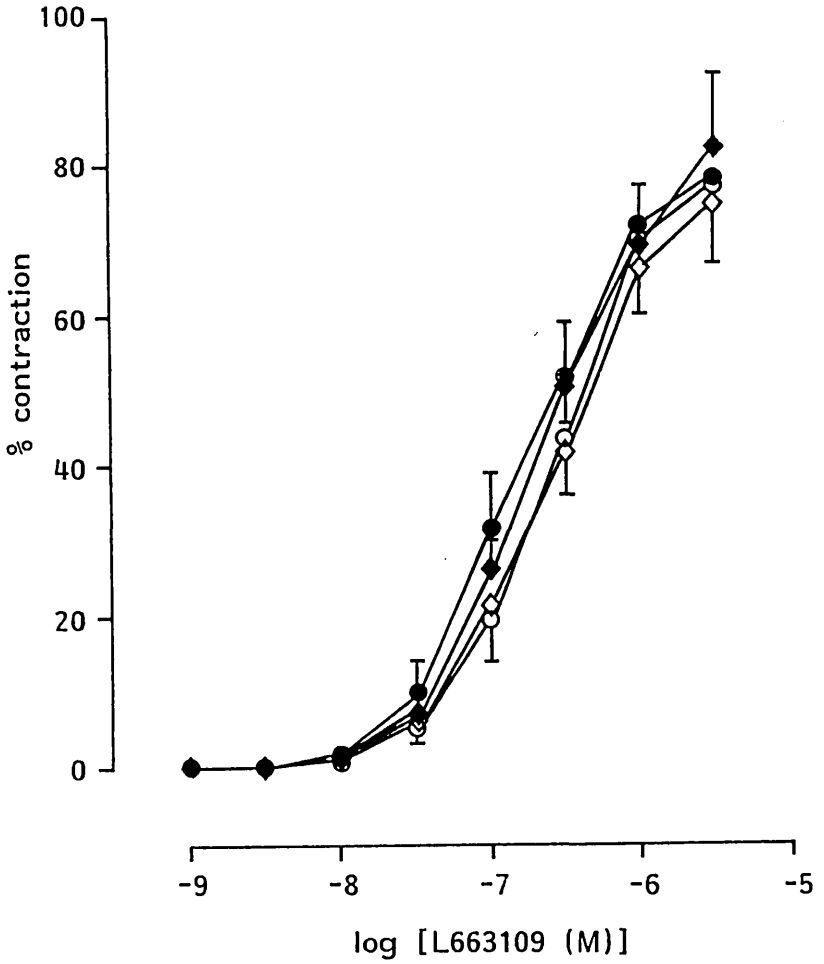


Figure 27. Responses of guinea-pig trachea to L663109. Contraction was induced in the absence (O intact, ● epithelium-denuded preparations) or presence of 10 uM phosphoramidon (◇ intact, ◆ epithelium-denuded preparations). Indomethacin (5 uM) was present throughout. Points represent means ± SE of n=8.

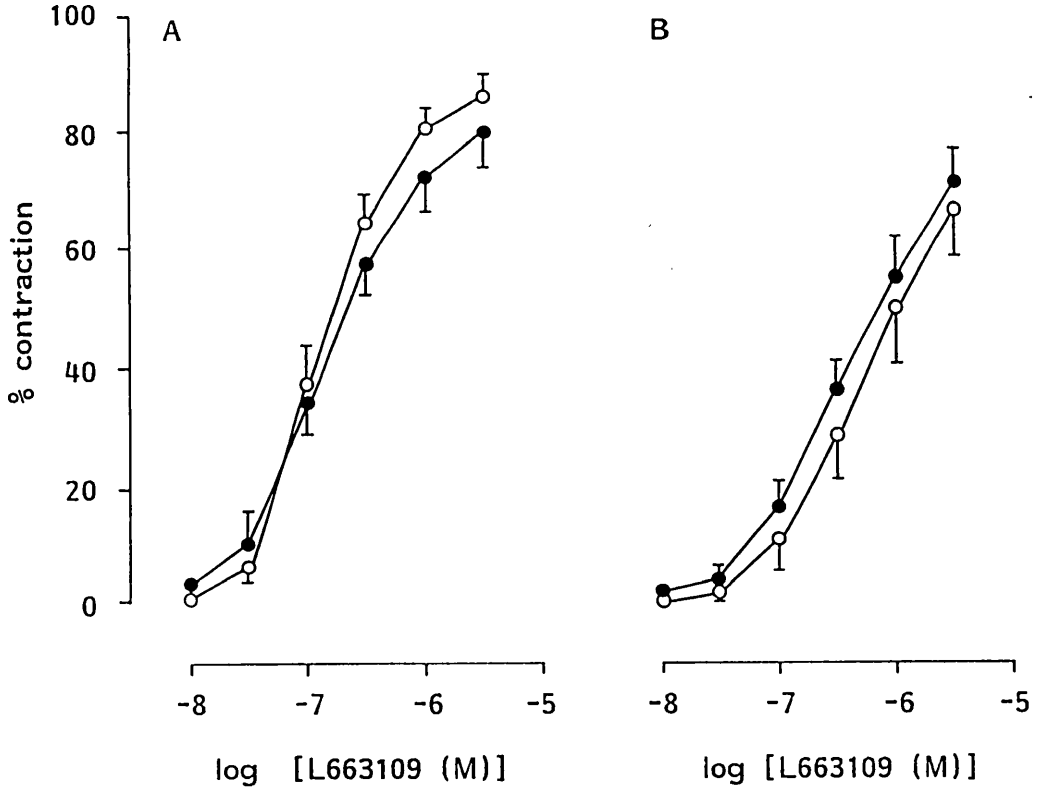


Figure 28. Responses of guinea-pig main bronchi (a) or hilar bronchi (b) to L663109. Contraction was induced in the absence (O) or presence (●) 10 uM phosphoramidon. Points represent means ± SE of n=5-7.

6.3.1.4. Selective NK2-receptor antagonists

L659837 (10 uM), L659874 (10 uM) and L659877 (1 uM) had no effect on resting tension or on NKA-induced contractions of guinea-pig trachea, main bronchi or hilar bronchi (table 10). L659877 (10 uM) induced small contractions in 1 out of 11 tracheal preparations (350 mg), 2 out of 6 main bronchi (88 $\pm$ 12 mg) and 3 out of 7 hilar bronchi (103 $\pm$ 32 mg). Contractions were transient and resting tension was restored to baseline levels within 15 min. L659877 (10 uM) inhibited NKA-contractions of guinea-pig airways, with 2.6-fold, 3.5-fold and 3.6-fold shifts in pD_2s for trachea, main bronchus and hilar bronchus respectively (figure 29; table 10). L659877 (10 uM) had no effect on contractile responses of guinea-pig trachea to carbachol (pD_2 7.16 $\pm$ 0.08, maximum contraction 2039 $\pm$ 398 mg untreated; pD_2 7.23 $\pm$ 0.05, maximum contraction 2229 $\pm$ 413 mg L659877-treated, n=4).

The effects of L659877 on e-NANC responses of guinea-pig bronchi induced by EFS (0.5 ms, 40 V, 8 Hz) is shown in figures 30 and 31. E-NANC responses of main bronchi were unaffected by 0.1 uM L659877, but were inhibited by 1 and 10 uM L659877 (40.4 $\pm$ 8.0 % and 83.3 $\pm$ 3.7 % inhibition, respectively, n=6). E-NANC responses of hilar bronchi were unaffected by 0.1 and 1 uM L659877, but

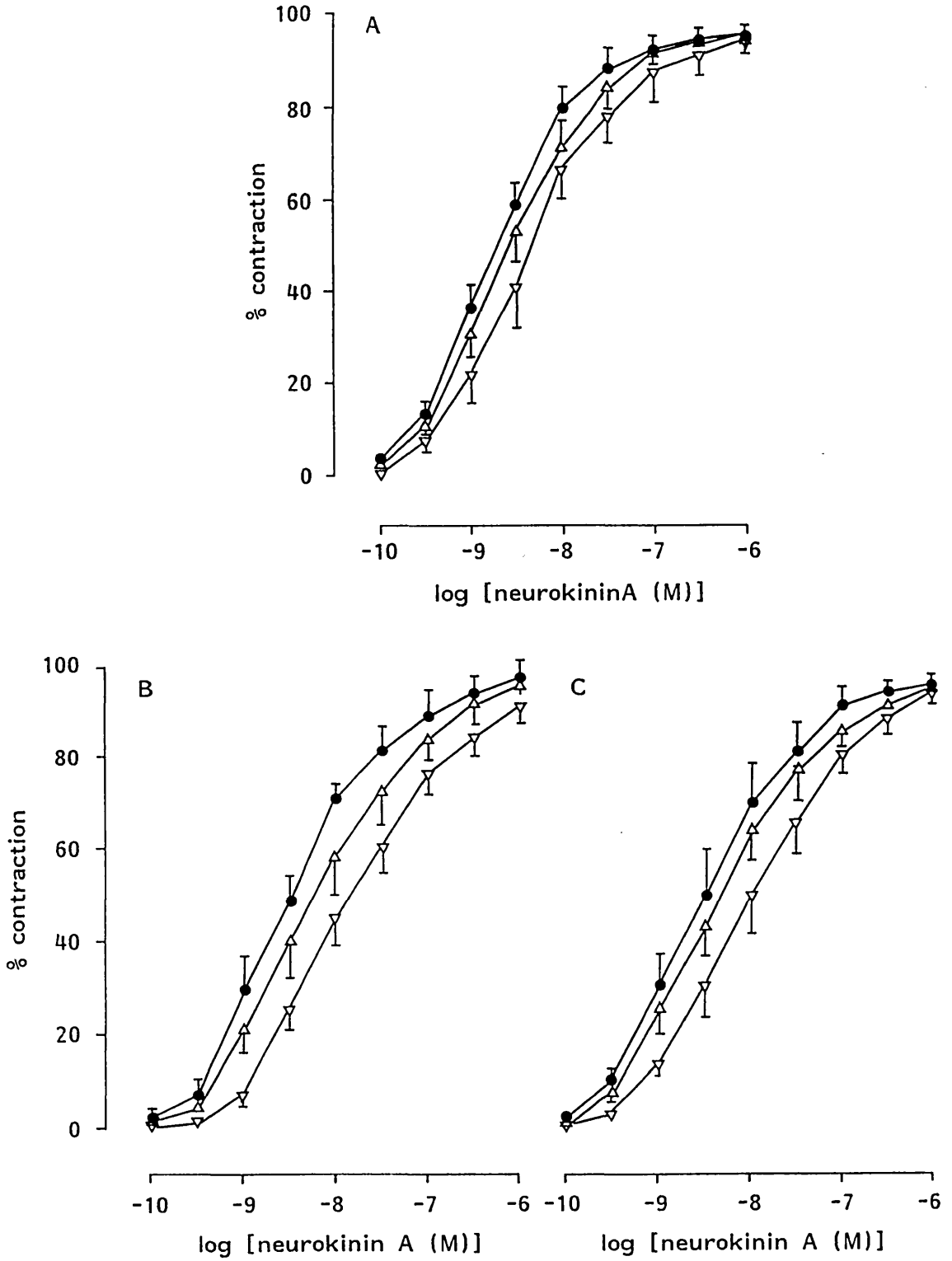


Figure 29. Effect of L659877 on NKA-induced contraction of (A) guinea-pig trachea, (B) main bronchi and (C) hilar bronchi. Responses were obtained in the presence of 1 uM (△) or 10 uM (▽) L659877, or 0.1 % DMSO (●). Indomethacin (5 uM) and phosphoramidon (10 uM) were present throughout. Points represent means ± SE of n=6.

Table 10. Effects of selective NK2-receptor antagonists on contractile responses of guinea-pig airways to NKA.

	pD_2		
	trachea	main bronchi	hilar bronchi
L659877			
DMSO	8.76 ± 0.12	8.42 ± 0.18	8.54 ± 0.08
1 uM	8.68 ± 0.13	8.16 ± 0.15	8.39 ± 0.07
10 uM	8.35 ± 0.06*	7.87 ± 0.10*	7.98 ± 0.06*
L659874			
DMSO	8.59 ± 0.09	8.29 ± 0.08	8.19 ± 0.11
10 uM	8.61 ± 0.07	8.09 ± 0.09	8.30 ± 0.09
L659837			
DMSO	8.45 ± 0.11	8.40 ± 0.07	8.08 ± 0.07
10 uM	8.20 ± 0.10	8.31 ± 0.06	8.11 ± 0.05

$pD_2 = -\log EC_{50}$.
All experiments carried out in the presence of 5 uM indomethacin and 10 uM phosphoramidon.

Figures represent means ± SE of n=4-7.

* $p<0.05$ Student's T-test, absence vs. presence of antagonist.

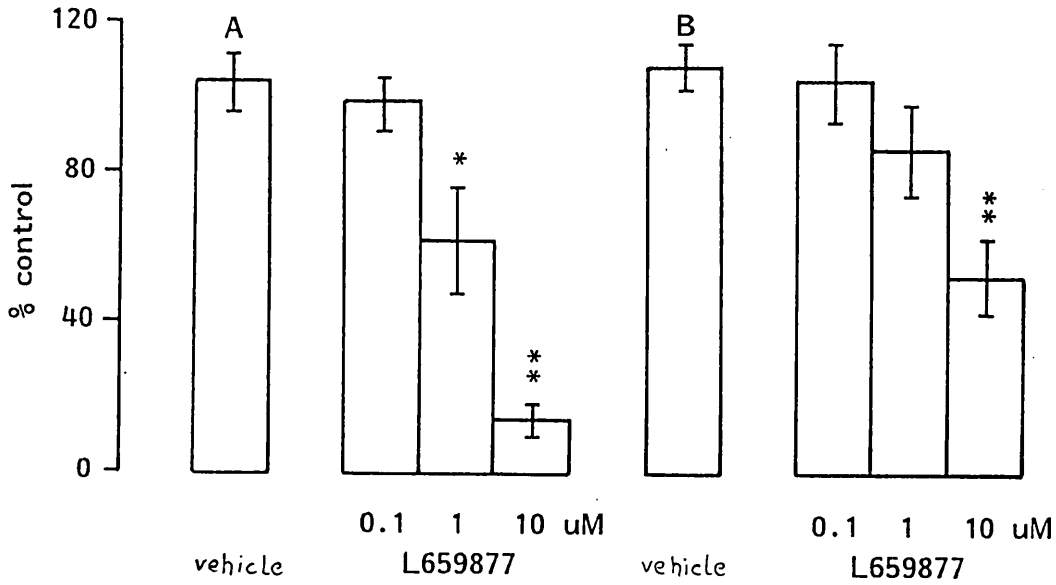


Figure 30. Effect of L659877 on e-NANC contraction of guinea-pig bronchi. E-NANC responses were induced by EFS (0.5 ms, 40 V, 8 Hz) of (A) main bronchi and (B) hilar bronchi. Indomethacin (5 uM) was present throughout. Responses in the presence of antagonist or solvent are expressed as % of control responses obtained in each preparation prior to the addition of antagonist or solvent. Responses are expressed as means $\pm$ SE of n=5-6. * $p<0.05$, ** $p<0.01$ compared to control responses.

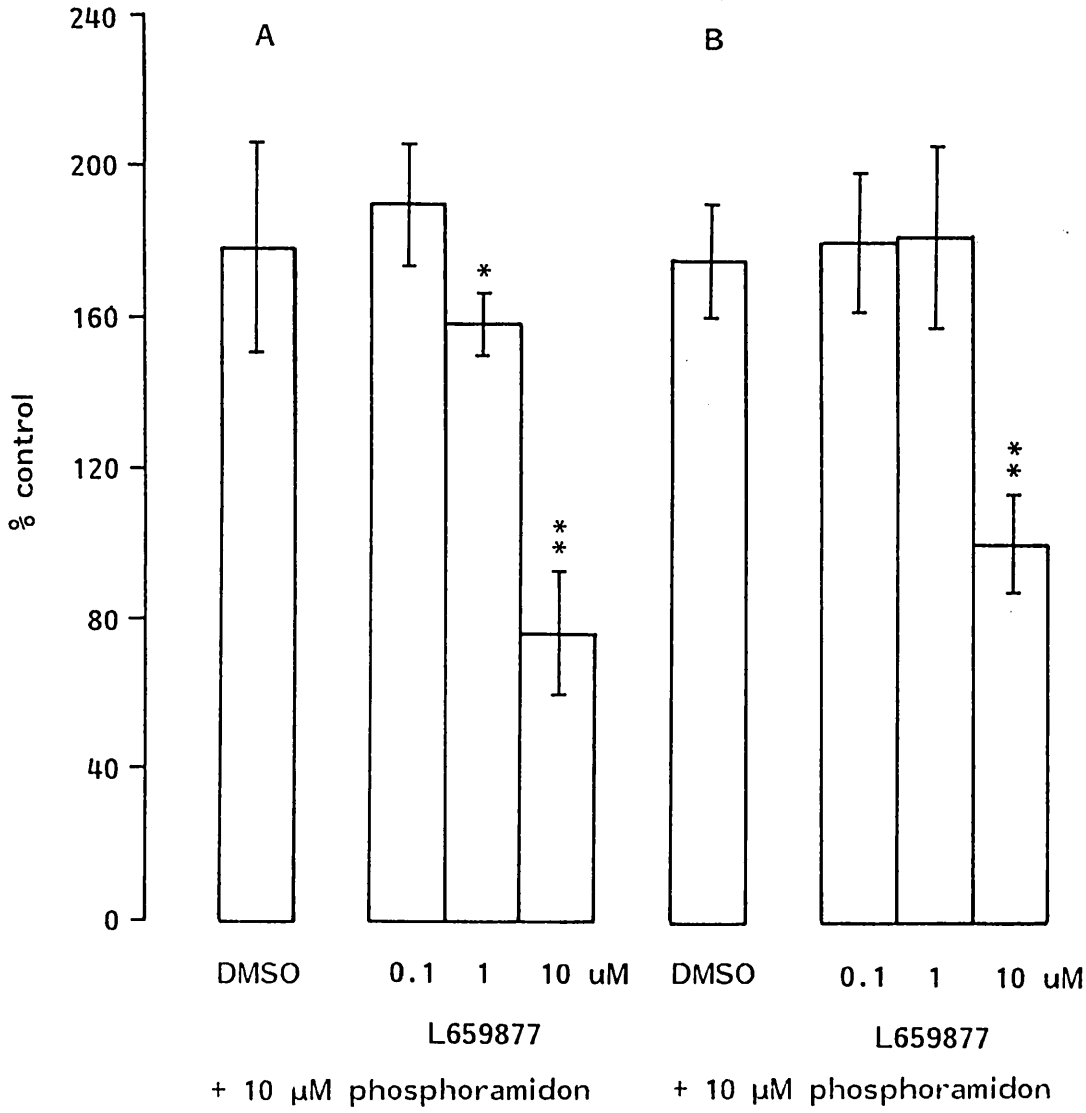


Figure 31. Effect of L659877 on potentiation of e-NANC contraction of guinea-pig bronchi by 10 uM phosphoramidon. E-NANC responses were induced by EFS (0.5 ms, 40 V, 8 Hz) of (A) main bronchi and (B) hilar bronchi. Indomethacin (5 uM) was present throughout. Responses are expressed as a % of control responses obtained prior to the addition of phosphoramidon and antagonist/solvent. Responses are expressed as means $\pm$ SE of n=5-6. * $p<0.05$, ** $p<0.01$ compared to responses obtained in the presence of DMSO.

were inhibited by 10 uM L659877 (47.7 ± 8.7 %, n=5). In the presence of 10 uM phosphoramidon, e-NANC responses of main bronchi were inhibited by 11 % and 56 % by 1 an 10 uM L659877 respectively, but not by lower concentrations (n=6). Similarly, e-NANC responses of hilar bronchi were inhibited by 42 % by 10 uM L659877, but not by lower concentrations (n=5).

Inhibition of e-NANC responses of guinea-pig bronchi by L659877 was similar to that of comparable NKA-induced contractions. EFS (0.5 ms, 40 V, 8 Hz) induced e-NANC contractions of hilar bronchi in the presence of 10 uM phosphoramidon which were 46.0 ± 5.6 % of $carb_{max}$. The concentration of NKA inducing 46 % of $carb_{max}$ in the presence of phosphoramidon was estimated to be 2 nM from a linear interpolation of the sigmoid curve fitted to mean NKA-induced contractions. In the presence of 10 uM L659877, 2 nM NKA induced a contraction of 26 % of $carb_{max}$, suggesting an inhibition of 43 % by L659877.

6.3.2. Human bronchi

6.3.2.1. Rank order of potency of tachykinins

The effect of tachykinins on human bronchial smooth muscle is shown in figure 32.

