" A STUDY OF THE EFFECTS OF THE NEUROKININ PEPTIDES ON RESPIRATORY FUNCTION IN SHEEP "

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To Mary, Paul and Eoin

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Title of Thesis A study of the effects of neurokinin peptides on respiratory function in sheep

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The respiratory responses, including changes in pulmonary resistance (RL) and dynamic compliance (Cdyn), to the neurokinin peptides substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) were assessed in anaesthetised normal Suffolk-cross and conscious Texel-cross asthmatic sheep.

In normal sheep (n=11) intravenous SP was a more potent bronchoconstrictor than NKA (n=9) and this was similar to findings in sheep with a naturally acquired airway allergy to Ascaris suum antigen (asthmatic) (n=5) where peptides were administered by inhalation. NKB (n=4) was assessed only in normal sheep and caused insignificant changes in bronchomotor tone. Intravenous SP and NKA, in normal anaesthetised sheep, caused a dose-dependent reduction in respiratory rate and this was similar for both peptides.

The bronchomotor response to SP in normal sheep demonstrated age-related changes. In sheep under 6 months of age there was a pronounced bronchoconstriction, with a subsequent reduction in the response as animals approach maturity. In old sheep, aged approximately four years, there was minimal bronchomotor response, however, there was dose-dependent apnoea.

The bronchomotor response to SP in anaesthetised normal sheep was significantly antagonised after pre-treatment with atropine (1mg/kg; n=6), hexamethonium (20mg/Kg; n=3) and the NK-1 antagonist CP 96,345 (0.1 and 0.5mg/Kg; n=5), but not by the H1 receptor antagonist chlorpheniramine (2mg/Kg; n=5) or the neurokinin antagonist spantide (10ug/kg/min; n=3). The anti-asthma drug nedocromil sodium (0.1 and 1.0mg/kg; n=4) had a variable effect on the response. In the isolated sheep trachealis muscle preparation the contractile effect of SP was inhibited by atropine (n=4) and the M1 receptor antagonist pirenzepine (n=8), with IC_{50} values of 5.6×10^{-8} and 5×10^{-10} M respectively, while spantide (n=7) and the NK-2 receptor antagonist L-659,874 (n=6) were ineffective.

In several normal sheep (n=10) intravenous SP consistently caused augmented breaths. Bilateral vagotomy (n=7) abolished, and cooling of the right cervical vagus (n=7), after section of the left vagus, to temperatures below 7° C significantly attenuated the bronchomotor response to SP in normal sheep.

The conclusion of this study is that the order of potency for the bronchomotor effects of the neurokinins is similar to rabbits and pigs but different from that reported for most other species, including man. The mechanism of action of SP is largely indirect, involving activation of vagal reflex mechanisms and/or modulation of ganglionic neurotransmission and acetylcholine release from cholinergic nerve endings.

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The assistance given to me by other members of the Department of Preclinical Sciences, both technical and academic, is also acknowledged. In particular I would like to thank Gordan Goodall for teaching me how to process airway tissue for histology and Dr GT Pearson for assisting me with the immunohistochemistry work. I would also like to thank Professor A Duggan for the provision of laboratory space.

I also wish to acknowledge the opportunity afforded to me by Dr W Abraham and his colleagues at the Department of Research Pulmonary Unit, Mount Sinai Medical Centre, Miami, Florida, to study the effects of neuropeptides in their conscious allergic sheep model.

Finally I am indebted to the Welcome Trust for the financial support that made this study possible.

For all those people who have given me support and assistance, however small, I am sincerely grateful.

DECLARATION

I declare that the contents of this thesis are my own work and have not been presented elsewhere for consideration for the degree of Doctor of Philosophy.