NKA and SP induced concentration-dependent contractions of human bronchi. Maximal contraction was not achieved in response to either tachykinin, therefore pD_2

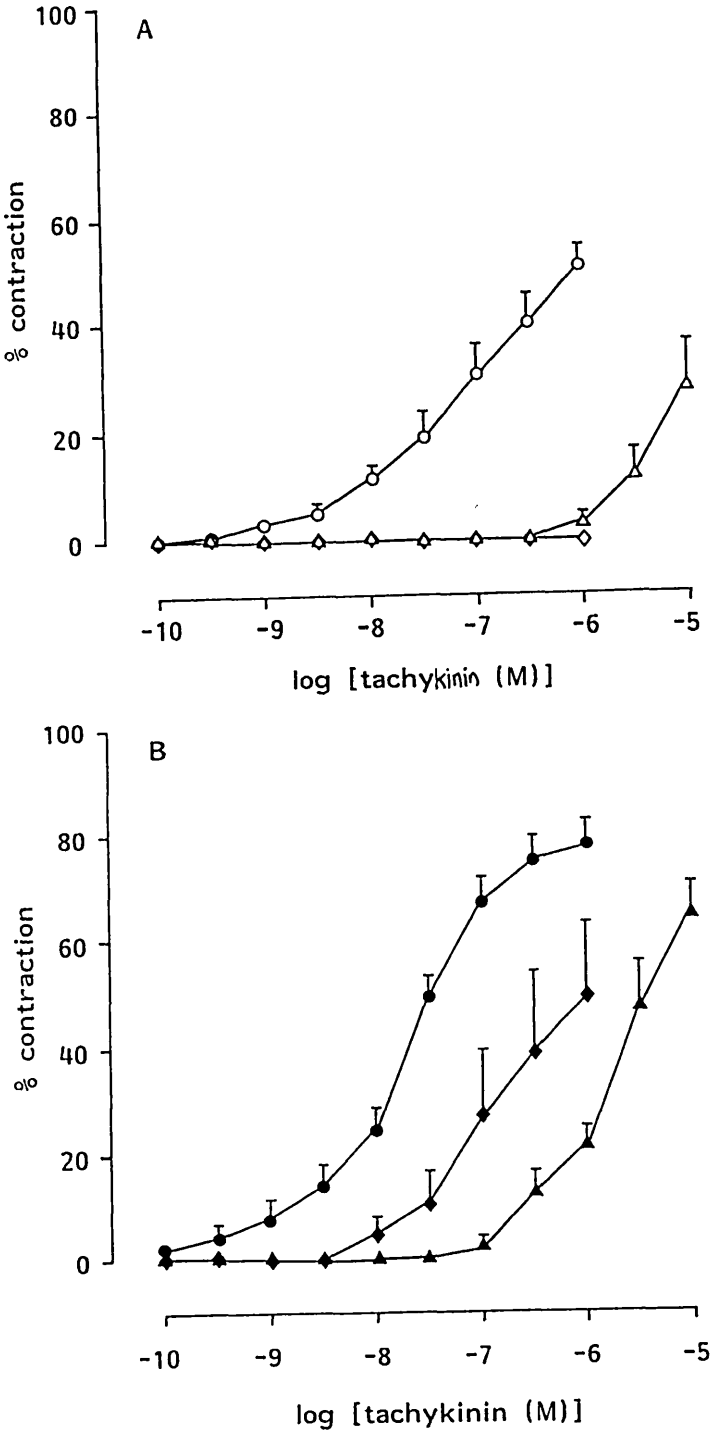


Figure 32. Responses of human bronchi to tachykinins. Tissues were (A) untreated (○ NKA, △ SP, ◇ NKB) or (B) pretreated with 10 uM phosphoramidon (● NKA, ▲ SP, ◆ NKB). Points represent means ± SE of n=4-8.

values could not be determined. A comparison of EC_{30}s revealed NKA to be 87 times more potent than SP (-log EC_{30} 6.91 $\pm$ 0.23 NKA, n=8; 4.97 $\pm$ 0.08 SP, n=6). NKB (up to 1 uM) had no effect on human bronchi (n=4). The rank order of tachykinin potency was therefore NKA > SP > NKB.

Phosphoramidon (10 uM) caused a 13- and 7-fold potentiation of contractile responses to NKA (-log EC_{30} 8.05 $\pm$ 0.11, n=8, $p<0.05$) and SP (-log EC_{30} 5.84 $\pm$ 0.19, n=6, $p<0.05$). In the presence of 10 uM phosphoramidon NKB also induced concentration-dependent contractions of human bronchi (-log EC_{30} 6.87 $\pm$ 0.22, n=4). In the presence of phosphoramidon the rank order of potency of tachykinins was NKA > NKB > SP.

6.3.2.2. Selective tachykinin-receptor agonists.

The effects of selective tachykinin-receptor agonists on human bronchial smooth muscle is shown in figure 33.

The NK1-receptor agonist [Sar^9,$Met(O_2)^{11}$]SP induced contractions of human bronchi (<5% of $carb_{max}$) at concentrations greater than 1 uM (n=4). Phosphoramidon (10 uM) potentiated contractions, although they remained less than 10 % of $carb_{max}$.

The NK2-receptor agonist [Nle^{10}]NKA(4-10) induced concentration-dependent contractions of human bronchi which were potentiated by 10 uM phosphoramidon (-log EC_{30} 7.22 $\pm$ 0.23 untreated, 7.86 $\pm$ 0.18 phosphoramidon-treated

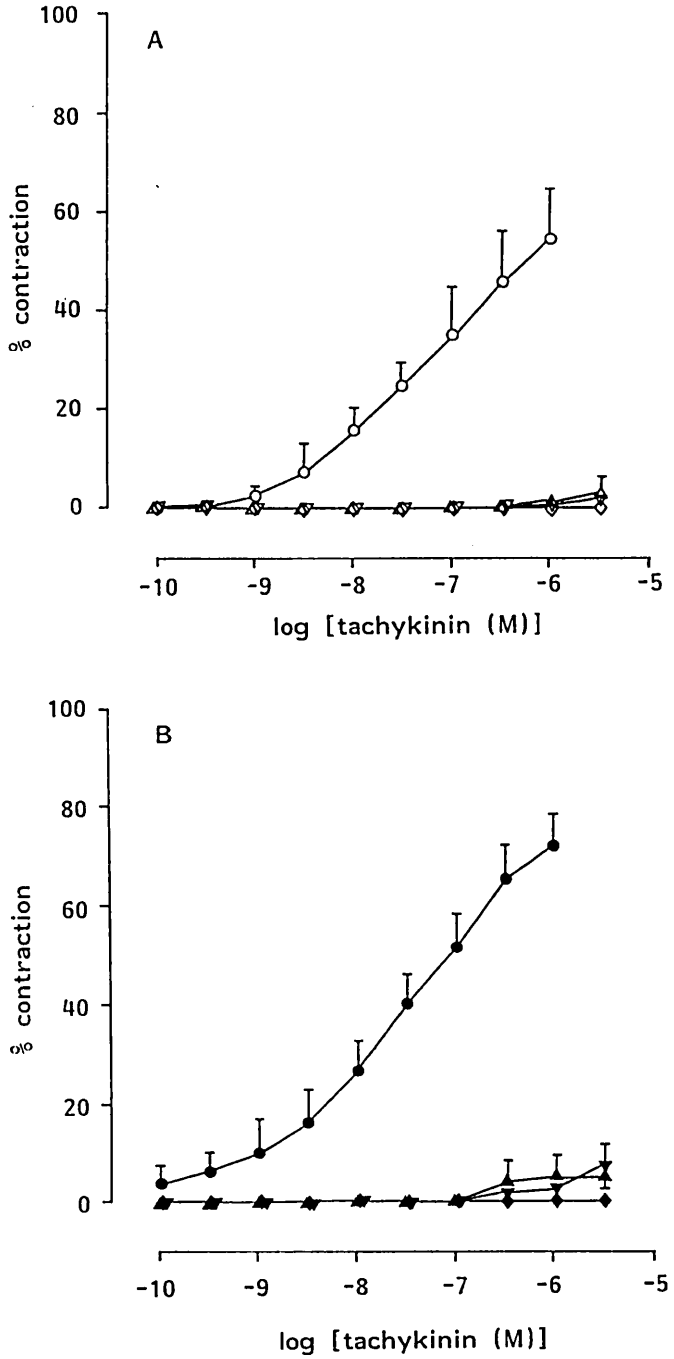


Figure 33. Responses of human bronchi to selective tachykinin-receptor agonists. Tissues were (A) untreated (△ [Sar^{9},Met(O$_2$)^{11}]SP; ○ [Nle^{10}]NKA(4-10); ▽ senktide; ◇ L663109) or (B) pretreated with 10 uM phosphoramidon (▲ [Sar^{9},Met(O$_2$)^{11}]SP; ● [Nle^{10}]NKA(4-10); ▼ senktide; ◆ L663109). Points represent means ± SE of n=4-7.

preparations, $p<0.05$, concentration-ratio 4.4, n=5) In the presence of phosphoramidon, [Nle^{10}]NKA(4-10)-induced contractions yielded a pD_2 of 7.63 $\pm$ 0.14.

The NK3-receptor agonist senktide induced small contractions of human bronchi (<5% of $carb_{max}$) at concentrations greater than 0.3 *u*M (n=7). Phosphoramidon (10 *u*M) potentiated contractions, although they remained less than 10 % of $carb_{max}$.

The putative NK4-receptor agonist L663109 had no effect on human bronchi, even in the presence of 10 *u*M phosphoramidon (n=4).

6.3.2.3. Selective NK2-receptor antagonists

The NK2-receptor antagonists L659877, L659837 and L659874 had no effect on resting tension but all caused a concentration-dependent inhibition of NKA-induced contractions of human bronchi (figure 34). pA_2 values obtained from Schild plots were 6.98 for L659877 (correlation coefficient 0.999, slope 0.81), 6.00 for L659837 (correlation coefficient 1.0, slope 0.93) and 5.69 for L659874 (correlation coefficient 0.99, slope 0.84).

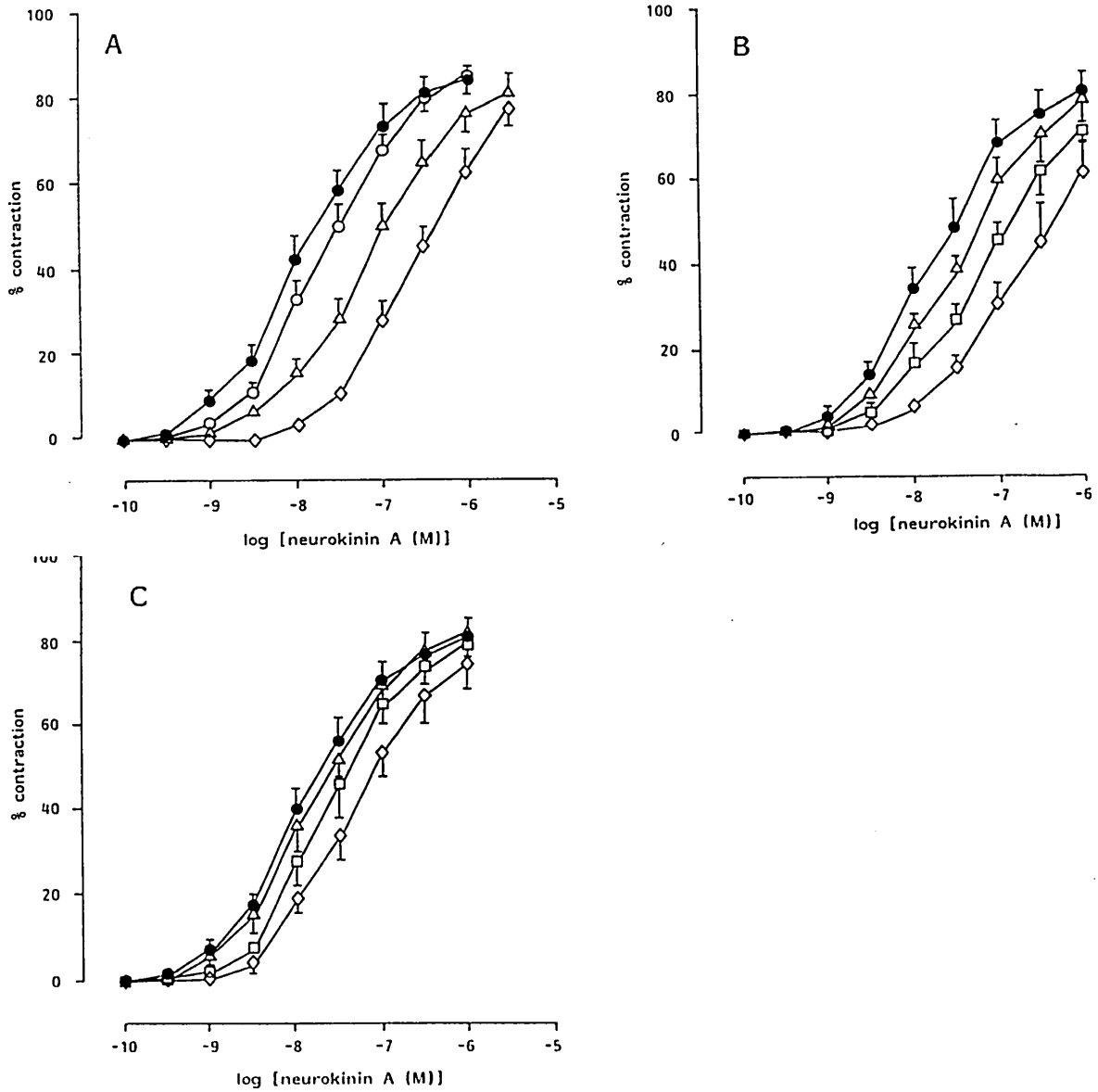


Figure 34. Effect of (A) L659877, (B) L659837 and (C) L659837 on NKA-induced contraction of human bronchi.
(A) Tissues were pretreated with (O) 0.1 uM, (Δ) 1 uM or (◇) 10 uM L659877, or with (●) 0.1 % DMSO (n=8-10).
(B) Tissues were pretreated with (O) 1 uM, (Δ) 3 uM or (◇) 10 uM LL659837, or with (●) 0.1 % DMSO (n=6-7).
(C) Tissues were pretreated with (O) 1 uM, (Δ) 3 uM or (◇) 10 uM L659874, or with (●) 0.1 DMSO (n=4-6).
Phosphoramidon (10 uM) was present throughout. Points represent means ± SE.

6.4. Discussion

Due to the unavailability of selective antagonists, the classification of tachykinin receptors is currently based on the rank order of agonist potencies in various mammalian tissues. Three distinct receptors can be differentiated according to the relative activity and binding affinity of tachykinins and tachykinin-related peptides (Lee *et al.*, 1982; Buck *et al.*, 1984; Burcher *et al.*, 1986; Regoli *et al.*, 1987). However several problems arise from this method of receptor classification. The mammalian tachykinins, NKA, NKB and SP, are not selective agonists but are active on all three receptor types. Preparations which contain more than one type of receptor may therefore yield orders of potency which are intermediate between those obtained on monoreceptor systems. Tachykinins may be metabolised by a variety of peptidases. The relative potency of tachykinins in different tissues may therefore be influenced by the distribution of peptidases and their affinity for tachykinins. As has previously been discussed, NEP may be a major inactivator of tachykinins in the airways and determination of orders of potency in the absence of peptidase inhibitors may yield misleading results. In the ferret trachea, the NEP inhibitor leucine-thiorphan changed the rank order of tachykinin potency from NKA > SP > NKB to NKA = NKB > SP (Sekizawa *et al.*, 1987b). The

development of stable selective agonists and antagonists is therefore of primary importance to the classification of tachykinin receptors.

The rank order of potency of tachykinins in guinea-pig airways was NKA > NKB > SP with NKA being 1 to 2 orders of magnitude more potent than SP. The order of potency was similar at all airway levels studied (trachea, main bronchus, hilar bronchus) and was maintained in the presence of the NEP inhibitor phosphoramidon. This order of potency is similar to that reported by others for the guinea-pig trachea (Advenier *et al.*, 1987) and corresponds to the NK2-receptor classification. Studies using selective agonists, however, have suggested that tachykinin-induced contractions of the guinea-pig trachea may be mediated by NK1 and NK2 receptors (Drapeau *et al.*, 1987; Regoli *et al.*, 1987; Ireland *et al.*, 1988). In our study, the NK1-receptor agonist [Sar^{9},$Met(O_2)^{11}$]SP (Drapeau *et al.*, 1987) and the NK2-receptor agonist [Nle^{10}]NKA(4-10) (Drapeau *et al.*, 1987) both induced potent contractions of guinea-pig trachea and bronchi, whereas the NK3-receptor agonist senktide (Wormser *et al.*, 1986) was virtually inactive. In the presence of phosphoramidon the potency of [Sar^{9}, $Met(O_2)^{11}$]SP and [Nle^{10}]NKA(4-10) was increased, but senktide remained relatively inactive. These results are therefore consistent with the presence of NK1- and NK2- receptors on guinea-pig airway smooth muscle.

Recently, the development of synthetic peptides which are potent agonists on the guinea-pig trachea but which are only weakly active on NK1-, NK2- and NK3-monoreceptor systems, has led to the suggestion that tachykinin-induced contraction of guinea-pig trachea may be mediated by a fourth type of tachykinin-receptor, the NK4-receptor (McKnight *et al.*, 1987 & 1988). One of these peptides, L663109, induced contractions of guinea-pig trachea and bronchi, although the potency in the trachea was about 100-fold lower than that reported by McKnight *et al.*, 1988. Contractions were not potentiated by phosphoramidon, sugesting that this peptide is not susceptible to degradation by NEP. Interestingly, L663109-induced contractions of the guinea-pig trachea were not potentiated by epithelium removal, and this supports the view that NEP in the epithelial layer is at least in part responsible for the modulatory influence of the epithelium on smooth muscle responses to tachykinins.

Tachykinin-receptor antagonists that are selective for the NK2-receptor (Williams *et al.*, 1988) have been shown to be ineffective on the guinea-pig trachea suggesting that NK2-receptors do not mediate tachykinin-induced contraction in this tissue (McKnight *et al.*, 1988). Our results support this contention since the selective NK2-receptor antagonists L659877, L659837 and L659874 (which have pA_2s of 8.0, 6.7 and 6.8 respectively on the rat vas deferens), had little effect on NKA-induced contraction of guinea-pig trachea and bronchus. Recent

autoradiographic and radioligand binding studies have shown a greater density of binding sites for SP than for NKA over airway smooth muscle and reveal that both NKA and SP bind NK1- rather than NK2-receptors (Burcher *et al.*, 1989). These findings are difficult to reconcile with the lack of effect of L663109 on an NK1-monoreceptor system such as the guinea-pig ileum treated with atropine (McKnight *et al.*, 1988).

Contractile responses of guinea-pig bronchi induced by the stimulation of e-NANC nerves are inhibited by nonselective tachykinin-receptor antagonists (Lundberg *et al.*, 1983c; Karlsson *et al.*, 1984; Leander *et al.*, 1984). In our experiments, e-NANC responses of guinea-pig bronchi were only inhibited by high concentrations of the NK2-receptor antagonist L659877. Inhibition was similar to that of comparable contractions induced by NKA suggesting that NK2-receptors may not mediate contractile responses of guinea-pig bronchi evoked by the stimulation of e-NANC nerves.

In human bronchi the rank order of potency of tachykinins is NKA > SP > NKB (Advenier *et al.*, 1987). This is confirmed by our experiments, with NKA being 2 orders of magnitude more potent than SP, and NKB being inactive. In the presence of phosphoramidon, NKA- and SP-induced contractions were potentiated and NKB became active. NKA retained the highest potency but the rank order of potency was changed to NKA > NKB > SP. The NK2-receptor agonist [Nle^{10}]NKA(4-10) was a potent agonist

on human bronchi and contractions were potentiated by phosphoramidon. The NK1-receptor agonist [Sar^9, $Met(O_2)^{11}$]SP, the NK3-receptor agonist senktide and the putative NK4-receptor agonist L663109 were virtually inactive on human bronchi, even in the presence of phosphoramidon. Contractile responses of human bronchi to tachykinins therefore appear to be mediated exclusively by NK2-receptors (Naline *et al.*, 1988). Furthermore, NKA-induced contractions of human bronchi were antagonised by the NK2-receptor antagonists L659877, L659837 and L659874. The contrasting findings on guinea-pig and human airways may reflect species differences or may be due to the different airway levels being studied. In the guinea-pig, although the potency of tachykinins decreased from the trachea to hilar bronchi, the receptor type(s) mediating contraction appeared to be the same. It is possible, however, that in more distal airways the distribution of receptor types may be altered.

In conclusion, the classification of tachykinin-receptor on guinea-pig ASM remains uncertain. Tachykinin-induced contraction of guinea-pig airways may not be mediated by NK2-receptors, and the existence of NK4-receptors on this tissue remains a possibility. Definitive receptor classification awaits the development of antagonists which selectively antagonise tachykinin-induced contractions of guinea-pig airways. Tachykinin-induced contraction of human ASM, on the other hand, appears to be mediated solely by NK2-receptors.

7. Modulation of VIP- and i-NANC nerve-induced relaxation of guinea-pig trachea by airway epithelium and NEP

7.1. Aim

VIP-induced relaxation of guinea-pig trachea is potentiated by NEP inhibition (Liu *et al.*, 1987). In the airways, NEP is localised to the interstitium, epithelium and smooth muscle (Sekizawa *et al.*, 1987), and we and others have suggested that epithelial modulation of tachykinin-induced contraction of ASM is at least in part due to the degradation of peptides by NEP in the epithelial layer. The present study was therefore designed to investigate the effect of epithelium removal and NEP inhibition on relaxation of guinea-pig trachea induced by exogenous VIP and by the stimulation of i-NANC nerves, for which VIP is a putative neurotransmitter.

7.2. Protocol

7.2.1. EFS parameters

Relaxant responses induced by the stimulation of i-NANC nerves by EFS were studied in guinea-pig trachea. Preparations were obtained from the central-cervical region of the trachea in order to avoid the induction of e-NANC responses to EFS. Tissues were incubated for 30 min with 1 *u*M atropine and 1 *u*M propranolol in order to block cholinergic and adrenergic responses to EFS. EFS was applied for 30 s at intervals of 15 min. Optimum parameters for the stimulation of i-NANC nerves were determined by constructing stimulus-response curves over a pulse duration range of 0.01-1 ms, voltage range of 5-60 V and frequency range 0.5-50 Hz. Tissues were incubated for 30 min with 3 *u*M TTX, and stimulus-response curves repeated. Spontaneous tone tended to decrease with time, therefore only one parameter was studied on one preparation. Relaxant responses were compared to relaxation induced by a maximally effective concentration of papaverine (100 *u*M) added at the end of each experiment.

For all subsequent studies a pulse duration of 0.1 ms, voltage of 40 V and frequency range of 0.5-50 Hz were used.

7.2.2. Epithelium removal and NEP inhibition.

Relaxation of intact and epithelium-denuded preparations obtained from the central-cervical region of the guinea-pig trachea, was induced by exogenous VIP or by EFS (0.1 ms, 40 V, 0.5-50 Hz) in the presence of 1 uM atropine and 1 uM propranolol. Parallel preparations were incubated for 30 min with or without 10 uM phosphoramidon, and cumulative C-R curves were constructed to VIP. In separate experiments, EFS frequency-response curves were constructed, tissues were incubated for 30 min with 10 uM phosphoramidon and frequency-response curves repeated. Relaxation was compared to relaxation induced by a maximally effective concentration of papaverine (100 uM).

7.3. Results

7.3.1. I-NANC nerve-induced relaxation

In the presence of 1 uM atropine and 1 uM propranolol, EFS of guinea-pig trachea induced an inhibitory response that was dependent on the pulse duration, voltage and frequency of stimulation, and was inhibited by 3 uM TTX (figure 35). Maximal responses were elicited by a pulse duration of 0.1 ms, voltage of 40 V and frequency of 25 Hz.

Epithelium removal had no effect on stimulus-response curves for pulse duration and voltage. However, epithelium removal significantly attenuated ($p<0.05$) relaxation induced by low frequencies of stimulation (0.5-2.5 Hz, 0.1 ms, 40 V), whilst having no effect on relaxation induced by higher frequencies. TTX (3uM) was more effective in blocking i-NANC responses in epithelium-denuded preparations than in intact preparations. Thus, TTX abolished relaxation of epithelium-denuded preparations induced by EFS of frequencies less than 25 Hz. In intact preparations, TTX-resistant relaxations were induced at all frequencies of stimulation, and in fact, relaxation induced by 0.5 Hz EFS was not significantly inhibited by TTX.

Epithelium removal also decreased the duration of relaxation induced by EFS. At a stimulation frequency of 10 Hz, which produces relaxations in intact and

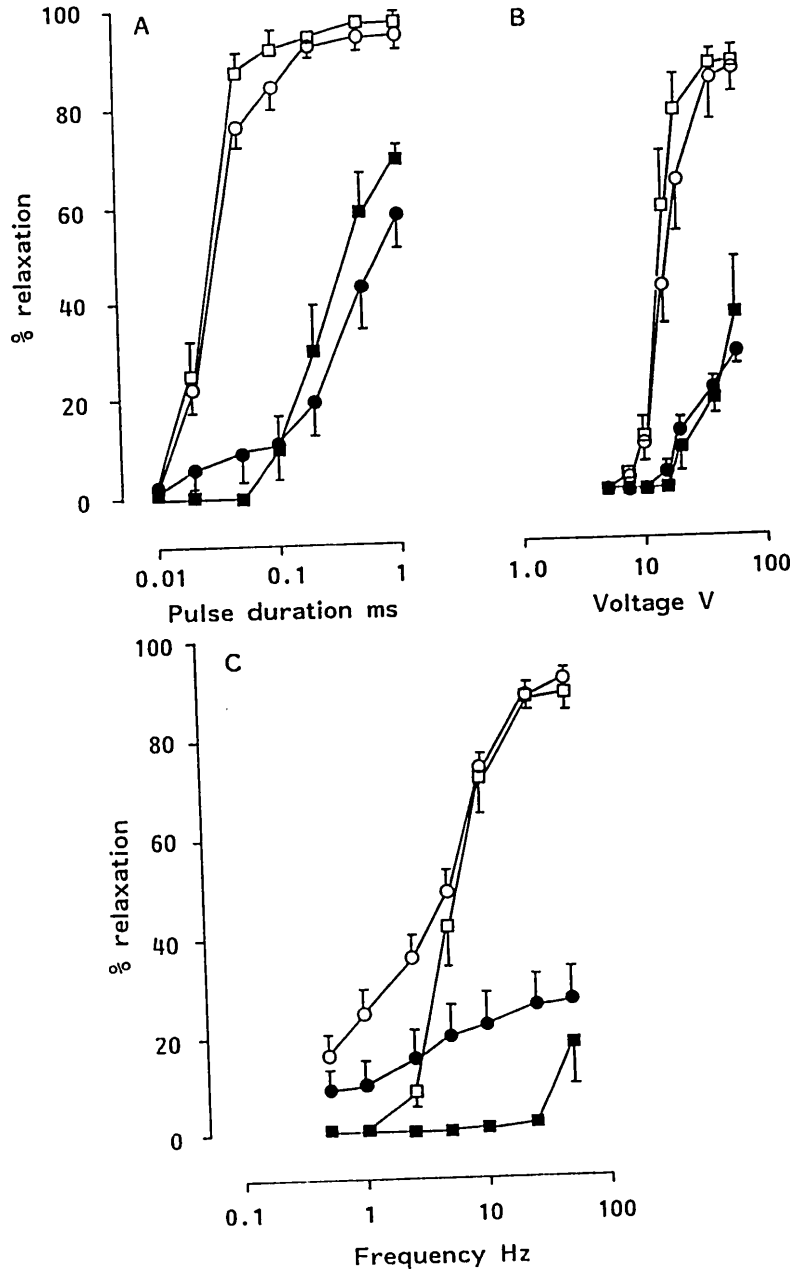


Figure 35. EFS parameters for the stimulation of i-NANC nerves in the guinea-pig trachea. (A) Effect of pulse duration on relaxation induced by EFS (40 V, 50 Hz). (B) Effect of voltage on relaxation induced by EFS (0.1 ms, 50 Hz). (C) Effect of frequency on relaxation induced by EFS (0.1 ms, 40 V). Responses were induced in the absence (O intact, □ epithelium-denuded preparations) and presence (● intact, ■ epithelium-denuded preparations) of 3 uM TTX. Atropine (1 uM) and propranolol (1 uM) were present throughout. Points represent means ± of n=5-8.

epithelium-denuded preparations of equal magnitude, the $t_{50\%}$ was 381 ± 34 s in intact preparations and 178 ± 20 s in epithelium-denuded preparations ($p<0.01$, n=8).

Incubation of intact and epithelium-denuded tracheal preparations with 10 uM phosphoramidon for 30 min had no effect on the magnitude of i-NANC responses induced by EFS (figure 36A). Phosphoramidon had no effect on the duration of relaxation induced by 10 Hz EFS in intact ($t_{50\%}$ 423 ± 42 s, n=8) or epithelium-denuded ($t_{50\%}$ 182 ± 17 s, n=8) preparations.

7.3.2. VIP-induced relaxation

Epithelium-removal caused a potentiation of relaxation of guinea-pig trachea induced by exogenous VIP (figure 36B). Mean pD_2s in intact (7.14 ± 0.09) and epithelium-denuded preparations (7.77 ± 0.17) were significantly different ($p<0.01$, n=6) and yielded a concentration-ratio of 4.0. Epithelium removal had no effect on maximal relaxation induced by VIP (1544 ± 193 mg intact, 1296 ± 153 mg epithelium-denuded preparations). Phosphoramidon caused a potentiation of relaxation induced by VIP in intact, but not epithelium-denuded, preparations (figure 36B). In intact preparations, mean pD_2s in untreated (7.14 ± 0.09) and phosphoramidon-treated (7.73 ± 0.11) preparations were significantly different ($p<0.01$, n=6) and yielded a concentration-ratio of 3.9. In epithelium-denuded preparations, mean pD_2s in untreated

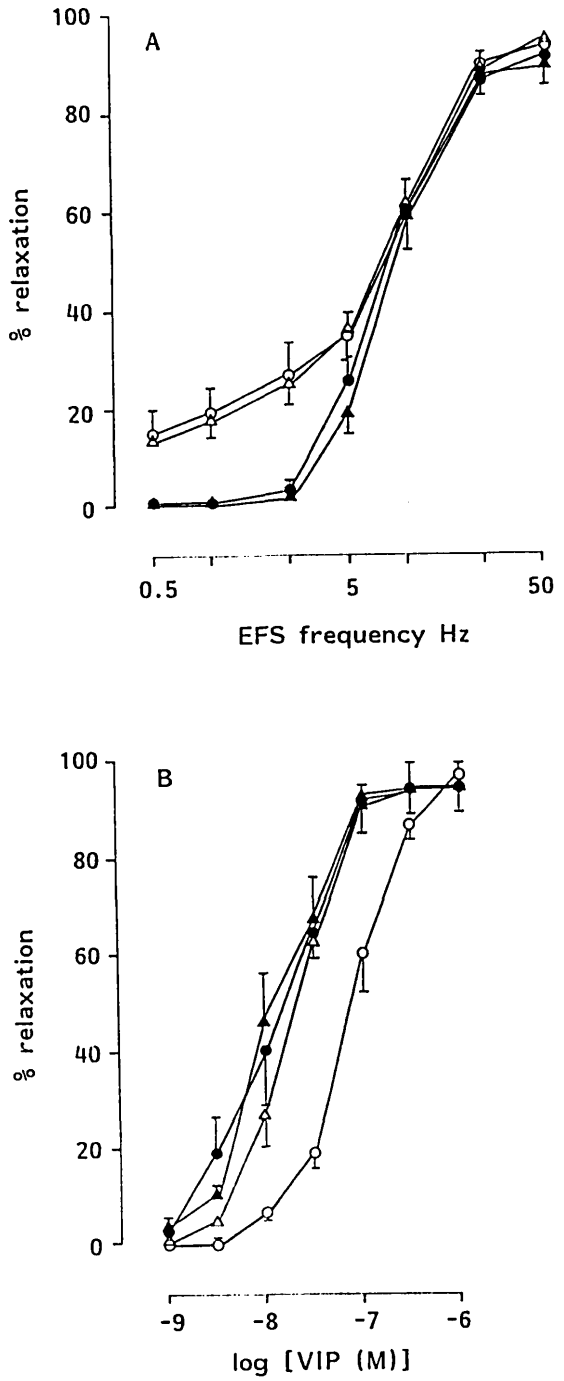


Figure 36. Effect of epithelium removal and NEP inhibition on responses of guinea-pig trachea to (A) i-NANC nerve stimulation and (B) VIP. I-NANC relaxation was induced by EFS (0.1 ms, 40 V, 0.5-50 Hz) in the presence of 1 uM atropine and 1 uM propranolol. Responses were obtained in the absence (○ intact, ● epithelium-denuded preparations) and presence (△ intact, ▲ epithelium-denuded preparations) of 10 uM phosphoramidon. Points represent means ± SE of n=8.

(7.77 ± 0.17) and phosphoramidon-treated (7.76 ± 0.26) preparations were not significantly different (n=6). Phosphoramidon had no effect on the maximal relaxation induced by VIP (1563 ± 184 mg intact, 1328 ± 321 mg epithelium-denuded preparations).

7.4. Discussion

Relaxant responses of airway smooth muscle are known to be modulated by the epithelium although the qualitative and quantitative nature of modulation appears to vary according to agonist, preparation and animal species (Fedan *et al*, 1988). Epithelium removal has been reported to attenuate relaxation of guinea-pig trachea to VIP with a decrease in potency and maximal relaxation (Chou & Said, 1987). In our experiments, however, the sensitivity of guinea-pig trachea to VIP was potentiated by epithelium removal, with no change in the maximum response induced by VIP. Widely varying effects of epithelium removal have also been described for other agonists. Epithelium removal has been reported to increase (Farmer *et al.*, 1986) or decrease (Chou & Said, 1987) the sensitivity of guinea-pig tracheal smooth muscle to sodium nitroprusside. Furthermore, the sensitivity of guinea-pig trachea to isoprenaline may be increased or unchanged by epithelium removal, with no effect or decreases in maximal responses (Farmer *et al.*, 1986; Goldie *et al.*, 1986; Holroyd *et al.*, 1986). Such contrasting findings are difficult to reconcile and we cannot explain the discrepancy in the observed effects of epithelium removal on relaxation induced by VIP.

Potentiation of relaxation by epithelium removal may arise from a variety of mechanisms. The epithelium may represent a diffusional barrier and removal of this

barrier could increase the accessibility of agonist molecules to the underlying smooth muscle. This, however, is unlikely since responses to VIP were measured under equilibrium conditions. Contractile responses of airway smooth muscle may be modulated by an inhibitory factor released by the epithelium (Flavahan *et al.*, 1985). In a similar fashion, relaxant responses may be modulated by an excitatory factor released by the epithelium, the loss of which would result in an apparent potentiation of relaxation. In the rat trachea, responses to antigenic stimulation may be modulated by an epithelial-dependent excitatory factor (Frossard & Muller, 1986). The inhibitory influence of epithelium removal on relaxation of guinea-pig trachea to isoprenaline is abolished by the extraneuronal uptake inhibitor corticosterone, suggesting that the epithelium may be a site of uptake of catecholamines (Farmer *et al.*, 1986). It is not known whether VIP and other neuropeptides are subject to neuronal or extraneuronal uptake, therefore uptake of VIP molecules by the epithelium remains a possibility. Epithelial modulation of contractile responses of guinea-pig tracheal smooth muscle to tachykinins may be due at least in part to metabolism by NEP in the epithelial layer (see section 4.). Relaxation of guinea-pig trachea in response to VIP is potentiated by an NEP inhibitor (Liu *et al.*, 1987) and ferret airway epithelium contains NEP (Sekizawa *et al.*, 1987b). In our experiments, phosphoramidon potentiated responses of

intact but not epithelium-denuded preparations to VIP, suggesting that VIP may be degraded by NEP located in the epithelial layer. Phosphoramidon in fact mimicked the effect of epithelium removal suggesting that epithelial modulation of VIP-induced relaxation may be entirely due to NEP activity in the epithelium.

Guinea-pig tracheal smooth muscle possesses an i-NANC innervation. EFS of guinea-pig trachea in the presence of atropine and propranolol induced a relaxation which was inhibited by TTX, confirming its neurogenic nature. Epithelium removal has been reported not to influence i-NANC responses evoked by EFS in the guinea-pig trachea (Thompson *et al.*, 1988). In our experiments, however, epithelium removal attenuated i-NANC responses evoked by low frequencies of EFS, with a reduction in both the magnitude and duration of relaxation. Differences in methodology (eg. spontaneous versus drug-induced tone, pretreatment with indomethacin, differences in stimulation parameters) may explain the discrepancy with our study. TTX was able to abolish relaxation to EFS in epithelium-denuded preparations, except at high frequencies where small TTX-resistant relaxations were seen. In intact preparations, however, TTX-resistant relaxations were seen at all frequencies of stimulation. The nature of TTX-resistant relaxations to EFS is unclear although several mechanisms have been suggested including a direct effect of EFS on smooth muscle membranes (Coburn & Tomita, 1973), release of relaxing mediators from

non-neuronal tissue (Middendorf & Russel, 1980) and a reduced sensitivity of i-NANC nerves to TTX (Fukuda & Kameyama, 1980; Katz & Miledi, 1969; Taylor *et al.*, 1984). The differential effect of TTX on relaxation of intact and epithelium-denuded preparations seen in our study has been reported in human airways and was suggested to be due to impaired diffusion of TTX to its site of action (Richardson & Beland, 1976). A more likely explanation is that it may be due to the release of a relaxant factor from the epithelium in response to EFS by a mechanism not susceptible to TTX. This could also explain the lower degree of relaxation to low frequencies of EFS observed in epithelium-denuded preparations compared to intact preparations since removal of the epithelium would eliminate the TTX-resistant component. Thus, the response of epithelium-denuded preparations to EFS in the presence of atropine and propranolol reflects the stimulation of i-NANC nerves, whereas the response of intact preparations may be due to the stimulation of i-NANC nerves and the release of a relaxant factor from the epithelium. It must be pointed out, however, that other investigators have shown that TTX abolishes i-NANC responses of intact guinea-pig tracheal preparations evoked by EFS voltage lower than 70 V, pulse duration lower than 0.5 ms and frequency lower than 25 Hz (Coburn & Tomita, 1973; Coleman & Levy, 1974; Richardson & Bouchard, 1975).

The demonstration that phosphoramidon potentiates relaxation induced by VIP raises the possibility that NEP may also modulate responses induced by the stimulation of i-NANC nerves. However, phosphoramidon had no effect on relaxant responses to EFS in terms of the magnitude of relaxation or duration of the response. One explanation for this discrepancy is that VIP is not the neurotransmitter released by i-NANC nerves. However, in view of the increasing evidence in favour of a role for VIP as an i-NANC neurotransmitter (Ellis & Farmer, 1989a & 1989b), other explanations must be sought. Phosphoramidon may not reach the site of release of the i-NANC neurotransmitter. This is unlikely since contractile responses of guinea-pig bronchi evoked by the stimulation of e-NANC nerves were potentiated by phosphoramidon (see section 4.3). Neurogenic responses to EFS are mediated by neurotransmitters released locally by nerve terminals in the smooth muscle layer and modulation of such responses by enzymatic degradation must occur in the vicinity of the neuroeffector junction. Thus, if NEP is not localised to this area it may not be involved in the degradation of the i-NANC neurotransmitter. This would be consistent with our suggestion that responses of guinea-pig trachea to exogenous VIP are primarily modulated by NEP localised to airway epithelium rather than smooth muscle. Furthermore, a differential distribution of NEP in smooth muscle may explain why e-NANC but not i-NANC responses of airway smooth muscle are potentiated by phosphoramidon.

Although e-NANC responses of guinea-pig airways to EFS are predominantly observed in the bronchi, they may sometimes be elicited in the trachea, particularly if preparations are obtained from ovalbumin-sensitised animals (Andersson & Grundstrom, 1983). Furthermore, SP-immunoreactive nerves are found in the tracheal smooth muscle layer (Wharton *et al.*, 19879; Lundberg *et al.*, 1984). Tracheal preparations in the present experiments were selected from the upper trachea, although it is possible that EFS may induce the release of endogenous tachykinins in these preparations, but responses may be masked by the inhibitory response to EFS. If the i-NANC neurotransmitter is degraded by NEP in the smooth muscle layer, potentiation of EFS-induced i-NANC responses by phosphoramidon may be masked by a parallel potentiation of e-NANC responses. It may therefore be interesting to examine the effect of phosphoramidon on i-NANC responses to EFS in the presence of a tachykinin-receptor antagonist.

In conclusion, airway epithelium exerts an inhibitory influence on responses of guinea-pig tracheal smooth muscle to VIP which may be due to the degradation of VIP by NEP localised to the epithelium. I-NANC responses do not appear to be modulated by endogenous NEP. Furthermore, EFS may induce the release of a relaxant factor from the epithelium.

8. Effect of phosphodiesterase inhibitors and guanylate/adenylate cyclase inhibitors on VIP- and i-NANC nerve-induced relaxation of guinea-pig trachea

8.1. Aim

Selective antagonists to VIP-receptors in the airways are unavailable, therefore the definitive identification of VIP as the neurotransmitter of i-NANC nerves is at present impossible. I-NANC responses to EFS can be induced in other isolated tissues, including the mouse anococcygeus and bovine retractor penis muscle. In these preparations the identity of the neurotransmitter released by i-NANC nerves is unknown, and may not be VIP. However the post-receptor mechanism mediating neurogenic relaxation is being compared to the mechanism of action of known agonists in an attempt to identify the i-NANC neurotransmitter.