The experimental work for this thesis was carried out with the assistance of

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SUMMARY

The neurokinins (tachykinins) are a group of closely related peptides with a common C-terminal amino acid sequence of Phe-X-Gly-Leu-Met-NH2 and have been identified in most mammalian body systems. The mammalian neurokinins include substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and neuroipeptide gamma. In the respiratory tract, they are located primarily in C-fibre afferent nerve fibres in smooth muscle, around mucus glands and blood vessels and in close association with the epithelial surface. Their endogenous release from these nerve fibres or their exogenous administration results in smooth muscle contraction, vasodilation, plasma leakage and oedema, and increased secretion of mucus. Because of these effects it has been postulated that the neurokinins, along with other neuropeptides in the airways, such as neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP), may have a role to play in the aetiopathogenesis of airway inflammation and airway smooth muscle reactivity, and in particular it has been suggested that they may have a role to play in asthma.

To this end the airway response to the neurokinins has been investigated in great detail in a number of species, including man, and it would appear that, in terms of bronchoconstriction, the order of potency for the neurokinins is NKA > SP > NKB, although there are species differences where SP is less potent than NKB.

With the advent of the use of sheep as an experimental animal in the investigation of airway pharmacology and physiology, and with the development of sheep models of human respiratory diseases such as asthma, the aim of this study was to investigate and characterise the bronchomotor response of sheep to the neurokinins.

The localisation of SP and the additional sensory neuropeptide calcitonin gene-related peptide was assessed in the airways of normal sheep, using indirect immunofluorescence histochemistry techniques. The distribution of these peptides roughly coincided with reports for other species, although the distribution was sparse. SP and CGRP-immunoreactive nerve fibres were found in airway smooth muscle, close to blood vessels and mucus glands. The distributions of NPY, vasoactive intestinal polypeptide (VIP) and enkephalin (ENK) were also assessed. No ENK-immunoreactive fibres were detected and only occassional weak-staining VIP fibres

were noted. However, there was a dense network of interlacing nerve fibres immunoreactive for NPY in airway smooth muscle.

The respiratory reponse to intravenous SP, NKA and NKB was assessed in normal anasethetised Suffolk-cross female sheep. NKB was found to be ineffective in causing broncho-constriction, while SP was more potent than NKA. However, in the peripheral airways SP and NKA were roughly equipotent in causing airway narrowing (as assessed by changes in dynamic compliance).

The bronchomotor response to inhaled SP and NKA was assessed in sheep (Texel-cross) with a naturally acquired airway allergy to Ascaris suum antigen (asthma model). An order of potency for both peptides similar to that for normal sheep was demonstrated in these asthmatic sheep.

The bronchomotor response to SP was also assessed in different age groups of normal sheep. The most pronounced bronchoconstriction occured in young sheep, under 12 months of age. In sheep over 12 months the response was markedly attenuated and in old sheep, aged approximately four years, there was minimal bronchoconstriction and a dose-dependent apnoea occured.

The pharmacological antagonism of the airway response to SP by the muscarinic receptor antagonist atropine, the ganglion blocker hexamethonium, nedocromil sodium, the NK-1 receptor antagonist CP 96,345-1, the neurokinin antagonist spantide, and the H1 receptor antagonist chlorpheniramine was assessed in normal sheep. Atropine, hexamethonium and CP 96,345-1 significantly antagonised the effect of SP. The effects of atropine, the M1 receptor antagonist pirenzepine, spantide and the NK-2 receptor antagonist L-659,874 on the contractile effect of SP on the isolate sheep trachealis muscle preparation were also assessed. Only atropine and pirenzepine significantly antagonised the effect of SP with pirenzepine being approximately 100 times more potent than atropine.

The effect of vagal cooling and vagotomy on the bronchoconstriction caused by SP was also determined. Vagotomy abolished the response to SP while the response was reduced in a temperatiure-dependent manner when the right vagus was cooled (after the left vagus had been sectioned).

From the results of this study it can be stated that the order of potency for the bronchomotor effects of neurokinins in sheep is different from that in other species and this also applies to asthmatic sheep. This, coupled with the age-related alteration of the response to SP, needs to be considered when using sheep as models for airway diseases in man. The mechanism of action of SP appears to be largely indirect, involving a reflex vagal bronchoconstriction mediated through the NK-1 receptor. There may be an additional indirect mechanism involving modulation of acetylcholine release form cholinergic nerve endings or modifying neurotransmission in airway parasympathetic ganglia. The mechanism of action of SP-induced bronchoconstriction in the sheep shows some similarity to that in the rabbit and pig, but is different from most other species including man.

THE AIM

The aims of the project, described in this thesis, are:

1. To investigate the bronchomotor response of normal sheep and sheep with a naturally acquired airway allergy to Ascaris suum antigen, to the neurokinin peptides, substance P, neurokinin A and neurokinin B.

2. To investigate the mechanisms involved in the bronchomotor response to the neurokinins in vivo and in vitro using a variety of pharmacologically active agents.

3. To investigate the the possible involvement of vagal reflex mechanisms in the control of the bronchomotor response to the neurokinins.

4. To examine the localisation of various neuropeptides in neuronal and non-neuronal structures within the sheep airway, using immunohistochemical techniques.

ABBREVIATIONS USED IN TEXT

#	animal identification number
AA	arachidonic acid
AB	augmented breath
ACE	angiotensin converting enzyme
ACh	acetylcholine
Aib	amnoisobutyric acid
Ala	alanine
Arg	arginine
BK	bradykinin
^{o}C	degrees centigrade
C	compliance
CaCl2	calcium chloride
Cdyn	dynamic compliance
CH	chlorpheniramine
cm	centimetre
cmH2O	centimetres of water
CRC	concentration response curve
CNS	central nervous system
CGRP	calcitonin gene-related peptide
D	diastolic blood pressure
DMNX	dorso-motor nucleus of the vagus
ENK	enkephalin
ENK-Li	enkephalin-like immunoreactivity
FEV1	forced expiratory volume in one second
FITC	fluorescein isothiocyanate
FRC	functional residual capacity
g	gramme
ug	microgrammes
GABA	gamma-amino butyric acid
$GABA_B$	gamma amino butyric acid B receptor
Glu	glutamine
Gly	glycine
GRP	gastrin releasing peptide
h	hour
H1,2	histamine receptors
HCl	hydrochloric acid
HR	heart rate
Hz	frequency (hertz)
I	inertance
Ile	isoleucine
iu	international units
j	imaginary complex number
KCl	potassium chloride
KH2PO4	potassium dihydrogen orthophosphate
Kg	kilogrammes
l	litre
l	mass density
Leu	leucine
Lys	lysine

Lys(Nic)	nicotinyllysine
M	molar concentration
M-1,2 or 3	muscarinic receptors
MBP	mean blood pressure
Met	methionine
met-ENK	met-enkephalin
met-ENK-Li	met-enkephalin-like immunoreactivity
mg	milligrammes
MgSO4	magnesium sulphate
min	minute
ml	millilitre
mmHg	millimetres of mercury
mmol	minimoles
ms	millisecond
umol	micromoles
NaCl	sodium chloride
ng	nanogrammes
NaHCO3	sodium hydrogen carbonate
NaH2PO4	sodium dihydrogen orthophosphate
NANC	non-adrenergic non-cholinergic
NANCis	non-adrenergic non-cholinergic inhibitory
NANCex	non-adrenergic non-cholinergic excitatory
NEP	neutral metallo-endopeptidase (enkephalinase)
NK-1,2 or 3	neurokinin receptors

(SP-P, SP-K, SP-E, NK-P, NK-A and NK-B are alternative historical terms for the NK receptors)

NKA	neurokinin A
NKB	neurokinin B
Nle	nor-leucine
nmol	nanomoles
NO	nitric oxide
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
P	pressure
pA2	concentration of antagonist reducing agonist response by 50%
PBS	phosphate buffered saline
Pel	elastic pressure
PGF2a	prostaglandin F2a
Pin	inertial pressure
Prs	resistive pressure
Pal	(3-pyridyl)alanine
Phe	phenylalanine
PHM	peptide histidine methionine
pmol	picomoles
Pro	proline
r	radius
R	resistance
RAR	rapidly adapting receptor