Smooth muscle relaxation is thought to be mediated by changes in intracellular levels of cyclic nucleotides. Receptor occupancy by the appropriate agonist is linked to the activation of adenylate cyclase or guanylate cyclase, leading to the accumulation of adenosine cyclic 3', 5'-monophosphate (cAMP) or guanosine cyclic 3', 5'-monophosphate (cGMP) respectively. Cyclic nucleotides are known to activate specific cAMP- or cGMP-dependent protein kinases which mediate relaxation through activation of a variety of cellular proteins. Relaxation of airway smooth

muscle induced by *beta*-agonists (Murad & Kimura, 1974), prostaglandin E_2 (Torphy *et al.*, 1985), forskolin (Torphy *et al.*, 1985) and VIP (Frandsen *et al.*, 1978) is associated with cAMP accumulation, whereas nitrocompounds, such as sodium nitroprusside (SNP) and nitric oxide, are thought to relax airway smooth muscle by a cGMP-mediated process (Katsuki and Murad, 1977). Thus, if VIP is the i-NANC neurotransmitter, relaxation of isolated smooth muscle preparations induced by i-NANC stimulation should also be mediated by cAMP. Neurogenic relaxation of the bovine retractor penis muscle is blocked by inhibitors of guanylate cyclase and is associated with an increase in the tissue content of cGMP but not cAMP suggesting that in this tissue i-NANC responses are not mediated by VIP but may involve a cGMP-dependent process (Bowman *et al.*, 1982; Bowman & Drummond, 1984).

Cyclic nucleotides are metabolised by specific intracellular phosphodiesterases (PDEs) and one of the criteria used in implicating cyclic nucleotides in the functional response to a particular agonist is that this response should be potentiated by appropriate PDE inhibitors. Thus, relaxation of airway smooth muscle to, for example, isoproterenol and SNP, should be facilitated by specific cAMP and cGMP PDE inhibitors respectively. We have therefore compared the effects of two selective PDE inhibitors, SK&F 94120 (a cAMP-PDE inhibitor) and zaprinast (a cGMP-PDE inhibitor) on relaxation of guinea-pig tracheal smooth muscle to isoproterenol, SNP,

VIP and EFS *in vitro*. We have also examined the effects of N-ethylmalimide (NEM), a putative adenylate cyclase inhibitor (Sigafoos and Abramowitz, 1986), and methylene blue, a putative guanylate cyclase inhibitor (Gruetter et al., 1981), on relaxation to isoproterenol, SNP, VIP and EFS.

8.2. Protocol

8.2.1. PDE inhibitors, NEM and methylene blue

Experiments were carried out in the presence of 5 uM indomethacin in order to prevent the generation of cyclooxygenase metabolites of arachidonic acid. Indomethacin was included in the KH solution at the beginning of the equilibration period and was present throughout.

Tissues were precontracted with 30mM KCl and relaxation was induced by exogenous agonists or by EFS. Cumulative C-R curves were constructed to isoproterenol, SNP and VIP. When relaxation was induced by isoproterenol tissues were incubated with 50 uM corticosterone for 30 min prior to KCl precontraction in order to block extraneuronal uptake of isoproterenol. When relaxation was induced by VIP tissues were incubated with 10 uM phosphoramidon for 30 min prior to KCl precontraction in order to prevent the breakdown of VIP by neutral endopeptidase (Liu *et al.*, 1987). EFS was applied for 30 s over a range of frequencies (0.1 ms, 40 V, 0.5-50 Hz). The order of frequencies was randomised in order to avoid any time-dependent variation in i-NANC responses. Atropine (1 uM) and propranolol (1 uM) were present throughout all procedures involving EFS in order to block cholinergic and adrenergic responses to EFS respectively. Phosphoramidon (10 uM) was also present in procedures involving EFS.

The effect of PDE inhibitors on responses to isoprenaline, SNP VIP and EFS was investigated by incubating parallel tissues for 30 min with SK&F 94120 (1-10 uM), zaprinast (3-30 uM) or vehicle (NaOH; final bath concentration 30 uM). For each trachea, one preparation served as control and the other three were treated with different concentrations of one PDE inhibitor. The effect of inhibitors of adenylate cyclase and guanylate cyclase on responses to isoprenaline, SNP, VIP and EFS was investigated by incubating parallel tissues with and without 3-100 uM NEM or 30-100 uM methylene blue.

8.2.2. Precontraction level

Since PDE inhibitors may themselves induce smooth muscle relaxation through the accumulation of cAMP and /or cGMP, they may also affect the level of precontraction produced by 30 mM KCl. Hence, any potentiation of relaxation observed in the presence of PDE inhibitors may be due to a decreased level of functional antagonism. In order to investigate this possibility we initially examined the effects of SK&F 94120 and zaprinast on contraction induced by KCl. Adjacent tissues were incubated with SK&F 94120 (1-10 uM), zaprinast (3-30 uM) or 30uM NaOH and cumulative C-R response curves constructed to KCl. Parallel tissue segments were

precontracted with 25 or 30 mM KCl in the absence of PDE inhibitors (see results) and relaxation was induced by isoproterenol, VIP or EFS as previously described.

8.2.3. TTX-resistant relaxation

To investigate the effect of TTX on relaxation to EFS parallel tissue segments were incubated with and without 3 uM TTX. Tissues were precontracted with 30 mM KCl and frequency-response curves were constructed to EFS. To investigate the effect of PDE inhibitors on TTX-resistant relaxation to EFS, tissues were incubated for 30 min with 3 uM TTX and with 10 uM SK&F 94120, 30 uM zaprinast or 30 uM NaOH. Frequency-response curves to EFS were then constructed. Atropine (1 uM) and propranolol (1 uM) were present throughout.

8.3. Results

8.3.1. Effect of PDE inhibitors on relaxation.

The effect of SK&F 94120 and zaprinast on relaxation induced by isoprenaline and SNP is shown in figures 37 and 38, and table 11. SK&F 94120 caused a potentiation of relaxation induced by isoprenaline (demonstrated as a leftward shift of C-R curves), but had no effect on relaxation induced by SNP. Significant increases in mean pD_2 values for relaxation to isoprenaline were obtained at 1, 3 and 10 uM SK&F 94120. Zaprinast had no effect on relaxation induced by isoprenaline but caused a potentiation of relaxation induced by SNP (demonstrated as a leftward shift of C-R curves). Significant increases in mean pD_2 values for relaxation to SNP were obtained at 10 and 30 uM zaprinast.

The effect of SK&F 94120 and zaprinast on relaxation of guinea-pig trachea induced by VIP and i-NANC nerve stimulation are shown in figures 39 and 40, and table 12. SK&F 94120 caused a potentiation of relaxation induced by VIP (demonstrated as a leftward shift of C-R curves), with significant increases in mean pD_2 values being attained at 3 and 10 uM SK&F 94120. Zaprinast had no significant effect on relaxation induced by VIP at any concentration.

SK&F 94120 caused a potentiation of relaxation induced by EFS. In the presence of SK&F 94120, EFS frequency-response curves did not always reach a plateau

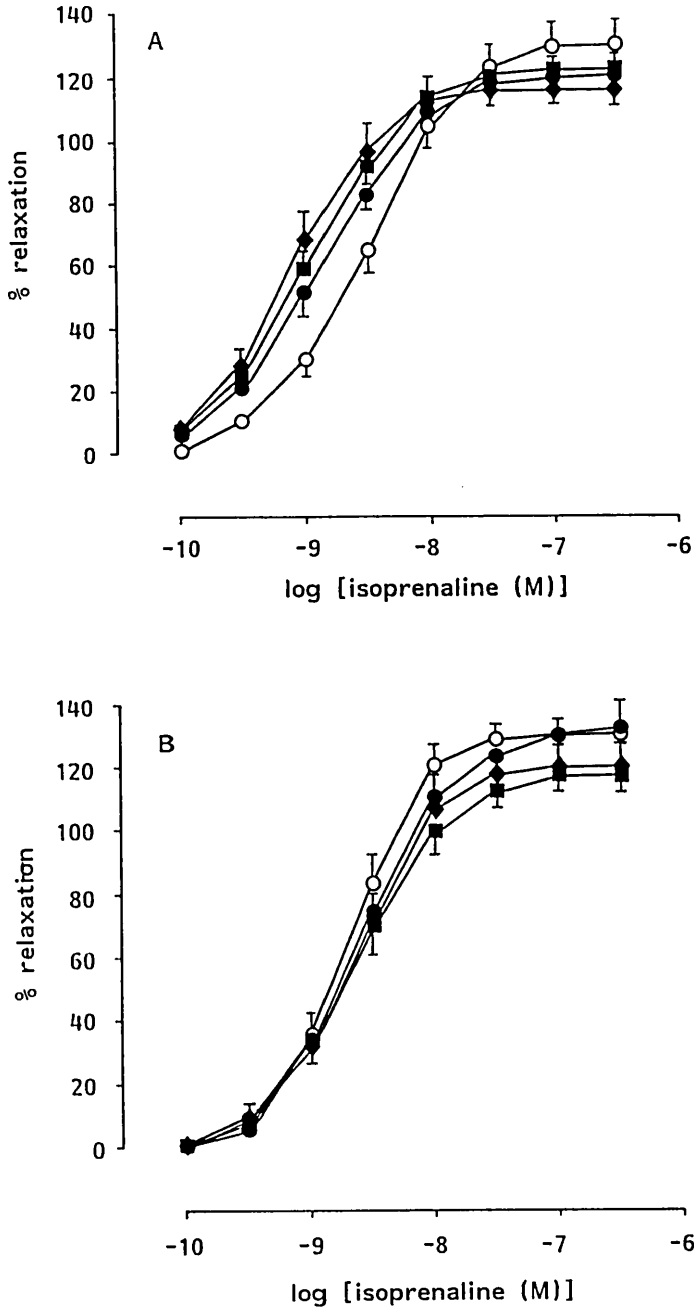


Figure 37. Effect of PDE inhibitors on relaxation of guinea-pig trachea to isoprenaline. In (A) tissues were incubated with 1 uM (●), 3 uM (■) or 10 uM (◆) SK&F 94120, or with 30 uM NaOH (O). In (B) tissues were incubated with 3 uM (●), 10 uM (■) or 30 uM (◆) zaprinast, or with 30 uM NaOH (O). Indomethacin (5 uM) and corticosterone (50 uM) were present throughout. Relaxation is expressed as % reversal of precontraction. Points represent means ± SE of n=6.

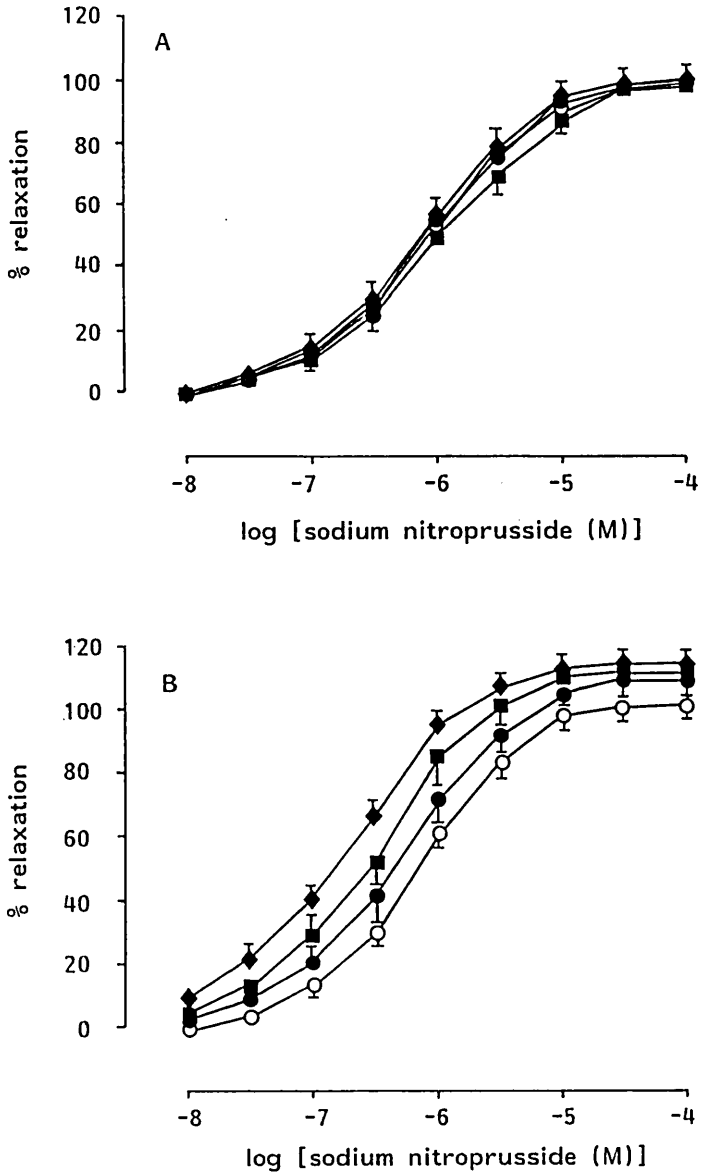


Figure 38. Effect of PDE inhibitors on relaxation of guinea-pig trachea to SNP. In (A) tissues were incubated with 1 uM (●), 3 uM (■) or 10 uM (◆) SK&F 94120, or with 30 uM NaOH (O). In (B) tissues were incubated with 3 uM (●), 10 uM (■) or 30 uM (◆) zaprinast, or with 30 uM NaOH (O). Indomethacin (5 uM) was present throughout. Relaxation is expressed as % reversal of precontraction. Points represent means ± SE of n=6.

Table 11. Effect of PDE inhibitors on relaxation of guinea-pig trachea induced by isoprenaline and SNP.

	isoprenaline		SNP	
PDE inhibitor	pD_2[1]	ratio[2]	pD_2	ratio[2]
30 uM NaOH	8.53 ± 0.11		6.06 ± 0.06	
1 uM SK&F 94120	8.89 ± 0.15**	2.29	6.08 ± 0.03	1.05
3 uM SK&F 94120	8.93 ± 0.11**	2.51	6.04 ± 0.05	0.95
10 uM SK&F 94120	9.19 ± 0.08**	3.72	6.08 ± 0.06	1.05
30 uM NaOH	8.71 ± 0.07		6.17 ± 0.05	
3 uM zaprinast	8.60 ± 0.11	0.78	6.28 ± 0.12	1.29
10 uM zaprinast	8.65 ± 0.11	0.87	6.44 ± 0.12*	1.86
30 uM zaprinast	8.70 ± 0.10	0.98	6.72 ± 0.06**	3.55

1. $pD_2 = -\log EC_{50}$.

2. Concentration-ratio, calculated as the EC_{50} in the presence of PDE inhibitor divided by the EC_{50} in the absence of PDE inhibitor.

* $p<0.05$, ** $p<0.01$ compared to responses in the absence of PDE inhibitor, unpaired Student's T-test.

pD_2s represent means ± SE of n=6-8.

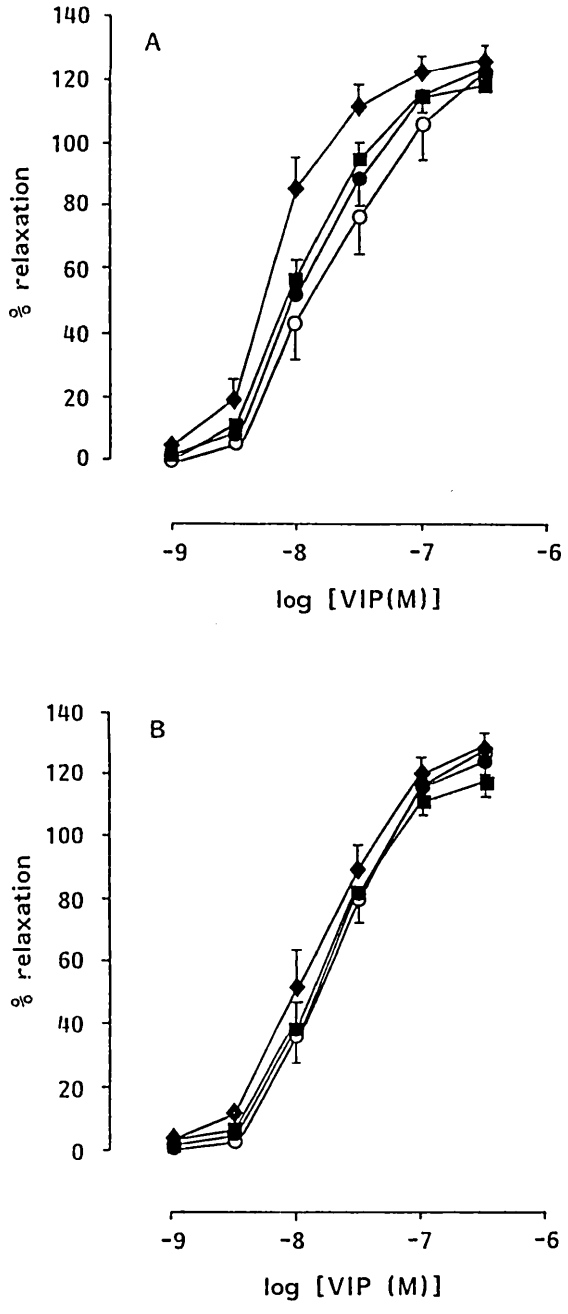


Figure 39. Effect of PDE inhibitors on relaxation of guinea-pig trachea to VIP. In (A) tissues were incubated with 1 uM (●), 3 uM (■) or 10 uM (◆) SK&F 94120, or with 30 uM NaOH (O). In (B) tissues were incubated with 3 uM (●), 10 uM (■) or 30 uM (◆) zaprinast, or with 30 uM NaOH (O). Indomethacin (5 uM) and phosphoramidon (10 uM) was present throughout. Relaxation is expressed as % reversal of precontraction. Points represent means ± SE of n=7-8.

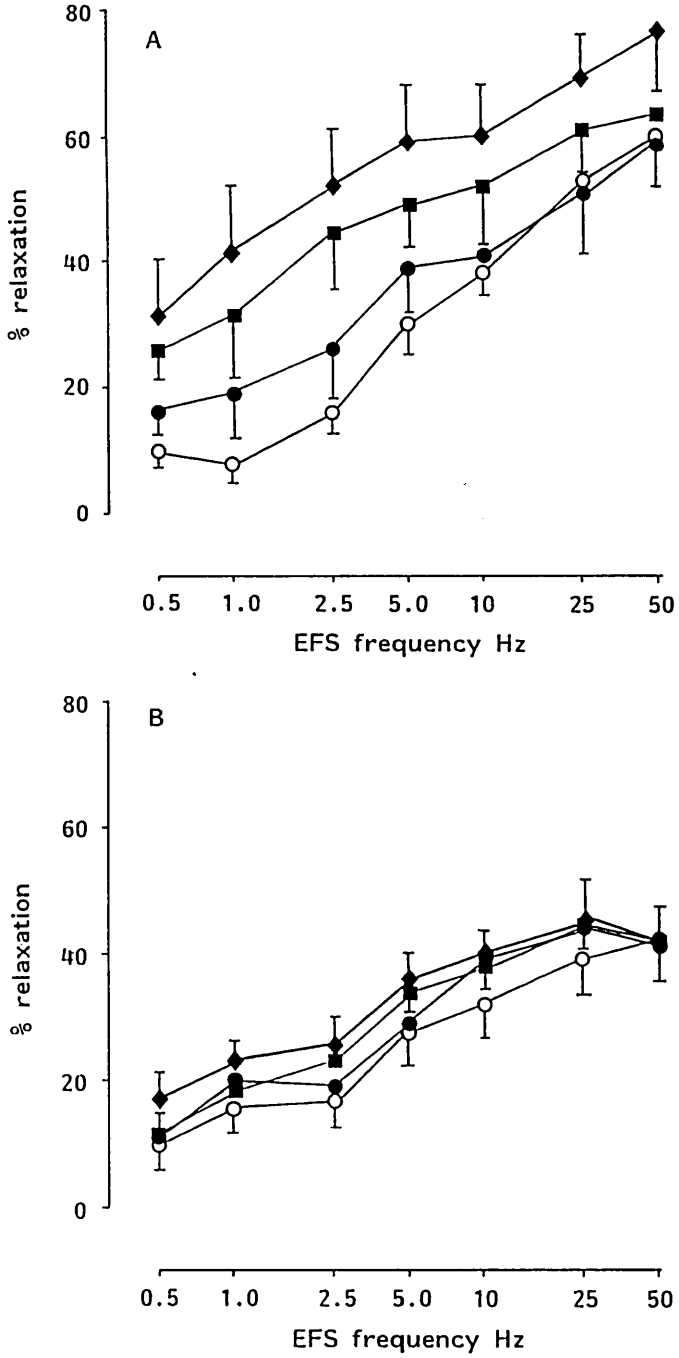


Figure 40. Effect of PDE inhibitors on relaxation of guinea-pig trachea to EFS (0.1 ms, 40 V, 0.5-50 Hz). In (A) tissues were incubated with 1 uM (●), 3 uM (■) or 10 uM (◆) SK&F 94120, or with 30 uM NaOH (O). In (B) tissues were incubated with 3 uM (●), 10 uM (■) or 30 uM (◆) zaprinast, or with 30 uM NaOH (O). Atropine (1 uM), propranolol (1 uM), indomethacin (5 uM) and phosphoramidon (10 uM) were present throughout. Relaxation is expressed as % reversal of precontraction. Points represent means ± SE of n=7-9.