RL	pulmonary resistance
RLiso	isovolume pulmonary resistance
S	systolic blood pressure
s	second
SAR	slowly adapting receptor
SD	single dose
SEM	standard error of the mean
SOM	somatostatin
SP	substance P
SP-Li	substance P-like immunoreactivity
T and TC	mast cell sub-groups
Trp	tryptophan
Tyr	tyrosine
V (or V')	flow
V"	flow acceleration
Val	valine
VE	expiratory flow
VI	inspiratory flow
VIP	vasoactive intestinal polypeptide
VIP-Li	vasoactive intestinal polypeptide-like immunoreactivity
VT	tidal volume
VTi	inspiratory tidal volume
VTe	expiratory tidal volume
w	2 pi times frequency
X	reactance

LIST OF FIGURES AND TABLES

(this includes a brief description of the figure content and not the complete legend)

MAIN TEXT FIGURES AND TABLES

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APPENDIX I FIGURES AND TABLES

CHAPTER 1. AIRWAY INNERVATION AND THE ROLE OF THE NEUROKININ PEPTIDES

This section will briefly outline the gross anatomy of the sheep respiratory system (Nickel et al, 1973), the current understanding of the complex neural control of mammalian bronchomotor tone and airway calibre, and the role of the classical and peptide neurotransmitters. With reference to the peptidergic neural systems, particular attention will be given to the neurokinin (tachykinin) family of peptides and our understanding of their pharmacology in the respiratory system. Adopting the current convention, the term neurokinin will be used instead of tachykinin.

1. Gross Anatomy of the Sheep Respiratory System:

The ruminants are obligate nasal breathers and have a relatively long nasal cavity. The larynx is similar to that described for other species, but the trachea shows interesting anatomical differences. The sheep trachea consists of 40-60 cartilage rings. These rings overlap in the cranial portion of the trachea giving a rigid and reasonably non-distendable structure. In the middle and caudal parts of the trachea the gap between the rings gradually widens. The tracheal lumen is circular for the entire length of the trachea and the left tip of each cartilage ring lies dorsal to the right tip. The trachealis muscle is located on the lumenal surface attached to the two ends of the cartilage ring.

The lungs of sheep, as in all ruminants, consist of the cranial and caudal lobes of left lung and the divided cranial, middle, caudal and accessory lobes of the right lung, but the lobes of sheep are poorly delineated. The cranial lobe bronchi leave directly from the trachea above the tracheal bifurcation, while the bronchi to the other lobes originate from the two mainstem bronchi, which are the divisions of the trachea at the carina.

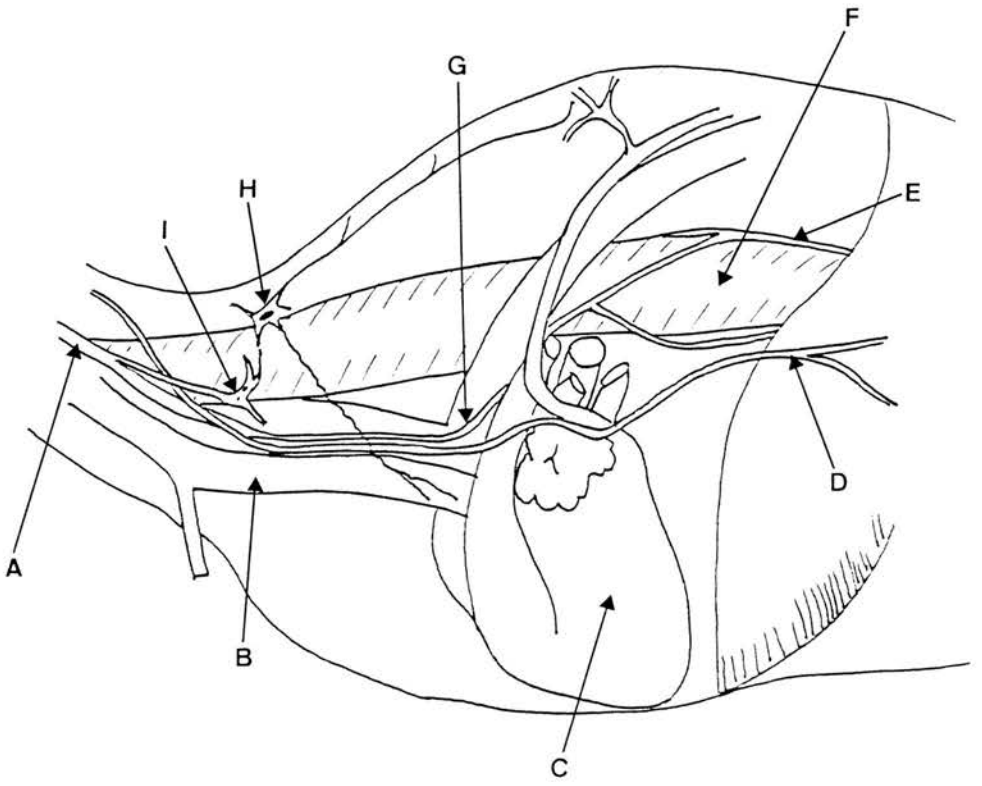


Figure 1. Gross anatomy of the sheep thorax. The lungs have been removed to show the principle structures of the thorax. A-vagosympathetic trunk; B-cranial vena cava; C-heart; D-phrenic nerve; E & G-vagus nerve; F-oesophagus; H-cervicothoracic ganglion; I-middle cervical ganglion. The external carotid artery is located immediately medio-ventral to the cervical vagus. The vagosympathetic trunk seperates into the vagus and sympathetic branches cranial to the middle cervical ganglion, close to the thoracic inlet.

Unlike the situation in cattle only the pulmonary arteries accompany the bronchi while the veins run intersegmentally. The bronchial circulation arises from the broncho-oesophageal artery and travels along the trachea, dividing at the tracheal bifurcation and entering the lungs along with the bronchi. There is no bronchial vein and systemic blood returns to the heart via the pulmonary veins.

The innervation of the sheep respiratory system is described later in this chapter. A drawing of the gross anatomy of the sheep thorax is shown in Figure 1.

2. Innervation of the airways and lung:

The innervation of the mammalian airways and lung is extremely complex and not completely understood (Figure 2). The simplistic model of neural control of the airways involving an excitatory vagal parasympathetic cholinergic system and an inhibitory sympathetic system is no longer valid (Coburn, 1988). The respiratory system involves at least three efferent and three afferent neural systems (Richardson, 1988). The majority of both afferent and efferent nerve fibres are present within the vagosympathetic trunk, but there is also some input at the spinal level.

2.1. Efferent Innervation:

The efferent components of the neural network includes the classical excitatory cholinergic and the inhibitory adrenergic systems, and the more recently identified mixed excitatory and inhibitory non-adrenergic non-cholinergic (NANC) system. While the excitatory cholinergic system has been demonstrated in all species studied, there are widespread differences in the type of inhibitory system found and in the localization of nerve types (Richardson, 1988).