Table 12. Effect of PDE inhibitors on relaxation of guinea-pig trachea to trachea to VIP and i-NANC nerve stimulation.

	VIP		i-NANC	
PDE inhibitor	pD_2[1]	ratio[2]	log EF_{35}[3]	ratio[2]
30 uM NaOH	7.59 ± 0.15		0.88 ± 0.09	
1 uM SK&F 94120	7.74 ± 0.15	1.41	0.60 ± 0.11	1.91
3 uM SK&F 94120	7.83 ± 0.11*	1.74	0.13 ± 0.10*	5.62
10 uM SK&F 94120	8.06 ± 0.11**	2.95	-0.17 ± 0.08*	11.22
30 uM NaOH	7.67 ± 0.06		1.06 ± 0.13	
3 uM zaprinast	7.73 ± 0.08	1.15	0.95 ± 0.09	1.29
10 uM zaprinast	7.76 ± 0.09	1.23	0.85 ± 0.11	1.62
30 uM zaprinast	7.86 ± 0.11	1.55	0.80 ± 0.12	1.82

1. pD_2 = -log EC_{50}.

2. Concentration-ratio, calculated as the EC_{50} in the presence of PDE inhibitor divided by the EC_{50} in the absence of PDE inhibitor.

3. EF_{35} = EFS frequency inducing 35 % reversal of KCl precontraction.

* $p<0.05$, ** $p<0.01$ compared to responses in the absence of PDE inhibitor, unpaired Student's T-test.

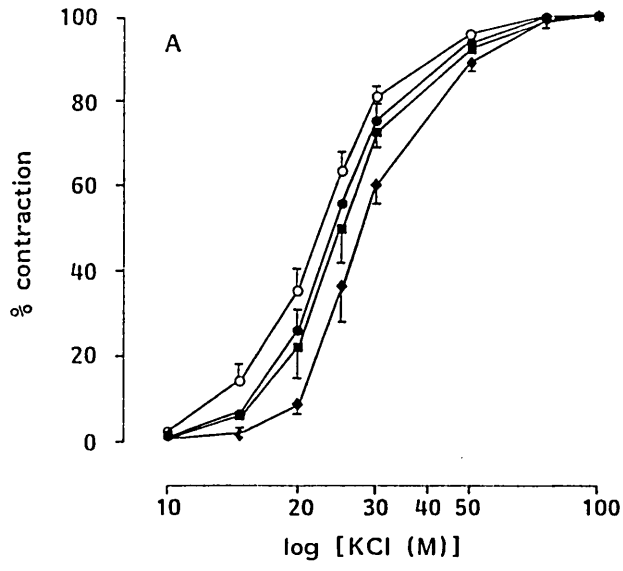
Figures represent means ± SE of n=7-9.

level within the frequency range used, therefore frequencies inducing 50 % of maximal relaxation to EFS could not be determined. Instead, comparisons were made of frequencies inducing 35 % reversal of KCl precontraction (EF_{35}). Mean EF_{35}s were significantly increased by 3 and 10 uM SK&F 94120, but were unaffected by zaprinast at any concentration. Zaprinast (30 uM) did, however, induce a small potentiation of relaxation induced by 1 and 5 Hz EFS ($p<0.05$).

8.3.2. Effect of PDE inhibitors on KCl contraction

SK&F 94120 caused a rightward shift of the C-R curves to KCl (figure 41) which was significant at 10 uM SK&F 94120 (mean pD_2 1.65 $\pm$0.01 NaOH-treated, 1.55 $\pm$ 0.01 SK&F 94120-treated, $p<0.01$, n=9) but not at lower concentrations. SK&F94120 (10 uM) had no significant effect on maximal contraction induced by KCl (2675 $\pm$ 220 mg NaOH-treated, 3104 $\pm$ 279 mg SK&F 94120-treated, n=9). Zaprinast (30 uM) had no effect on contraction induced by KCl (pD_2 1.64 $\pm$ 0.02, maximal contraction 2889 $\pm$ 243 mg NaOH-treated preparations; pD_2 1.65 $\pm$ 0.01, maximal contraction 2765 $\pm$ 155 mg zaprinast-treated preparations, n=6), (figure 41).

In the absence of SK&F 94120, 30 mM KCl produced a contraction which was 81.0 $\pm$ 2.3 % of its maximum contraction. In the presence of 10 uM SK&F 94120, 30 mM KCl induced 60.6 $\pm$ 4.9 % of its maximum contraction. In



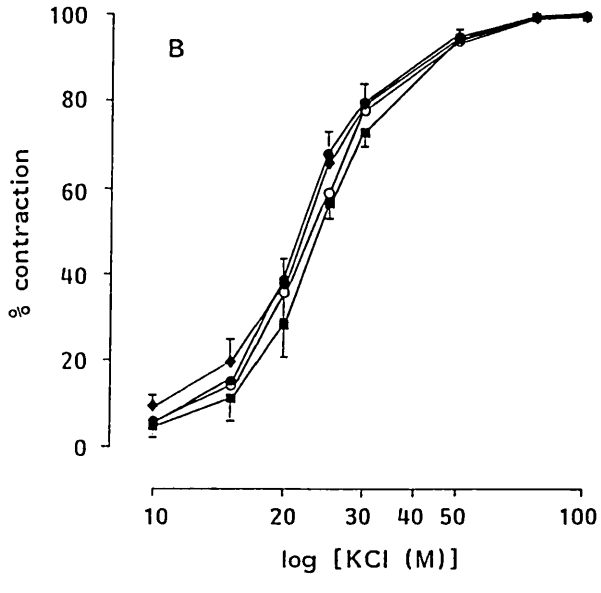


Figure 41. Effect of PDE inhibitors on contraction of guinea-pig trachea to KCl. In (A) tissues were incubated with 1 uM (●), 3 uM (■) or 10 uM (◆) SK&F 94120, or with 30 uM NaOH (O). In (B) tissues were incubated with 3 uM (●), 10 uM (■) or 30 uM (◆) zaprinast, or with 30 uM NaOH (O). Indomethacin (5 uM), atropine (1 uM) and propranolol (1 uM) were present throughout. Contraction is expressed as % of maximum contraction in each preparation. Points represent means ± SE of n=9.

order to determine whether the potentiation of relaxation induced by SK&F 94120 could be due to a reduction in the level of precontraction, a concentration of KCl was selected which induced a contraction (in the absence of SK&F 94120) similar to that produced by 30 mM KCl in the presence of 10 uM SK&F 94120. Thus, 25 mM KCl induces 61 % of maximal KCl contraction.

8.3.3. Effect of precontraction level on relaxation

There was no significant difference in relaxation induced by isoproterenol, VIP or EFS between tissues precontracted by 25 mM KCl (61 % of maximal KCl contraction) and 30 mM KCl (81 % of maximal KCl contraction) (table 13).

8.3.4. TTX-resistant relaxation

TTX attenuated relaxations induced by EFS (figure 42). Attenuation was significant for stimulation frequencies greater than 1 Hz. TTX-resistant relaxations were not significantly potentiated by either 10 uM SK&F 94120 or 30 uM zaprinast (figure 42).

Table 13. Effect of KCl precontraction on responses of guinea-pig trachea to isoprenaline, VIP and EFS.

	Precontraction	
	25 mM KCl	30 mM KCl
isoprenaline (pD_2[1])	8.67 ± 0.14	8.43 ± 0.07
VIP (pD_2[1])	7.91 ± 0.16	7.69 ± 0.06
EFS (log EF_{50}[2])	0.59 ± 0.06	0.49 ± 0.05
EFS (log EF_{35}[3])	0.91 ± 0.06	0.79 ± 0.09

1. pD_2 = -log EC_{50}

2. EF_{50} = frequency inducing 50 % of maximal relaxation to EFS.

3. EF_{35} = frequency inducing 35 % reversal of KCl precontraction.

Figures represent means ± SE of n=7-10

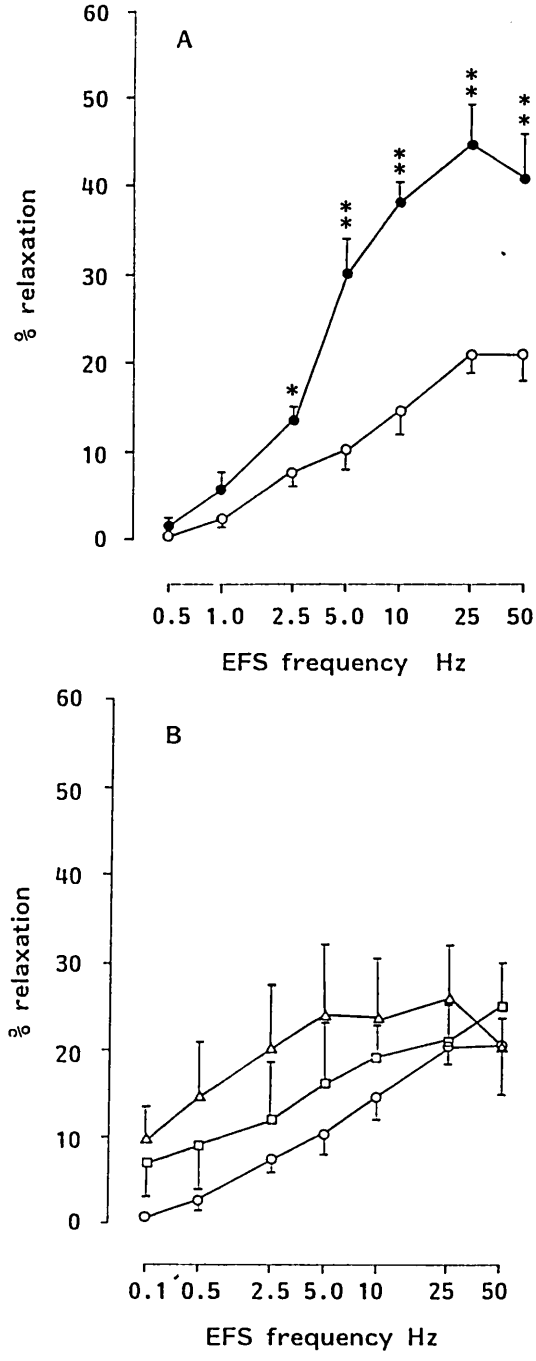


Figure 42. TTX-resistant responses of guinea-pig trachea to EFS (0.1 ms, 40 V, 0.5-50 Hz). In (A) relaxation was induced in the absence (●) and presence (O) of 3 uM TTX. In (B) relaxation was induced in the presence of 3 uM TTX plus 10 uM SK&F 94120 (Δ), 30 uM zaprinast (□) or 30 uM NaOH (O). Atropine (1 uM), propranolol (1 uM) were present throughout. Relaxation is expressed as % reversal of KCl precontraction. Points represent means ± SE of n=6-8, * p0.05, ** p<0.01 absence vs. presence of TTX.

8.3.5. Effect of NEM on relaxation

NEM (30 uM) had no effect on resting tension or on contractile responses to 30 mM KCl (1889 ± 267 mg untreated, 1823 ± 336 mg NEM-treated preparations, n=8). NEM (30 uM) had no effect on relaxation induced by isoprenaline (pD_2 8.38 ± 0.06, maximal response 126 ± 8 % untreated preparations; pD_2 8.46 ± 0.05, maximal response 119 ± 10 % NEM-treated preparations, n=4) or SNP (pD_2 6.36 ± 0.03, maximal response 102± 8 % untreated preparations; 6.46 ± 0.07, maximal response 111 ± 6 % NEM-treated preparations, n=4).

NEM (100 uM) induced a contractile response in 1 out of 6 preparations (468 mg), and reduced contractile responses to 30 mM KCl (1970 ± 432 mg untreated, 389 ± 107 mg NEM-treated, n=6). NEM (100 uM) abolished responses to isoprenaline (n=3) and SNP (n=3).

8.3.6. Effect of methylene blue on relaxation

Methylene blue (30 uM) induced a contractile response (312 ± 21, n=22) but had no significant effect on contractile response to KCl (2030 ± 226 mg untreated, 1736 ± 162 mg methylene blue-treated, n=22). Methylene blue (30 uM) had no effect on relaxation induced by isoprenaline (pD_2 8.72 ± 0.16 untreated, 8.57 ± 0.13 methylene blue-treated, n=5) but inhibited relaxation induced by SNP (pD_2 6.08 ± 0.11 untreated, 5.61 ± 0.02 methylene

blue-treated, $p<0.05$, concentration-ratio 2.95, n=6), (figure 43). Methylene blue (30 uM) had no effect on relaxation induced by VIP (pD_2 7.72 $\pm$ 0.09 untreated, 7.68 $\pm$ 0.08 methylene blue-treated, n=5) or on relaxation induced by EFS (log EF_{50} 0.54 $\pm$ 0.05 untreated, 0.48 $\pm$ 0.07 methylene blue-treated, n=6), (figure 43).

Methylene blue (100 uM) induced a contractile response (2432 $\pm$ 653 mg, n=3) and was not used in further experiments.

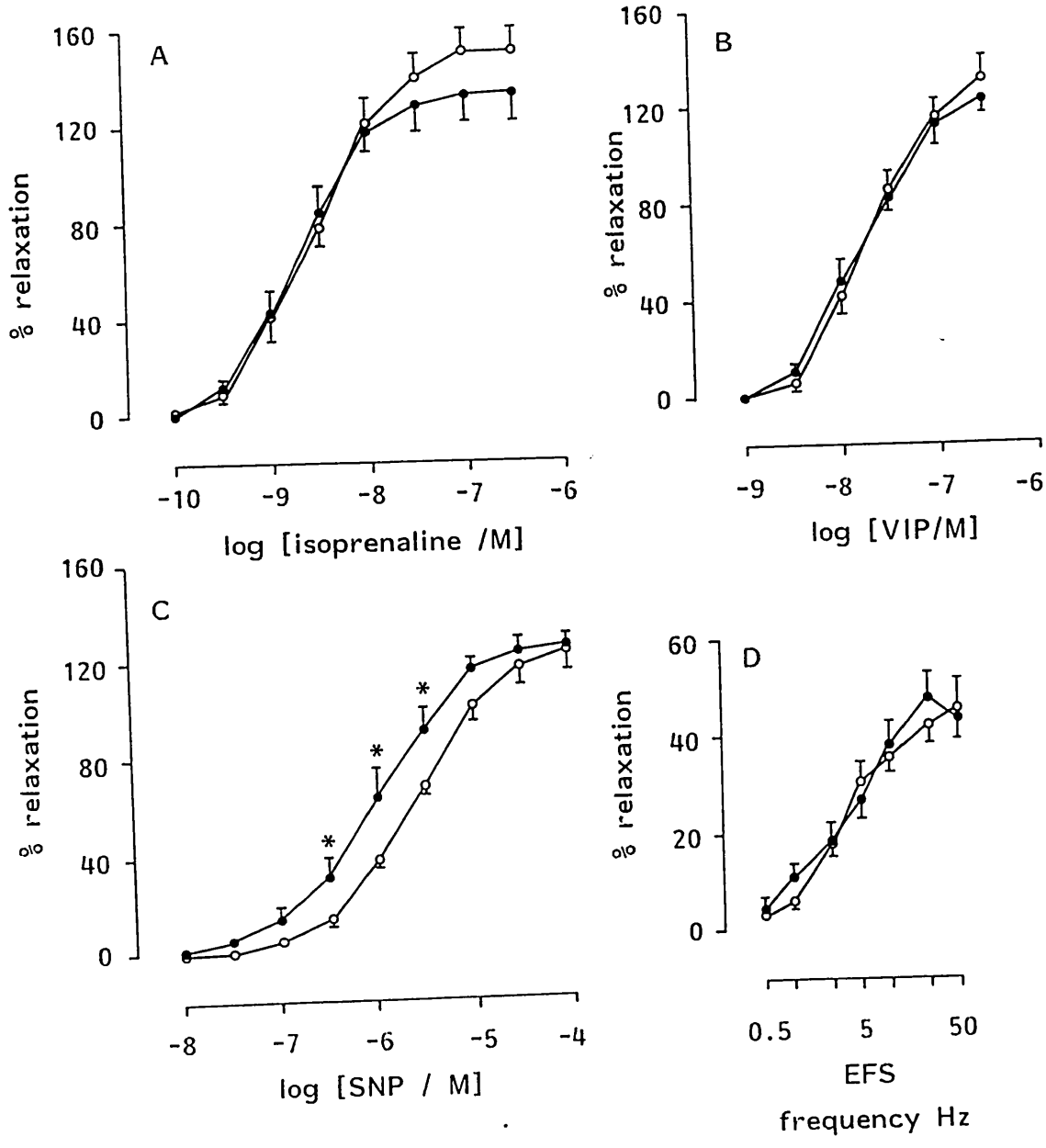


Figure 43. Effect of methylene blue on responses of guinea-pig trachea to (A) isoprenaline, (B) VIP, (C) SNP and (D) EFS (0.1 ms, 40 V, 0.5-50 Hz). Relaxation was induced in the absence (O) or presence (●) of 30 uM methylene blue. Indomethacin (5 uM) was present throughout. Corticosterone (50 uM) was present in (A). Phosphoramidon (10 uM) was present in (B) and (D). Atropine (1 uM) and propranolol (1 uM) were present in (D). Relaxation is expressed as % reversal of KCl precontraction. Points represent means $\pm$ SE of n=5-6, * $p<0.05$ absence vs. presence of methylene blue.

8.4. Discussion

Cyclic nucleotide PDEs can be differentiated into several isoenzymes according to their kinetic characteristics, substrate specificity and sensitivity to activators and inhibitors (Weishaar *et al.*, 1985). In various tissues, zaprinast selectively inhibits the low Km PDE I which hydrolyses cGMP and sometimes cAMP, whereas SK&F 94120 selectively inhibits the low Km PDE III which hydrolyses cAMP (Bergstrand *et al*, 1977; Gristwood *et al.*, 1985; Weishaar *et al.*, 1986). PDE inhibitors induce smooth muscle relaxation, although a good correlation between relaxation and elevation of tissue cyclic nucleotide levels is not always observed. SK&F 94120, for example, induces relaxation of guinea-pig trachea at concentrations (1 uM) that have no effect on cAMP levels (Bryson & Rodgers, 1987). PDE inhibitors may therefore induce effects by mechanisms unrelated to inhibition of cyclic nucleotide metabolism, and this possibility cannot be excluded in our experiments.

In canine airways, SK&F 94120 caused a potentiation of isoprenaline-induced relaxation and cAMP accumulation but had no effect on SNP-induced relaxation or cGMP accumulation, whereas zaprinast potentiated the functional and biochemical responses to SNP but not isoprenaline (Torphy *et al.*, 1987). Although cyclic nucleotide levels were not measured in our experiments, similar functional

effects of SK&F 94120 and zaprinast were observed. SK&F 94120 also potentiated relaxation induced by the stimulation of i-NANC nerves and it is tempting to speculate that this effect is mediated by a potentiation of cAMP accumulation. Zaprinast induced a small potentiation of relaxation induced by EFS although overall there was no significant shift in the frequency-response curve. This, however does not preclude cGMP as a mediator of such relaxation since cGMP may be metabolised by a PDE isoenzyme which is not inhibited by zaprinast. In canine tracheal smooth muscle cGMP levels are regulated by calmodulin-sensitive (PDE Ic) and calmodulin-insensitive (PDE Ia) isoenzymes, and zaprinast is about 30-fold selective for PDE Ia over PDE Ic (Cieslinski *et al.*, 1988). If similar isoenzymes exist in guinea-pig tracheal smooth muscle, significant inhibition of PDE Ia should be achieved by all concentrations of zaprinast used in our experiments, but only high concentrations would inhibit PDE Ic. In human tracheal smooth muscle, a further calmodulin-sensitive PDE (PDE 1b) hydrolyses cGMP but is not inhibited by zaprinast (Cieslinski *et al.*, 1988). If such a calmodulin-sensitive enzyme is the major regulator of cGMP breakdown following stimulation of i-NANC nerves, then zaprinast would not be expected to potentiate relaxation induced by EFS. EFS-induced relaxation of the lower esophageal sphincter is associated with an increase

in cGMP content (Torphy *et al.*, 1986), but neither the relaxation nor the cGMP accumulation is potentiated by zaprinast (Rattan & Moummi, 1989; Barnette *et al.*, 1989).

NEM has been purported to be an inhibitor of adenylate cyclase (Sigafoos & Abramowitz, 1986) and in the lower esophageal sphincter inhibits relaxation induced by forskolin, VIP and EFS. In our experiments, however, a selective inhibition of isoprenaline-induced relaxation could not be demonstrated and NEM was therefore not a useful adenylate cyclase inhibitor in our system. Methylene blue has been purported to be an inhibitor of particulate and soluble guanylate cyclase (Gruetter *et al.*, 1981) and in our experiments induced a small but significant inhibition of SNP-induced relaxation but not of isoprenaline- or VIP-induced relaxation. SNP is an activator of the soluble guanylate cyclase, therefore any elevation of cGMP levels by EFS may not necessarily occur in the same subcellular compartment. Methylene blue, however, had no effect on relaxation induced by the activation of i-NANC nerves suggesting that guanylate cyclase does not mediate this response. In contrast, oxyhaemoglobin, another inhibitor of guanylate cyclase, has been shown attenuate i-NANC relaxation of guinea pig tracheal smooth muscle (Ko & Lai, 1988), and methylene blue inhibits EFS-induced relaxation of lower esophageal sphincter (Rattan & Moummi, 1989).

Potentiation of responses to exogenous agonists by PDE inhibitors results from the modulation of cyclic nucleotide levels (or other mechanisms) in the smooth muscle. Potentiation of responses to nerve stimulation, however may result not only from postjunctional events but also from modulation of neurotransmitter release. The effects of PDE inhibitors on neurotransmitter release from mammalian peripheral nerves is not known, although there is evidence to suggest that cAMP may mediate modulation of neurotransmitter release from invertebrate nerve endings (Kupfermann, 1980). Furthermore, EFS of isolated tissue will excite all neural elements present. Guinea-pig airways possess an excitatory non-adrenergic non-cholinergic (e-NANC) innervation, which is predominant in bronchial smooth muscle, but may occasionally be observed in the trachea (Grundstrom *et al.*, 1981). Responses to EFS therefore represent a balance between excitatory and inhibitory components. Potentiation of EFS-induced relaxation by SK&F 94120 could therefore be due to (i) postjunctional potentiation of i-NANC responses, (ii) postjunctional attenuation of e-NANC responses, (iii) prejunctional potentiation of i-NANC neurotransmitter release, or (iv) prejunctional inhibition of e-NANC neurotransmitter release. Our experiments were conducted on cervical trachea where e-NANC responses are least likely to be observed. However, we cannot exclude modulation of e-NANC responses as the mechanism by which SK&F 94120 potentiates EFS-induced relaxation,

particularly in the presence of phosphoramidon which is known to potentiate e-NANC responses to EFS (Sekizawa *et al.*, 1987b). SK&F 94120 was able to potentiate responses to VIP, therefore if EFS-induced relaxation is mediated by the release of VIP from i-NANC nerves, then a parallel potentiation of EFS-induced relaxation would be expected.

SK&F 94120 reduced the level of precontraction induced by KCl. It is well established that when airway smooth muscle is precontracted with a muscarinic agonist, the degree of relaxation induced by a *beta*-agonist is inversely proportional to the degree of muscarinic tone (van den Brink, 1973). This functional antagonism may in part be due to an inhibitory effect of muscarinic agonists on drug-induced cAMP accumulation and cAMP-dependent protein kinase activation (Torphy *et al.*, 1983). If relaxation following precontraction by KCl is also subject to functional antagonism, then the potentiation by SK&F 94120 of relaxation to at least isoprenaline may simply be due to a reduction in the level of KCl precontraction and consequently a reduction in the degree of functional antagonism. A comparison of relaxation following precontraction by 30 versus 25 mM KCl (in the absence of PDE inhibitors) indicated that relaxation responses to isoproterenol, VIP and EFS were not significantly potentiated by decreasing the level of precontraction f m 81 to 61 % of maximal KCl contraction. Thus, changes in

precontraction levels are unlikey to account for the effects of SK&F 94120 on relaxation to isoproterenol, VIP and EFS.

Relaxation to EFS in the presence of atropine and propranolol was attenuated by TTX suggesting that it is at least in part neurogenic in origin. KCl itself is able to induce the release of i-NANC neurotransmitter by depolarising nerve membranes (Gibson & James, 1977), although in our experiments EFS was still able to induce neural responses in the presence of KCl. Stimulation parameters used in the present study are similar to those used by others to elicit TTX-sensitive i-NANC relaxations, but TTX-resistant relaxations appeared to be larger than those generally reported for the guinea-pig trachea (Coburn & Tomita, 1973; Coleman & Levy, 1974; Richardson & Bouchard, 1975). Indeed a great variability is seen between studies in the proportion of TTX-sensitive and resistant relaxations obtained. Several factors may contribute to this including the type of precontraction (spontaneous or drug induced), the degree of precontraction or the physical characteristics of the stimulation apparatus (Altiere *et al.*, 1984). The large TTX-resistant component of relaxation to EFS seen in our study may be due to the use of KCl as a precontracting agent, however further experiments would be required to investigate this possibility. The presence of a TTX-resistant component to EFS-induced relaxation raises the possibility that PDE inhibitors may modulate this

component rather than neurally-mediated relaxation. This is unlikely since SK&F 94120 and zaprinast had no effect on TTX-resistant relaxations induced by EFS.

In conclusion, we have shown that relaxation of guinea-pig trachea induced by the stimulation of i-NANC nerves is potentiated by inhibition of cAMP breakdown. Although we cannot determine the mechanism underlying this effect our results are consistent with the view that VIP may be the neurotransmitter released by i-NANC nerves.

9. Conclusion

In vitro experiments on isolated smooth muscle preparations allow the characterisation of the neural and humoral control of smooth muscle function, and of the modulation of control mechanisms by endogenous and exogenous factors. Modulation of the neural input to smooth muscle can occur at several sites: 1) prejunctionally, through the modulation of neurotransmitter release, 2) at the neuroeffector junction, through an interaction with the neurotransmitter (eg. metabolism of the neurotransmitter) or 3) postjunctionally, through alterations in the number, affinity or efficacy of neurotransmitter receptors. Experiments described in this thesis suggest that cholinergic neurotransmission in human airway smooth muscle may be modulated by catecholamines. We have used an indirect method of comparing responses induced by endogenous and exogenous acetylcholine to suggest that catecholamines may inhibit acetylcholine release from cholinergic nerve terminals, although verification would require actual measurement of neurotransmitter release. This is important since modulation of cholinergic neurotransmission in canine airways by noradrenaline has been suggested by a similar approach to ours (Vermeire & Vanhoutte, 1979) but cannot be demonstrated by acetylcholine release measurements (Martin & Collier, 1986).

Characterisation of the neural control of airway smooth muscle requires the identification of neurotransmitters and their receptors. A variety of peptides have been localised to nerves supplying the airways and NANC responses induced in isolated preparations may be mediated by neuropeptides. NKA and SP are putative transmitters for the e-NANC innervation of guinea-pig airways, although tachykinins may be co-localised with other peptides such as calcitonin-related peptide (Lundberg *et al.*, 1985) and galanin (Cheung *et al.*, 1985). We have attempted to characterise the receptors mediating tachykinin-induced and e-NANC nerve-induced contraction of ASM, although selective antagonists to tachykinin-induced contractions of guinea-pig airways are still unavailable.

VIP and PHI are the putative neurotransmitters of the i-NANC innervation of ASM, and in the absence of VIP-receptor antagonists we have attempted to find a common role for cyclic nucleotides in mediating VIP- and i-NANC nerve-induced relaxation of guinea-pig trachea. Neurogenic and VIP-induced relaxation was potentiated by an cAMP-PDE inhibitor although the mechanism could not be elucidated. Measurements of tissue cyclic nucleotide levels may provide more conclusive evidence for a role for cAMP in mediating both forms of relaxation. Furthermore, we could not differentiate between potentiation of responses induced by i-NANC nerves and attenuation of e-NANC nerves by PDE inhibitors, therefore experiments

should have been conducted in the presence of a tachykinin-receptor antagonist in order to eliminate e-NANC responses to EFS.

The biological actions of neurotransmitters may be terminated in a variety of ways, including neuronal and extraneuronal uptake, diffusion away from the receptor, internalization of neurotransmitter-receptor complexes and enzymatic degradation of neurotransmitter molecules. Much research has recently been focused on the metabolism of neuropeptides by a variety of membrane-bound and soluble peptidases. In the airways tachykinins and VIP may be degraded by NEP, and we have demonstrated that responses induced by these peptides applied exogenously may be potentiated by NEP inhibition. Observations based on the modulation of responses to exogenous agonists cannot always be extrapolated to modulation of neurogenic responses. We have suggested that degradation of exogenous VIP occurs primarily at epithelial sites, and indeed relaxation induced by the stimulation of i-NANC nerves was not potentiated by NEP inhibition. It is difficult to understand the relevance, however, of peptide degradation at epithelial sites to the response of smooth muscle to peptides *in vivo*. The differential effect of NEP inhibition on responses induced by the stimulation of e-NANC and i-NANC nerves may be of physiological importance since any changes in peptidase activity may alter the balance of excitatory and inhibitory influences on airway tone.

10. References

ADVENIER, C., NALINE, E., DRAPEAU, G. AND REGOLI, D.: Relative potencies of neurokinins in guinea-pig trachea and human bronchus. Eur. J. Pharmacol. 139: 133-137, 1987.

AIZAWA, H., MIYAZAKI, N., SHIGEMATSU, N. AND TOMOOKA, M: A possible role of airway epithelium in modulating hyperresponsiveness. Br. J. Pharmacol. 93: 139-145, 1988.

ALTIERE, R. J., SZAREK, J. L. AND DIAMOND, L.: Neural control of relaxation in cat airways smooth muscle. J. Appl. Physiol. 57: 1536-1544, 1984.

ALTIERE, R. J. AND DIAMOND, L.: Effect of *alpha*-chymotrypsin on the nonadrenergic inhibitory system in cat airways. Eur. J. Pharmacol. 114: 75-78, 1985.

ANDERSSON, R. G. G. AND GRUNDSTROM, N.: The excitatory non-cholinergic non-adrenergic nervous system of the guinea-pig airways. Eur. J. Respir. Dis. 64: 141-157, 1983.

ARIENS, E. J. AND SIMONIS, A. M.: Physiological and pharmacological aspects of adrenergic receptor classification. Biochem. Pharmacol. 32: 1539-1545, 1983.

BAKER, D. G., BASBAUM, C, B., HERBERT, D. A. AND MITCHELL, R. A.: Transmission of airway ganglia of ferrets: inhibition by norepinephrine. Neurosci. Let. 41: 139-143, 1983.

BAKER, D. G. AND DON, H.: Catecholamines abolish vagal but not acetylcholine tone in the cat trachea. J. Appl. Physiol. 63: 2490-2498, 1987.

BARNES, P. J., KALINER, J., HAMILTON,C. AND DOLLERY, C.: Demonstration of *alpha*$_1$-adrenoceptor in guinea-pig lung using [^{3}H]prazosin. Life Sci. 25: 1207-1214, 1979.

BARNES, P. J., FITZGERALD, G., BROWN, M. AND DOLLERY, C.: Nocturnal asthma and changes in circulating epinephrine, histamine and cortisol. N. Eng. J. Med. 303: 263-267, 1980a.

BARNES, P. J., KARLINER, J. S. AND DOLLERY, C. T.: Human lung adrenoceptors studied by radioligand binding. Clin. Sci. 58: 457-461, 1980b.

BARNES, P. J., BROWN, M. J., SILVERMAN, M. AND DOLLERY, C. T.: Circulating catecholamines in exercise and hyperventilation-induced asthma. Thorax 36: 435-440, 1981.

BARNES, P. J., BASBAUM, C. B., NADEL, J. A. AND ROBERTS, J. M.: Localization of *beta*-adrenoceptors in mammalian lung by light microscopic autoradiography. Nature 299: 444-447, 1982a.

BARNES, P. J., IND, P. W. AND BROWN, M. J.: Plasma histamine and catecholamines in stable asthmatic subjects. Clin. Sci. Lond. 62: 661-665, 1982b.

BARNES, P. J., SKOOGH, B.-E., NADEL, J. A. AND ROBERTS, J. M.: Postsynaptic *alpha*$_2$-adrenoceptors predominate over *alpha*$_1$-adrenoceptors in canine tracheal smooth muscle and mediate neuronal and humoral *alpha*-adrenergic contraction. Mol. Pharmacol. 23: 570-575, 1982c.

BARNES, P. J., NADEL, J. A., ROBERTS, J. M. AND BASBAUM, C. B.: Muscarininc receptors in lung and trachea: autoradiographic localization using [^{3}H]quinuclidinyl benzylate. Eur. J. Pharmacol. 86: 103-106, 1983a.

BARNES, P. J., NADEL, J. A., SKOOGH, B.-E. AND ROBERTS, J. M.: Characterisation of *beta*-adrenoceptor subtypes in canine airway smooth muscle by radioligand binding and physiological responses. J. Pharmacol. Exp. Therap. 225: 456-461, 1983b.

BARNES, P. J., SKOOGH, B.-E., BROWN, J. K. AND NADEL, J. A.: Activation of *alpha*-adrenergic responses in tracheal smooth muscle: a postreceptor mechanism. J. Appl. Physiol. 54: 1469-1476, 1983c.

BARNES, P. J., CUSS, F. M. AND PALMER, J. B.: The effect of airway epithelium on smooth muscle contractility in bovine trachea. Br. J. Pharmacol. 86: 685-691, 1985.

BARNES, P. J.: Asthma as an axon reflex. The Lancet 242-244, 1986.

BARNES, P. J: Muscarinic receptor subtypes: implications for lung disease (editorial). Thorax 44: 161-167, 1989.

BARNETTE, M., TORPHY T. J., GROUS, M., FINE, C. AND ORMSBEE, H. S: Cyclic GMP: a potential mediator of neurally- and drug-induced relaxation of opossum lower esophageal sphincter. J. Pharmacol. Exp. Therap. 249: 524-528, 1989.

BERGSTRAND, H., KRISTOFFERSON, J., LUNDQUIST, B. AND SCHURMANN, A.: Effects of antiallergic agents, compound 48/80 and some reference inhibitors on the activity of partially purified human lung tissue adenosine cyclic 3', 5'- monophosphate and guanosine cyclic 3', 5'- monophosphate phosphodiesterases. Mol. Pharmacol. 13: 38-42, 1977.

BERKIN, K. G., INGLIS, G. C., BALL, S. G. AND THOMSON, N. C.: Airway responses to low concentrations of adrenaline and noradrenaline in normal subjects. Q. J. Exp. Physiol. 70: 203-209, 1985.

BLACK, J. L., JOHNSON, P. R. A. AND ARMOUR, C. L: Potentiation of the contractile effects of neuropeptides in human bronchus by an enkephalinase inhibitor. Pulm. Pharmacol. 1: 21-23, 1988.

BLACKMAN, J. G. AND MCCAIG, D. J: Studies on an isolated innervated preparation of guinea-pig trachea. Br. J. Pharmacol. 80: 703-710, 1983.

BOWMAN, A., GILLESPIE, J. S. AND POLLOCK, D.: Oxyhaemoglobin blocks non-adrenergic non-cholinergic inhibition in the bovine retractor penis muscle. Eur. J. Pharmacol. 85: 221-224, 1982.

BOWMAN, A. AND DRUMMOND, A. H.: Cyclic GMP mediates neurogenic relaxation in the bovine retractor penis muscle. Br. J. Pharmacol. 81: 665-674, 1984.

BRINK, C., GRIMAUD, C., GUILLOT, C. AND OREHEK, J: The interactions between indomethacin and contractile agents on human isolated airway smooth muscle. Br. J. Pharmacol. 69: 383-388, 1980.

BRYSON, S. E. AND RODGER, I. W.: Effects of phosphodiesterase inhibitors on normal and chemically-skinned isolated airway smooth muscle. Br. J. Pharmacol. 92: 673-681, 1987.

BUCK, S. H., BURCHER, E., SHULTS, C. W., LOVENBERG, W. AND O'DONOHUE, T. L.: Novel pharmacology of substance K-binding sites: a third type of tachykinin receptor. Science 226: 987-989, 1984.

BURCHER, E., BUCK, S. H., LOVENBERG, W. AND O'DONOHUE, T. L: Characterization and autoradiographic localisation of multiple tachykinin binding sites in gastrointestinal tract and bladder. J. Pharmacol. Exp. Ther. 236: 819-831, 1986.

BURCHER, E., WATKINS, D. J. AND O'FLYNN, N. M.: Both neurokinin A and substance P bind to NK1 receptors in guinea-pig lung. Pulm. Pharmacol. 1: 201-203, 1989.

BURNSTOCK, G.: Purinergic nerves. Pharmacol. Rev. 24: 509-581, 1972.

BUTLER, G. B., ADLER, K. B., EVANS, J. N., MORGAN, D. W. AND SZAREK, J. L.: Modulation of rabbit airway smooth muscle responsiveness by respiratory epithelium: involvement of an inhibitory metabolite of arachidonic acid. Am. Rev. Respir. Dis. 135: 1099-1104, 1987.

CABEZAS, G. A., GRAF, P. D. AND NADEL, J. A.: Sympathetic versus parasympathetic nervous regulation of airways in dogs. J. Appl. Physiol. 31: 651-655, 1971.

CAMERON, A. A., JOHNSTON, C. F., KIRKPATRICK, C. T. AND KIRKPATRICK, M. C. A.: The quest for the inhibitory transmitter in bovine tracheal smooth muscle. Q. J. Exp. Physiol. 68: 413-426, 1983.

CARSTAIRS, J. R., NIMMO, A. J. AND BARNES, P. J.: Autoradiographic visualisation of *beta*-adrenoceptor subtypes in human lung. Am. Rev. Respir. Dis. 132: 541-547, 1985.

CAUGHEY, G. H., LEIDIG, F., VIRO, N. F. AND NADEL, J. A.: Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase. J. Pharmacol. Exp. Therap. 244: 133-137, 1988.

CHESROWN, S. E., VENUGOPALAN, C. S., GOLD, W. M. AND DRAZEN, J. M.: *In vivo* demonstration of nonadrenergic inhibitory innervation of the guinea-pig trachea. J. Cli. Invest. 65: 314-320, 1980.

CHEUNG, A., POLAK, J. M. AND BAUER, F. E.: Distribution of galanin immunoreactivity in the respiratory tract of pig, guinea pig, rat, and dog. Thorax 40: 889-896, 1985.

CHOU, J. AND SAID, S. I.: Removal of epithelium attenuates airway relaxation and enhances constriction. Fed. Proc. 46: 659 (abstract), 1987.

CHRISTOFIDES, N. D., YIANGOU, Y., PIPER, S., GHATEI, M., SHEPPARD, N., TATEMOTO, K., POLAK, J. M. AND BLOOM, S. R.: Distribution of PHI in the mammalian respiratory tract and some aspects of its pharmacology. Endocrinology 115: 1956-1963, 1984.

CHUBB, I. W., HODGSON, A. J. AND WHITE, G. H.: Acetylcholinesterase hydrolyses substance P. Neuroscience, 5: 2065-2072, 1980.

CIESLINSKI, L. B., REEVES, M. L. AND TORPHY, T. J.: Cyclic nucleotide phosphodiesterases (PDEs) in canine and human tracheal smooth muscle (TSM). FASEB J. 2: 1065 (abstract), 1988.

COBURN, R. F. AND TOMITA, T.: Evidence for nonadrenergic inhibitory nerves in the guinea-pig trachealis muscle. Am. J. Physiol. 224: 1072-1080, 1973.

COE, C. AND BARNES, P. J.: An inhaled anticholinergic drug reduces nocturnal asthma. Chest 90: 485-488, 1986.

COLEBATCH, M. J. H. AND HALMAGYI, D. F. J.: Effect of vagotomy and vagal stimulation on lung mechanics and circulation. J. Appl. Physiol. 18: 881-887, 1963.

COLEMAN, R. A.: Effects of some purine derivatives on the guinea-pig trachea and their interactions with drugs that block adenosine uptake. Br. J. Pharmacol. 57: 51-57, 1976.

COLEMAN, R. A. AND LEVY, G. P.: A non-adrenergic inhibitory pathway in guinea-pig trachea. Br. J. Pharmacol. 69: 167-174, 1974.

COLERIDGE, H. M. AND COLERIDGE, J. C. G.: Reflexes from the tracheobronchial tree and lungs. In Handbook of physiology, the respiratory system. Vol 2: Control of breathing. Ed. N. Cherniack, J. G. Widdicombe. Washington DC: American Physiological Society: 395-429, 1986.

COLLIER, H. O. J. AND JAMES, G. W. L.: Humoral factors affecting pulmonary inflation during acute anaphylaxis in the guinea-pig *in vivo*. Br. J. Pharmacol. 30: 283-301, 1967.

DANIEL, E. E., KANNAN, M., DAVIES, C. AND POSEY-DANIEL, V.: Ultrastructural studies on the neuromuscular control of human tracheal and bronchial muscle. Respir. Physiol. 63: 109-128, 1986.