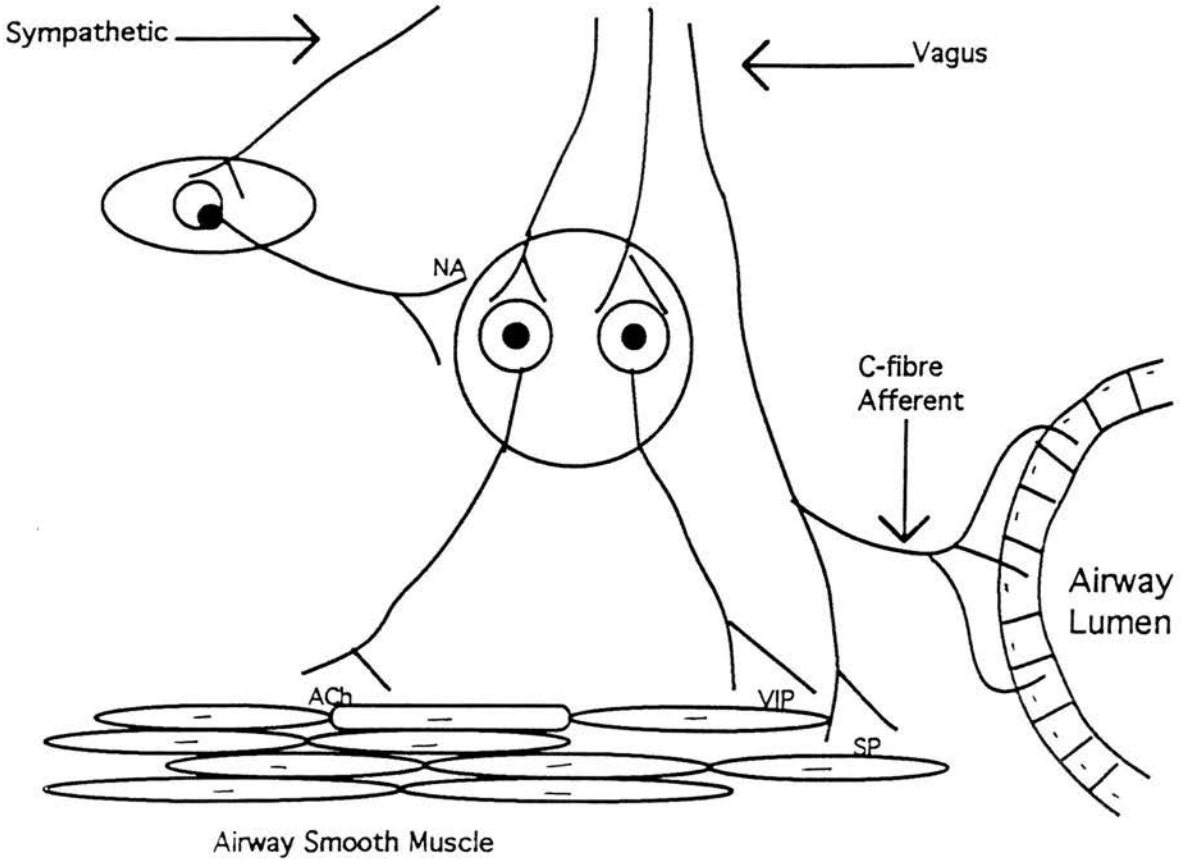


Figure 2. Diagrammatic illustration of the innervation of the respiratorty system and some of the recognised and putative peptide and non-peptide neurotransmitters involved. The afferent input from the airways include stretch receptor inputs from smooth muscle (not shown) and C-fibre afferent inputs from the airway epithelium. The efferent output includes cholinergic and non-cholinergic non-adrenergic (NANC) systems, both originating in the medulla, carried in the vagus and innervating airway parasympathetic ganglia. Post ganglionic nerve fibres innervate airway smooth muscle. The system is also modulated by adrenergic innervation of parasympathetic ganglia. ACh-acetylcholine; NA-nor-adrenaline; SP-substance P; CGRP-calcitonin gene-related peptide; VIP-vasoactive intestinal polypeptide.

2.1.1. Excitatory Cholinergic System:

The parasympathetic nervous system is the most important neural system innervating the airways. It is the major system causing bronchoconstriction and is responsible for the resting bronchomotor tone (Nadel & Barnes, 1984). The system predominantly innervates the smooth muscle of the larger airways and appears to be absent from the peripheral airways and the respiratory bronchioles (Barnes et al, 1983). The efferent motor pathways travel to the lung in the vagus as preganglionic nerve fibres, innervating the mural parasympathetic ganglia from which short postganglionic nerve fibres emerge to innervate the smooth muscle. It is because of this inaccessibility of parasympathetic airway ganglia that little is known of their function (Skoogh, 1986). The parasympathetic cholinergic neural network is the main excitatory neural system innervating airway smooth muscle. Cholinergic post-ganglionic nerve fibres release acetylcholine from nerve terminals which bind to post-synaptic muscarinic cholinoceptors causing smooth muscle contraction.