DANSER, A. J. H., VAN DER ENDE, R., LORENZ, R. R., FLAVAHAN, N. A. AND VANHOUTTE, P. M.: Prejunctional $beta_1$-adrenoceptors inhibit cholinergic neurotransmission in canine bronchi. J. Appl. Physiol. 62: 785-790, 1987.

DAVIS, C., KANNAN, M. S., JONES, T. R. AND DANIEL, E. E.: Control of human airway smooth muscle: *in vitro* studies. J. Appl. Physiol. 53: 1080-1087, 1982.

DAVIS, C. AND KANNAN, M. S.: Sympathetic innervation of human tracheal and bronchial smooth muscle. Resp. Physiol. 68: 53-61, 1987.

DE JONGSTE, J. C., MONS, H., BONTA, I. L. AND KERREBIJN, K. F.: Nonneuronal components in the response of fresh human airways to electrical field stimulation. J. Appl. Physiol. 63: 1558-1566, 1987.

DEY, R. D., SHANNON, W. A. AND SAID, S. I.: Localization of VIP-immunoreactive nerves in airways and pulmonary vessels of dogs, cats and human subjects. Cell Tissue Res. 220: 231-238, 1981.

DEVILLIER, P., ADVENIER, C., DRAPEAU, G., MARSAC, J. AND REGOLI, D.: Comparison of the effects of epithelium removal and of an enkephalinase inhibitor on the neurokinin-induced contractions of guinea-pig isolated trachea. Br. J. Pharmacol. 94: 675-684, 1988.

DIAMOND, L. AND O'DONNELL, M.; A nonadrenergic vagal inhibitory pathway to feline airways. Science 208: 185-188, 1980.

DIAMOND, L. AND ALTIERE, J. A.: Airway nonadrenergic noncholinergic inhibitory nervous system, In The airways: neural control in health and disease, ed. M. A. Kaliner and P. J. Barnes, M. Dekker, New York, 343, 1988.

DION, S., DRAPEAU, G., RHALEB, N.-E., D'ORLEANS-JUSTE, P. AND REGOLI, D.: Receptors for substance P and neurokinins. Correlation between binding and biological activities. Eur. J. Pharmacol. 138: 125-128, 1987.

DJOKIC, T. D., NADEL, J. A., DUSSER, D. J., SEKIZAWA, K., GRAF, P. D. AND BORSON, D. B.: Inhibitors of neutral endopeptidase potentiate electrically and capsaicin-induced noncholinergic contraction in guinea-pig bronchi. J. Pharmacol. Exp. Ther. 248: 7-11, 1989.

DOIDGE, J. M. AND SATCHELL, D. G.: Adrenergic and non-adrenergic inhibitory nerves in mammalian airways. J. Auton. Nerv. Syst. 5: 83-99, 1982.

DOODS, H. N., MATHAY, M.-J., DAVIDESKO, D., VAN CHARLDORP, K. J., DE JONGE, A. AND VAN ZWIETEN, P. A.: Selectivity of muscarinic antagonists in radioligand and *in vivo* experiments for the putative M1, M2 and M3 receptors. J. Pharmacol. Exp. Ther. 242: 257-262, 1987.

DRAPEAU, G., D'ORLEANS-JUSTE, P., DION, S., RHALEB, N. E., RONISSI, R. E. AND REGOLI, D.: Selective agonists for substance P and neurokinin receptors. Neuropeptides 10: 43-54, 1987.

DROSCHODT-LANCKMAN, D. AND STROSBERG, A. D.: *In vitro* degradation of the C-terminal octapeptide of cholecystokinin by "enkephalinase A". FEBS Lett. 152: 109-113, 1983.

DUSSER, D. J., NADEL, J. A., SEKIZAWA, K., GRAF, P. D. AND BORSON, D. B.: Neutral endopeptidase and angiotensin converting enzyme inhibitors poteniate kinin-induced contraction of ferret trachea. J. Pharmacol. Exp. Therap. 244: 531-536, 1988a.

DUSSER, D. J., UMENO, E., GRAF, P. D., DJOKIC, T., BORSON, D. B. AND NADEL, J. A.: Airway neutral endopeptidase-like enzyme modulates tachykinin-induced bronchoconstriction *in vivo*. J. Appl. Physiol. 65: 2585-2591, 1988b.

EL-BERMANI, A. I.: Pulmonary noradrenergic innervation of rat and monkey: a comparative study. Thorax 33: 167-174, 1978.

ELLIS, J. L. AND FARMER, S. G.: The effects of vasoactive intestinal peptide (VIP) antagonists, and VIP and histidine isoleucine antisera on non-adrenergic, non-cholinergic relaxations of tracheal smooth muscle. Br. J. Pharmacol. 96: 513-520, 1989a.

ELLIS J. L. AND FARMER, S. G.: Effects of peptidases on non-adrenergic, non-cholinergic inhibitory responses of tracheal smooth muscle: a comparison with effects on VIP- and PHI-induced relaxation. Br. J. Pharmacol. 96: 521-526, 1989b.

ERDOS, E. G.: Kininases. In: Handbook of experimental pharmacology. Ed. Erdos E. G., Heidelberg: Springer-Valag, 25 Suppl: 427-487, 1979.

ERDOS, E. G. AND YANG, H. Y. T.: An enzyme in microsomal fraction of kidney that inactivates bradykinin. Life Sci. 6: 569-574, 1967.

FARMER, J. B. AND COLEMAN, R. A.: A new preparation of the isolated intact trachea of the guinea-pig. J. Pharm. Pharmacol. 22: 46-50, 1970.

FARMER, S. G., FEDAN, J. S., HAY, D. W. P. AND RAEBURN, D.: The effects of epithelium removal on the sensitivity of guinea-pig isolated trachealis to bronchodilator drugs. Br. J. Pharmacol. 89: 407-414, 1986.

FAULKNER, D., FRYER, A. D. AND MCLAGAN, J.: Postganglionic muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. Br. J. Pharmacol. 88: 181-187, 1986.

FEDAN, J. S., HAY, D.W. P., FARMER, S. G. AND RAEBURN, D.: Epithelial cells: modulation of airway smooth muscle reactivity, In Asthma: basic mechanisms and clinical management, ed. P. J. Barnes, I. W. Rodgers, N. C. Thomson, Academic Press, London, 143, 1988.

FINE, J. M., GORDON, T. AND SHEPPARD, D.: Epithelium removal alters responsiveness of guinea-pig trachea to substance P. J. Appl. Physiol. 66: 232-237, 1989.

FLAVAHAN, N. A., AARHUS, L. L., RIMELE, T. J. AND VANHOUTTE, P. M.: Respiratory epithelium inhibits bronchial smooth muscle tone. J. Appl. Physiol. 58: 834-838, 1985.

FOSTER, R. W.: A note on the electrically transmurally stimulated isolated trachea of the guinea-pig. J. Pharm. Pharmacol. 16: 125-128, 1964.

FRANCONI, G. M., GRAF, P. D., LAZARUS, S. C., NADEL, J. A. AND CAUGHEY, G. H.: Mast cell tryptase and chymase reverse airway smooth muscle relaxation induced by vasoactive intestinal peptide in the ferret. J. Pharmacol. Exp. Therap. 248: 947-951, 1989.

FRANDSEN, E. K., KRISHNA, G. A. AND SAID S. I.: Vasoactive intestinal polypeptide promotes cyclic adenosine 3', 5' -monophosphate accumulation in guinea pig trachea. Br. J. Pharmacol. 62: 367-369, 1978.

FROSSARD, N. AND MULLER, F.: Epithelial modulation of tracheal smooth muscle responses to antigenic stimulation. J. Appl. Physiol. 61: 1449-1456, 1986.

FUKUDA, J. AND KAMEYAMA, M.: Tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in tissue cultured spinal ganglia neurons from adult mammals. Brain Res. 182: 191-197, 1980.

GAFFORD, J. T., SKIDGEL, R. A., ERDOS E. G. AND HERSH, L. B.: Human kidney "enkephalinase", a neutral metalloendopeptidase that cleaves active peptides. Biochemistry 22: 3265-3271, 1983.

GAMSE, R., LEMBECK, F. AND CUELLO, A. C.: Substance P in the vagus nerve. Naunyn-Schmiedeberg's Arch. Pharmacol. 306: 37-44, 1979a.

GAMSE, R., MOLNAR, A. AND LEMBECK, F.: Substance P release from spinal cord slices by capsaicin. Life Sci. 25: 629-636, 1979b.

GIBSON, A. AND JAMES, T. A.: The nature of potassium-chloride induced relaxations of the rat anococcygeus muscle. Br. J. Pharmacol. 60, 141-145, 1977.

GIBSON, A., MIRZAZADEH, S., TUCKER, J. F. AND WILSEY, C.: The cGMP-phosphodiesterase inhibitor M&B 22,948 potentiates NANC relaxations of the mouse anococcygeus. Br. J. Pharmacol. 95: 507P, 1988.

GOLDIE, R. G., PATERSON, J. W. AND WALE, J. L.: Pharmacological response of human and porcine lung parenchyma, bronchus and pulmonary artery. Br. J. Pharmacol. 76: 515-521, 1982.

GOLDIE, R. G., PAPADIMITRIU, J. M., PATERSON, J. W., RIGBY, P. J., SELF, H. M. AND SPINA, D.: Influence of the epithelium on responsiveness of guinea-pig isolated trachea to contractile and relaxant agonists. Br. J. Pharmacol. 87: 5-14, 1986.

GRIECO, M. H. AND PIERSON, R. N.: Mechanisms of bronchoconstriction due to *beta*-adrenergic blockade. J. Allergy Clin. Immunol. 48: 143-152, 1971.

GRISTWOOD, R. W., OWEN, D. A. A. AND REEVES, M. L.: Phosphodiesterase in the guinea-pig cardiac ventricle: Specific inhibition of type III activity by SK&F 94120. Br. J. Pharmacol. 85: 224P, 1985.

GRUETTER, C. A., KADOWITZ, P. J. AND IGNARRO, L.: Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerine, sodium nitrate and amyl nitrite. Can. J. Physiol. Pharmacol. 59: 150-156, 1981.

GRUNDSTROM, N., ANDERSSON, R. G. G. AND WIKBERG, J. E. S.: Prejunctional $alpha_2$-adrenoceptors inhibit contraction of tracheal smooth muscle by inhibiting cholinergic neurotransmission. Life Sci. 28: 2981-2986, 1981a.

GRUNDSTROM, N., ANDERSSON, R. G. G. AND WIKBERG, J. E. S: Pharmacological characterisation of the guinea-pig tracheobronchial smooth muscle. Acta Pharmacol. et Toxicol. 49: 150-157, 1981b.

GRUNDSTROM, N. AND ANDERSSON, R. G. G.: Inhibition of the cholinergic neurotransmission in human airways via prejunctional *alpha*-2-receptors. Acta Physiol. Scand. 125: 513-517, 1985.

HAMMER, P. AND GIACHETTI, A.: Muscarinic receptor subtypes: M1 and M2 biochemical and functional characterisation. Life Sci. 31: 2991-2998, 1982.

HANSON, G. R. AND LOVENBERG, W.: Elevation of substance P-like immunoreactivity in rat central nervous system by protease inhibition. J. Neurochem. 35: 1370-1374, 1980.

HAY, D. W. P., FARMER, S. G., RAEBURN, D., ROBINSON, V. A., FLEMING, W. W. AND FEDAN, J. S.: Airway epithelium modulates the reactivity of guinea-pig respiratory smooth muscle. Eur. J. Pharmacol. 129: 11-18, 1986.

HAY, D. W. P., FARMER, S. G., RAEBURN, D., MUCCITELLI, R. M., WILSON, K. A. AND FEDAN, J. S.: Differential effects of epithelium removal on the responsiveness of guinea-pig tracheal smooth muscle to bronchoconstrictors. Br. J. Pharmacol. 92: 381-388, 1987a.

HAY, D. W. P., MUCCITELLI, R. M., HORSTEMEYER, D. L., WILSON, K. A. AND RAEBURN, D.: Demonstration of the release of an epithelium-derived inhibitory factor from a novel preparation of guinea-pig trachea. Eur. J. Pharmacol. 136: 247-250, 1987b.

HAYE-LEGRAND, J., CERRINA, J, RAFFESTIN, B., LABAT, C., BOULET, C., BAYOL, A., BENVENISTE, J. AND BRINK, C.: Histamine contraction of isolated human airway muscle preparations: role of prostaglandins. J. Pharmacol. Exp. Ther. 239: 536-541, 1986.

HOGG, J. C. AND EGGLESTON, P. A.: Is asthma an epithelial disease? Am. Rev. Respir. Dis. 129: 207-208, 1984.

HOLROYD M. C.: The influence of epithelium on the responsiveness of guinea-pig isolated trachea. Br. J. Pharmacol. 87: 501-507, 1986.

HOOPER, N. M., KENNY, A. J. AND TURNER, A. J.: The metabolism of neuropeptides. Neurokinin A (substance K) is a substrate for endopeptidase-24.11 but not for peptidyl dipeptidase A (angiotensin converting enzyme). Biochem. J. 231: 357-361, 1985.

HOOPER, N. M. AND TURNER, A. J.: Neurokinin B is hydrolysed by synaptic membranes and by endopeptidase-24.11 ("enkephalinase") but not by angiotensin converting enzyme. FEBS 190: 133-136, 1985.

HUA, X.-Y., THEODORSSON-NORHEIM, E., BRODIN, E., LUNDBERG, J. M. AND HOKFELT, T.: Multiple tachykinins (neurokinin A, neuropeptide K and substance P) in capsaicin-sensitive sensory neurons in the guinea-pig. Regul. Pept. 13: 1-19, 1985.

IND, P. W., CAUSON, R. C., BROWN, M. J. AND BARNES, P. J.; Circulating catecholamines in acute asthma. Br. Med. J. 290: 267-279, 1985.

IND, P. W., DIXON, C. M. S., FULLER, R. W. AND BARNES, P. J.: Anticholinergic blockade of *beta*-blocker-induced bronchoconstriction. Am. Rev. Respir. Dis. 139: 1390-1394, 1989.

IRELAND, S. J., JORDAN, C. C., STEPHENS-SMITH, M. L. AND WARD, P.: Receptors mediating the contractile response to neurokinin agonists in the guinea-pig trachea. Regul. Pept. 22: 93 (abstract), 1988.

IRVIN, C. G., BOILEAU, R., TREMBLAY, J., MARTIN, R. R. AND MACKLEM, P. T.: Bronchodilatation: noncholinergic, nonadrenergic mediation demonstrated *in vivo* in the cat. Science 207: 791-792, 1980.

ITO, Y. AND TAJIMA, K.: Dual effects of catecholamines on pre- and post- junctional membranes in the dog trachea. Br. J. Pharmacol. 75: 433-440, 1982.

ITO, Y. AND TAKEDA, K.: Non-adrenergic inhibitory nerves and putative transmitters in the smooth muscle of cat trachea. J. Physiol. 330, 497-511, 1982.

ITO, Y.: Pre- and post-junctional actions of procaterol, a $beta_2$-adrenoceptor stimulant, on dog tracheal tissue. Br. J. Pharmacol. 95: 268-274, 1988.

JACOBOWITZ, D. KENT, K. M., FLEISCH, J. M. AND COLLIER, T.: Histofluorescent study of catecholamine-containing elements in cholinergic ganglia from the cat and dog lung. Proc. Soc. Exp. Biol. Med. 144: 464-466, 1973.

JANCSO, G., KIRALY, E. AND JANCSO, A.: Pharmacologically induced selective degeneration of chemosensitive primary sensory neurons. Nature 270: 741-743, 1977.

JESSELL, T. M., IVERSEN L. L. I. AND CUELLO, A. C.: Capsaicin-induced depletion of substance P from primary sensory neurons. Brain Res. 152: 183-188, 1978

JOHNSON, A. R., ASHTON, J., SCHULZ, W. W. AND ERDOS, E. G.: Neutral metalloendopeptidase in human lung tissue and cultured cells. Am. Rev. Respir. Dis. 132: 564-568, 1985.

JONES, T. R., KANNAN, M. S. AND DANIEL, E. E.: Ultrastructural study of guinea-pig tracheal smooth muscle and its innervation. Can. J. Physiol. Pharmacol. 58: 974-983, 1980.

KARLSBERG, R. P., CRYER, P. E. AND ROBERTS, R.: Serial plasma catecholamine response early in the course of clinical acute myocardial infarction: Relationship to infarct extent and mortality. Am. Heart J. 102: 24-29, 1981.

KARLSSON, J. A. AND PERSSON, C. G. A.: Evidence against vasoactive intestinal polypeptide (VIP) as a dilator and in favour of substance P as a constrictor in airway neurogenic responses. Br. J. Pharmacol. 79: 634-636, 1983.

KARLSSON, J. A., FINNEY, M. J. B., PERSSON, C. G. A. AND POST, C.: Substance P antagonists and the role of tachykinins in non-cholinergic bronchoconstriction. Life Sci. 35: 2681-2691, 1984.

KARLSSON, J. A. AND PERSSON, C. G. A.: Neither vasoactive intestinal peptide (VIP) nor purine derivatives may mediate non-adrenergic tracheal inhibition. Acta Physiol. Scand. 122: 589-598, 1984.

KATSUKI, S. AND MURAD, F.: Regulation of adenosine cyclic 3', 5'-monophosphate levels and contractility in bovine tracheal smooth muscle. Mol. Pharmacol. 13: 330-341, 1977.

KATZ, B. AND MILEDI, R.: Tetrodotoxin-resistant electrical activity in presynaptic terminals. J. Physiol. 203: 459-487, 1969.

KERR, M. A. AND KENNY, A. J.: The purification and specificity of a neutral endopeptidase from rabbit kidney brush border. Biochem J. 137: 477-489, 1974.

KIMURA, S., OKADA, M., SUGITA, Y., KANAZAWA, I. AND MUNEKATA, E.: Novel neuropeptides, neurokinin *A* and *B*, isolated from porcine spinal cord. Proc. Jpn. Acad. 59(B): 101-104, 1983.

KNEUSSL, M. P. AND RICHARDSON, J. B.: *Alpha*-adrenergic receptors in human and canine tracheal and bronchial smooth muscle. J. Appl. Physiol. 45: 307-311, 1978.

KO, W.-C. AND LAI, Y.-L.: The tracheal nonadrenergic noncholinergic inhibitory system during antigen challenge. Respir. Physiol. 74: 129-138, 1988.

LAITINEN, L. A., HEINO, M., LAITINEN, A., KAVA, T. AND HAAHTELA, T.: Damage of the airway epithelium and bronchial reactivity in patients with asthma. Am. Rev. Respir. Dis. 131: 599-606, 1985a.

LAITINEN, A., PARTANEN, M., HERVONEN, A. AND LAITINEN, L. A.: Electron microscopic study of the innervation of human lower respiratory tract. Evidence of adrenergic nerves. Eur. J. Respir. Dis. 67: 209-215, 1985b.

LAMMERS, J.-W., MINETTE, P., MCCUSKER, M. AND BARNES, P. J.: The role of pirenzepine-sensitive (M1) muscarinic receptors in vagally mediated bronchoconstriction in humans. Am. Rev. Respir. Dis. 139: 446-449, 1989.

LEANDER, S., GRUNDSTROM, N., ANDERSSON, R. G. G. AND HAKANSON, R.: Neuronally mediated non-cholinergic contraction of guinea-pig bronchial smooth muscle is inhibited by a substance antagonist. Agents & Actions 14: 315-318, 1984.

LEIKAUF, G. D., UEKI, I. F., NADEL, J. AND WIDDICOMBE, J. H.: Release of cyclooxygenase products from cultured epithelium derived from human and dog trachea. Fed. Proc. 44: 1920, 1985.

LEE, C.-M., IVERSEN, L. L., HANLEY, M. R. AND SANDBERG, B. E. B.: The possible existence of multiple receptors for substance P. Naunyn-schiedeberg's Arch. Pharmacol. 318: 281-287, 1982.

LIU, L.W., SATA, T., KUBOTA, E., PAUL, E. AND SAID, S. I.: airway relaxant effect of vasoactive intestinal peptide (VIP): selective potentiation by phosphoramidon, an enkephalinase inhibitor. Am. Rev. Respir. Dis. 135: 86A, 1987.

LLORENZ, C. AND SCHWARTZ, J.-C.: Enkephalinase activity in rat peripheral organs. Eur. J. Pharmacol. 69: 113-116, 1981.

LUNDBERG, J. M., HOKFELT, T., NILSSON, G., TERENIUS, L., REHFELD, J. F., ELDE, R. AND SAID, S.: Peptide neurons in the vagus, splanchnic and sciatic nerves. Acta Physiol. Scand. 104: 499-501, 1978.

LUNDBERG, J. M. AND SARIA, A.: Bronchial smooth muscle contraction induced by stimulation of capsaicin-sensitive neurons. Acta Physiol. Scand. 116: 473-476, 1982.

LUNDBERG, J. M., BRODIN, E. AND SARIA, A.: Effects and distribution of vagal capsaicin-sensitive substance P neurons with special reference to the trachea and lungs. Acta Physiol. Scand. 119: 243-252, 1983a.

LUNDBERG, J. M., MARTLING, C. R. AND SARIA, A.: Substance P and capsaicin-induced contraction of human bronchi. Acta Physiol. Scand. 119: 49-53, 1983b.

LUNDBERG, J. M., SARIA, A., BRODIN, E., ROSELL, S. AND FOLKERS, K.: A substance P antagonist inhibits vagally induced increase in vascular permeability and bronchial smooth muscle contraction in the guinea-pig. Proc. Natl. Acad. Sci. USA 80: 1120-1124, 1983c.

LUNDBERG, J. M., HOKFELT, T., MARTLING, C.-R., SARIA, A. AND CUELLO, C.: Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. Cell Tissue Res. 235: 251-261, 1984a.

LUNDBERG, J. M., JAHRENKRUG, J., HOKFELT, T., MARTLING, C.-R., LARSSON, O., TATEMOTO, K. AND ANGGARD, A.; Coexistence of peptide HI (PHI) and VIP in nerves regulating blood flow and bronchial smooth muscle tone in various mammals including man. Peptides 5: 593-606, 1984b.

LUNDBERG, J., FRANCO-CEREDA, A., HUA, X., HOKFELT, T. AND FISCHER, J. A.: Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivity in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. Eur. J. Pharmacol. 108: 315-319, 1985.

LUNBLAD, K. A. L. AND PERSSON, G. A.: The epithelium and the pharmacology of guinea-pig tracheal tone *in vitro*. Br. J. Pharmacol. 93: 909-917, 1988.

MAK, J. C. W. AND BARNES, P. J.: Muscarinic receptor subtypes in human and guinea-pig lung. Eur. J. Pharmacol. 164: 223-230, 1989a.

MAK, J. C. W. AND BARNES, P. J.: Autoradiographic visualisation of muscarinic receptor subtypes in human and guinea-pig lung. Am. Rev. Respir. Dis. 139: 74A, 1989b.

MAMBER, L. AND GERSHON, M. D.: A reciprocal adrenergic-cholinergic axoaxonic synapse in the mammalian gut. Am. J. Physiol. 236: E738-E745, 1979.

MARTIN, J. G. AND COLLIER, B.: Acetylcholine release from canine isolated airway is not modulated by norepinephrine. J. Appl. Physiol. 61: 1025-1030, 1986.

MARTLING, C.-R., THEODORSSON-NORHEIM, E. AND LUNDBERG, J. M.: Occurrence and effects of multiple tachykinins: substance P, neurokinin A and neuropeptide K in human lower airways. Life Sci. 40: 1633-1643, 1987.

MATSAS, R., FULCHER, I. S., KENNY, A. J. AND TURNER, A. J.: Substance P and [Leu]enkephalin are hydrolysed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. Proc. Natl. Acad. Sci. USA 80: 3111-3115, 1983.

MATSUMOTO, N., INOUE, H., ICHINOSE, M., ISHII, M., INOUE, C., SASAKI, H. AND TAKISHIMA, T.: Effective sites by sympathetic *beta*-adrenergic and vagal nonadrenergic inhibitory stimulation in constricted airways. Am. Rev. Respir. Dis. 132: 1113-1117, 1985.

MATSUZAKI, Y., HAMASAKI, Y. ANS SAID, S. I.: Vasoactive intestinal peptide: a possible transmitter of non-adrenergic relaxation of guinea pig airways. Science 210: 1252-1253, 1980.

MCKNIGHT, A. T., MAGUIRE, J. J., VARNEY, M. A.: Characterisation of receptors for tachykinins in guinea-pig isolated trachea. Br. J. Pharmacol. 91: 360 (abstract), 1987.

MCKNIGHT, A. T., MAGUIRE, J. J., VARNEY, M. A., WILLIAMS, B. J. AND IVERSEN, L. L.: Characterization of tachykinin receptors using selectivity of agonists and antagonists: evidence for an NK-4 type. Regul. Pep. 22: 126 (abstract) 1988.

MIDDENDORF, W. F. AND RUSSELL, J. A.: Innervation of airway smooth muscle in the baboon: evidence for a nonadrenergic inhibitory system. J. Appl. Physiol. 48: 947-956, 1980.

MINAMINO, N., KANGAWA, K., FUKUDA, A. AND MATSUO, H.: Neuromedin L: a novel mammalian tachykinin identified in porcine spinal cord. Neuropeptides 4: 157-166, 1984.

MINETTE, P. AND BARNES, P. J.: Prejunctional inhibitory muscarinic receptors on cholinergic nerves in human and guinea-pig airways. J. Appl. Physiol. 64: 2532-2537, 1988.

MOLNAR, A. MAKARA, G. B., GYORGY, L. AND UNYI, G.: The bronchoconstriction action of capsaicin in the guinea-pig. Acta Physiol. Acad. Hung. 36: 413-421, 1969.

MURAD, F. AND KIMURA, H.: Cyclic nucleotide levels in incubations of guinea pig trachea. Biochim. Biophys. Acta 343: 275-286, 1974.

MURLAS, C., NADEL, J. A. AND ROBERTS, J. M.: The muscarinic receptors of airway smooth muscle: their characterisation *in vitro*. J. Appl. Physiol. 52: 1084-1091, 1982.

MURLAS, C.: Effects of mucosal removal on guinea-pig airway smooth muscle responsiveness. Cli. Sci. 70: 571-575, 1986.

NALINE, E., ADVENIER, C., DEVILIER, P., DRAPEAU, G., DION, S. AND REGOLI, D.: Characterization of neurokinin effects and receptors in human isolated bronchi. Regul. Pep. 22: 133 (abstract), 1988.

NILSSON, G., DAHLBERG, K., BRODIN, E., SUNDLER, F. AND STRANDBERG, K.: Distribution and constrictor effect of substance P in guinea-pig tracheobronchial tissue. In: Substance P, eds. Von Euler, U. S. and Pernow, B. Raven Press, N. Y.: 75-81, 1977.

O'DONNELL, S. R., SAAR, N. AND WOOD, L. J.: The density of

adrenergic nerves at various levels in the guinea-pig lung. Clin Exp. Pharmacol. Physiol. 5: 325-332, 1978.

OLSEN, C. R., COLEBATCH, H. J. H., MEBEL, P. E., NADEL, J.

A. AND STAUB, N. C.: Motor control of pulmonary airways studied by nerve stimulation. J. Appl. Physiol. 20: 202-208, 1965.

OREHEK, J., DOUGLAS, J. S. AND BOUHUYS, A.: Contractile responses of the guinea-pig trachea *in vitro*: modification by prostaglandin synthesis inhibiting drugs. J. Pharmacol. Exp. Ther. 194: 554-564, 1975.

PANDOL, S. J., DHARMSATHAPHORN, K., SCHOEFFIELD, M. S., VALE, W. AND RIVIER, J.; Vasoactive intestinal peptide receptor antagonist [4-Cl-D-Phe^{6},Leu^{17}]-VIP. Am. J. Physiol. 250: G553-G557, 1986.

PAPKA, R. E., FURNESS, J. B., DELLA, N. G. AND COSTA, M.: Depletion of substance P-immunoreactivity and acetylcholinesterase activity from nerve fibres in the guinea-pig heart. Neurosci. Lett 27: 47-53, 1981.

PARTANEN, M., LAITINEN, A., HERVONEN, A., TOIVANEN, M. AND LAITINEN, L. A.: Catecholamine- and acetylcholinesterase-containing nerves in human lower respiratory tract. Histochem. 76: 175-188, 1982.

PIPER, P. J., COLLIER, H. O. J. AND VANE, J. R.: Release of catecholamines in the guinea-pig by substances involved in anaphylaxis. Nature 213: 838-840, 1967.

PIPER, P. J. AND WALKER, J. L: The release of spasmogenic substance from chopped human lung tissue and its inhibition. Br. J. Pharmacol. 47: 291-304, 1973.

POLAK, J. M. AND BLOOM, S. R.: Regulatory peptides in the respiratory tract of man and their animals. Exp. Lung Res. 3: 313-328, 1982.

RAEBURN, D., HAY, D. W. P., FARMER, S. G. AND FEDAN, J. S.: Epithelium removal increases the reactivity of human isolated tracheal muscle to methacholine and reduces the effect of verapamil. Eur. J. Pharmacol. 123: 451-453, 1986.

RATTAN, S. AND MOUMMI, C: Influence of stimulation and inhibitors of cyclic nucleotide on lower esophageal sphincter. J. Pharmacol. Exp. Therap. 248: 703-709, 1989.

REGOLI, D., DRAPEAU, G., DIO, S. AND D'ORLEANS-JUSTE, P.: Pharmacological receptors for substance P and neurokinins. Life Sci. 40: 109-117, 1987.

RICHARDSON, J. B. AND BOUCHARD, T.: Demonstration of a nonadrenergic inhibitory nervous system in the trachea of the guinea pig. J. Allergy Clin. Immunol. 56: 473-480, 1975.

RICHARDSON, J. AND BELAND, J.: Nonadrenergic inhibitory nervous system in human airways. J. Appl. Physiol. 41: 764-771, 1976.

RICHARDSON, J. B.: Nerve supply to the lungs. Am Rev. Respir. Dis. 119: 785-802, 1979.

RICHARDSON, J. B. AND FERGUSON, C. C.: Neuromuscular structure and function in the airways. Fed. Proc. 38: 202-208, 1979.

RUGG, E. L., BARNETT, D. B. AND NAHORSKI, S. R.: Coexistence of *beta*1 and *beta*2 adrenoceptors in mammalian lung: evidence from direct binding studies. Mol. Pharmacol. 14: 996-1005, 1978.

SAID, S. I., KITAMURA, S., YOSHIDA, T., PRESKITT, J. AND HOLDEN, L. D.: Humoral control of airways. Ann. N. Y. Acad. Sci. 221: 103-114, 1974.

SANT'AMBROGIO, G.: Information arising from the tracheobronchial tree of mammals. Physiol. Rev. 62: 531-569, 1982.

SARIA, A., MARTLING, C.-R., DALSGAARD, C.-J. AND LUNDBERG, J. M.: Evidence for substance P immunoreactive spinal afferents that mediate bronchoconstriction. Acta Physiol. Scand. 125: 407-414, 1985.

SATCHELL, D.: Non-adrenergic, non-cholinergic nerves in mammalian airways: their function and the role of purines. Comp. Biochem. Physiol. 72C: 189-196, 1982.

SATCHELL, D. G.: Adenosine deaminase antagonises inhibitory responses to adenosine and non-adrenergic, non-cholinergic inhibitory nerve stimulation in isolated preparations of guinea-pig trachea. Br. J. Pharmacol. 83: 323-325, 1984.

SEKIZAWA, K., TAMAOKI, J., NADEL, J. A. AND BORSON, D. B.: Enkephalinase inhibitor potentiates substance P- and electrically induced contraction in ferret trachea. J. Appl. Physiol. 63, 1401, 1987a.

SEKIZAWA, K., TAMAOKI, J., GRAF, P. D., BASBAUM, C. B., BORSON, D. B. AND NADEL, J. A.: Enkephalinase inhibitor potentiates mammalian tachykinin-induced contraction in ferret trachea, J. Pharmacol. Exp. Ther. 243, 1211, 1987b.

SHEPPARD, M. N., KURIAN, S. S., HENZEN-LONGMANS, S. C., MICHETTI, F., COCCHIA, D., COLE, P., RUSH, R. A., MARANGOS, P. J., BLOOM, S. R. AND POLAK, J. M.: Neuron-specific enolase and S-100. New markers for delineating the innervation of the respiratory tract in man and other animals. Thorax 38: 333-340, 1983.

SHEPPARD, D., THOMPSON, J. E., SCYPINSKI, L., DUSSER, D., NADEL, J. A. AND BORSON, D. B.: Toluene diisocyanate increases airway responsiveness to substance P and decreases airway enkephalinase. J. Clin. Invest. 81: 1111-1115, 1988.

SHORE, S. A., STIMLER-GERARD, N. P., COATS, S. R. AND DRAZEN, J. M.: Substance P-induced bronchoconstriction in the guinea-pig: Enhancement by inhibitors of neutral metalloendopeptidase and angiotensin converting enzyme. Am. Rev. Respir. Dis. 137: 331-336, 1988.

SIGAFOOS, J. F. AND ABRAMOWITZ, J: Effects of N-ethylmaleimide on gonadotropin and *beta*-adrenergic receptor function coupled to rabbit luteal adenylyl cyclase. Endocrinology 119: 1432-1438, 1986.

SILVA, D. G. AND ROSS, G.: Ultrastructural and fluorescence histochemical studies on the innervation of the tracheo-bronchial muscle of normal cats treated with 6-hydroxydopamine. J. Ultrstruct. Res. 47: 310-328, 1974.

SKIDGEL, R. A., ENGELBRECHT, S., JOHNSON A. R. AND ERDOS, E. G.: Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase. Peptides 5: 769-776, 1984.

SKOOGH, B.-E.: Transmission through airway ganglia. Eur. J. Respir. Dis. 131 (Suppl.): 159-170, 1983.

SKOOGH, B.-E.: Parasympathetic ganglia in the airways. Bull. Eur. Physiopathol. Respir. 22, Suppl. 7: 143-147, 1986.

SKOOGH, B.-E. AND SVEDMYR, N.: $Beta_2$-adrenoceptor stimulation inhibits ganglionic transmission in ferret trachea. Pulm. Pharmacol. 1: 167-172, 1989.

STIMLER-GERARD, N. P.: Neutral endopeptidase-like enzyme controls the contractile activity of substance P in guinea-pig lung. J. Clin. Invest. 79: 1819-1825, 1987.

STRETTON, C. D. AND BARNES, P. J.: Cholecystokinin-octapeptide constricts guinea-pig and human airways. Br. J. Pharmacol. 97: 675-682, 1989.

TATEMOTO, K., LUNDBERG, J. M., JORNVALL, M. AND MUTT, V.: Neuropeptide K: Isolation, structure and biological activities of a novel brain tachykinin. Biochem. Biophys. Res. Commun. 128: 947-953, 1985.

TAYLOR, S. M., PARE, P.D. AND SCHELLENBERG, R.R.: Cholinergic and nonadrenergic mechanisms in human and guinea pig airways. J. Appl. Physiol. 56: 958-965, 1984.

TERENGHI, G. MCGREGOR, G. P., BHUTTACHARJI, S. WHARTON, J., BLOOM, S. R. AND POLAK, J. M.: Vagal origin of substance P-containing nerves in the guinea-pig lung. Neuroscience Lett. 36: 229-236, 1983.

THOMPSON, J. E. AND SHEPPARD, D.: Phosphoramidon potentiates the increase in lung resistance mediated by tachykinins in guinea-pigs. Am. Rev. Respir. Dis. 137: 337-340, 1988.

THOMPSON, D. C, WELLS, J. L., ALTIERE, R. J. AND DIAMOND, L.: The effect of epithelium removal on non-adrenergic, non-cholinergic inhibitory responses in the isolated central airways of the cat and guinea pig, Eur. J. Pharmacol. 145: 231-237, 1988.

TORPHY, T. J., RINARD, G. A., RIETOW, M. G. AND MAYER, S. E.: Functional antagonism in canine tracheal smooth muscle: inhibition by methacholine of the mechanical and biochemical responses to isoproterenol. J. Pharmacol. Exp. Ther. 227: 694-699, 1983.

TORPHY, T. J., ZHENG, C., PETERSON, S. M., FISCUS, R. R., RINARD, G. A. AND MAYER, S. E.: Inhibitory effect of methacholine on drug-induced relaxation, cyclic AMP accumulation, and cyclic AMP-dependent protein kinase activation in canine tracheal smooth muscle. J. Pharmacol. Exp. Ther. 233: 409-417, 1985.

TORPHY, T. J., FINE, C. F., BURMAN, M., BARNETTE, M. S. AND ORMSBEE, H. S.: Lower esophageal sphincter relaxation is associated with increased cyclic nucleotide content. Am. J. Physiol. 251: G786-G793, 1986.

TORPHY, T. J., BURMAN, M., HUANG, L. B. F., HOROHONICH, S. AND CIESLINSKI, L. B.: Regulation of cyclic AMP content and cAMP-dependent protein kinase activity in airway smooth muscle. Prog. Clin. Biol. Res. 245: 263-275, 1987.

TSCHIRHART, E. AND LANDRY, Y.: Airway epithelium releases a relaxant factor: demonstration with substance P. Eur. J. Pharmacol. 132: 103-104, 1986.

VAN DEN BRINK, F. G.: The model of functional interaction. II. Experimental verifications of a new model: the antagonism of *beta*-adrenoceptor stimulants and other agonists. Eur. J. Pharmacol. 22: 279-286, 1973.

VAN KOPPEN, C. J., BLANKESTEIJN, W. M. AND KLAASEN, A. B. M.: Autoradiographic visualisation of muscarinic receptors in human bronchi. J. Pharmacol. Exp. Therap. 224: 760-764, 1987.

VENUGOPALAN, C. S., SAID, S. I. AND DRAZEN, J. M.: Effect of vasoactive intestinal peptide on vagally mediated tracheal pouch relaxation. Respir. Physiol. 56: 205-216, 1984.

VERMEIRE, P. A. AND VANHOUTTE, P. M.: Inhibitory effects of catecholamines in isolated canine bronchial smooth muscle. J. Appl. Physiol. 46: 787-791, 1979.

VON EULER, U. S. AND GADDUM, J. H.: An unidentified depressor substance in certain tissue extracts. J. Physiol. (London) 72: 74-87, 1931.

WAELBROECK, M., ROBBERECHT, P., COY, D. M., CAMUS, J.-C., DE NEEF, P. AND CHRISTOPHE, J.: Interaction of growth hormone-releasing factor (GRF) and 14 GRF analogs with vasoactive intestinal peptide (VIP) receptors of rat pancreas. Discovery of [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 as a VIP antagonist. Endocrinology 116: 2643-2644, 1985.

WARREN, J. B. AND DALTON, N.: A comparison of the bronchodilator and vasopressor effects of exercise levels of adrenaline in man. Clin. Sci. 64: 475-479, 1983.

WASSERMAN, M. A., GRIFFIN, R. L. AND MALO, P. E.: Comparative *in vitro* tracheal relaxant effects of porcine and hen VIP. In. Vasoactive Intestinal Peptide. Ed. Sami. S. Said, Raven Press, N. Y.: 177-184, 1982.

WEISHAAR, R. E., CAIN, M. H. AND BRISTOL, J. A.: A new generation of phosphodiesterase inhibitors: multiple molecular forms of phosphodiesterase and the potential for drug selectivity. J. Med. Chem. 28: 537-545, 1985.

WEISHAAR, R. E., BURROWS, S. D., KOBYLARZ, D. C., QUADE, M. M. AND EVANS, D. B.: Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. Isolation, characterization, and effects of various reference phosphodiesterase inhibitors and cardiotonic agents. Biochem. Pharm. 35: 787-800, 1986.

WHARTON, J., POLAK, J. M., BLOOM, S. R., WILL, J. A., BROWN, M. R. AND PEARSE, A. G. E.: Substance P-like immunoreactive nerves in mammalian lung. Invest. Cell. Pathol. 2: 3-10, 1979.

WILLIAMS, B. J., CURTIS, N. R., MCKNIGHT, A. T., MAGUIRE, J., FOSTER, A. AND TRIDGETT, R.: Development of NK2-selective antagonists. Regul. Pept. 22: 189 (abstract), 1988.

WORMSER, V., LAUFER, R., HART, Y., GHOREV, M., GILON, C. AND SELINGER, Z.: Highly selective agonists for substance P receptor subtypes. The EMBO J. 5: 2805-2808, 1986.

XU, G. L., SIVARAJAH, K., WU, R., NETTESHEIM, P. AND ELING, T.: Biosynthesis of prostaglandins by isolated and cultured airway epithelial cells. Exp. Lung. Res. 10: 101-108, 1986.

Publications

The following publications have arisen from the work described in this thesis:

Frossard N., Rhoden K.J. & Barnes P.J.: Influence of epithelium on guinea-pig airway responses to tachykinins: role of endopeptidase and cyclooxygenase. J. Pharmacol. Exp. Ther. 248: 292-299, 1989.

Rhoden K.J., Meldrum L.A. & Barnes P.J.: Inhibition of cholinergic neurotransmision in human airways by beta2-adrenoceptors. J. Appl. Physiol. 65: 700-705, 1988.

Rhoden K.J. & Barnes P.J.: Potentiation of nonadrenergic neural relaxation in guinea pig airways by a cAMP phosphodiesterase inhibitor. J. Pharmacol. Exp. Ther. (In press).

Rhoden K.J. & Barnes P.J.: Epithelial modulation of NANC and VIP-induced reponses: role of neutral endopeptidase. Eur J Pharmacol (In press).

Papers submitted:

Rhoden K. J. & Barnes P. J.: Characterisation of tachykinin-receptors on guinea-pig and human airway smooth muscle. (Submitted to Br. J. Pharmacol.)