Three distinct classes of muscarinic receptors have been identified using specific pharmacological antagonists and at least five receptor classes have been cloned (Barnes, 1990). The muscarinic receptors have been classed into M1 (neuronal), M2 (cardiac) and M3 (smooth muscle, secretory) (Doods et al, 1987). The existence of muscarinic receptor subtypes in airways and lung has been known for several years, but there seems to be species variability in their localization and expression. The M1 receptor predominates in human lung tissue, but is primarily localised to submucous glands and the alveolar septum, and there are smaller numbers of M3 receptors (Mak & Barons, 1989; Barnes, 1990). Human muscarinic receptors are found in central and peripheral airway smooth muscle, whereas in the ferret they are predominantly localised in the central airways

(Barnes et al, 1983). In the guinea pig there are very few M1 receptors as assessed by binding for the M1 antagonist [^{3} H]pirenzepine (Mak & Barnes, 1989).

In bovine and canine tracheal smooth muscle the major type of receptor has been reported to be M2 (Roffel et al, 1989; Fernandes et al, 1992), while in man the predominant receptor mediating smooth muscle contraction is the M3 subtype (Roffel et al, 1989). However, antagonists with a greater specificity for the M3 receptor were not assessed in the bovine study and bovine trachealis muscle is believed to have M3 receptors (Roffel et al, 1989). In the rabbit airway it has been shown that the M1 receptor mediates bronchoconstriction caused by vagal stimulation, but is not present on smooth muscle (Bloom et al, 1987).

Using data from functional and binding studies it appears that bronchomotor tone can be modulated by activation of the three muscarinic receptor subtypes (Barnes, 1990; Minette & Barnes, 1990). M1 receptors are present in ganglia and modulate ganglionic neurotransmission, since the M1 receptor antagonist pirenzepine has been shown to inhibit vagally mediated bronchoconstriction at the ganglia level (Beck et al, 1987; Bloom et al, 1987; Yang & Biggs, 1991). The M2 receptor acts as an autoreceptor, at pre-synaptic sites, modulating and inhibiting ACh release from cholinergic nerve endings. Activation of the M2 receptor has no effect on vagally induced bronchoconstriction and its presence has been demonstrated in man, the guinea pig, dog and rat (Faulkner et al, 1986; Beck et al, 1987; Ito & Yoshitomi, 1988; Minette & Barnes, 1988; Aas & Maclagan, 1990; DelMonte et al, 1990; Doleman et al, 1991; Yang & Biggs, 1991). The M2 receptor has been shown to functionally antagonise isoproterenol-induced airway relaxation in the dog (Fernandes et al, 1992) and endogenous noradrenaline release from the guinea pig trachea (Racke et al, 1992). Finally, the M3 receptor is the receptor present

on smooth muscle (Mak & Barnes, 1990) and controls the smooth muscle contractile response to ACh. In addition, there are differences in muscarinic receptor types subserving the other respiratory functions of cholinergic neuro-transmission, including mucus secretion and pulmonary arterial vasodilation (McCormack et al, 1988; Gater et al, 1989).

Modulation of airway cholinergic neurotransmission by non-cholinergic mechanisms has also been clearly demonstrated in canine bronchi (Flavahan & Vanhoutte, 1985; Vanhoutte & Flavahan, 1988). Noradrenaline prejunctionally inhibits the release of acetylcholine from cholinergic nerve endings, as demonstrated by its ability to inhibit the contraction of airway smooth muscle caused by vagal stimulation but not that caused by exogenous acetylcholine (Vermiere & Vanhoutte, 1979). It also depresses neurotransmission in parasympathetic ganglia, although this has not yet been demonstrated in airway ganglia (Skogh, 1988). The inhibitory effect of noradrenaline is mediated through beta adrenoceptors on cholinergic nerve endings and pre-synaptic alpha and beta adrenoceptors in parasympathetic ganglia (Brown & Caulfield, 1981; Soh, 1988).

Vasoactive intestinal polypeptide (VIP) and nitric oxide (NO), the putative neurotransmitters of the non-adrenergic non-cholinergic inhibitory system (NANCis), modulate cholinergic neurotransmission in the ferret and guinea pig trachea and in isolated human central and peripheral airways (Sekizawa et al, 1988; Belvisi et al, 1991; Belvisi et al, 1992). In the ferret trachea VIP potentiates the response to electrical field stimulation at low concentrations (1nM) and inhibits at higher concentrations (20mM) (Sekizawa et al, 1988). The action of VIP is presumed to be pre-synaptic as it does not affect smooth muscle contraction caused by exogenous ACh, but the exact site of action

of NO is not yet known (Belvisi et al, 1991).

Pre-ganglionic non-adrenergic inhibitory and putative peptidergic non-cholinergic excitatory nerves have also been demonstrated in the vagus (Chesrow et al, 1980; Yip et al, 1981) and peptides might also be involved in modulation of ganglionic neurotransmission. In the guinea pig gut substance P (SP) and VIP stimulate ACh release from myenteric neurons by a calcium-dependent mechanism, and in the lung neurokinins contract airway smooth muscle by facilitating ACh release from postganglionic parasympathetic nerve endings and by a direct action on the smooth muscle itself (Yau et al, 1986; Hall et al, 1989). However, this effect of neurokinins is not mediated through prejunctional M2 receptors, as the facilitatory effect of gallamine, the M2 antagonist, on neural bronchoconstriction is not affected by depletion of sensory neuropeptides with capsaicin (DelMonte et al, 1990).

2.1.2. Inhibitory Nor-adrenergic and Non-adrenergic
Non-cholinergic (NANC) Systems:

There has been considerable difficulty in conclusively demonstrating a functional adrenergic neural network in human airways (Richardson & Beland, 1976; Davis et al, 1982; Nadel & Barnes, 1984; Richardson, 1988), despite the demonstration of inhibitory beta adrenoceptors in the proximal airways (Doidge & Satchell, 1982). However, several investigators have demonstrated adrenergic nerves in human airways (Doidge & Satchell, 1982; Partanen et al, 1982; Laitinen et al, 1985a), with the nerve fibres primarily found in the periphery, in close association with blood vessels, and with sparse innervation of smooth muscle (Pack & Richardson, 1984). A close anatomical relationship between parasympathetic ganglia and sympathetic nerves has been demonstrated in human airways (Partanen et al, 1982). Noradrenaline has also been shown to inhibit cholinergic

neurotransmission in airway parasympathetic ganglia in the ferret and cat (Baker et al, 1982), suggesting a functional role for the adrenergic system in controlling bronchomotor tone.

There may also be an association between the noradrenergic system and the neurokinins. SP has been demonstrated in the superior cervical ganglion of the rat and is found co-localised with tyrosine hydroxylase, suggesting sympathetic neurons can express both noradrenaline and SP simultaneously (Bohn et al, 1984). Haruki et al (1983) have demonstrated that SP-containing neurons in the nodose ganglia can anastamose with the post-ganglionic sympathetic trunk associated with the superior cervical ganglia, and an association between SP-containing and catecholamine-containing neurons has been demonstrated in the CNS (Pickel, 1979). SP, in addition to CGRP, VIP and NPY, has also been demonstrated in association with catecholamines in laryngeal nerve paraganglia, which are carotid body-like structures believed to be involved in the hypoxic ventilatory response (Dahlqvist et al, 1992). Whether or not similar associations exist in the rest of the respiratory system is not known, but it would appear that noradrenaline, either circulating or released from sympathetic nerves, could modify neurotransmission in peptidergic neurons and their nerve fibres. Conversely, SP, NKA and NKB have been shown to modulate activity in sympathetic neurons and can have a long lasting effect on the excitability of these cells (Konishi et al, 1992a,b). Of further interest is the sprouting of SP-containing nerve fibres and an increased SP content found in sympathetic ganglia after chemical sympathectomy (Benarroch et al, 1992), and the primary role of SP in sympathetic ganglia may be to interact with adrenergic mechanisms in maintaining arterial blood pressure. While SP could affect all preganglionic sympathetic nerve fibres, morphological studies suggest SP probably only modulates the activity of a small number of neurons (Pillows et al, 1992).

Nevertheless, the NANCis system appears to be the most important inhibitory neural system in man, since functional non-adrenergic relaxation of airway smooth muscle can be demonstrated (Richardson & Ferguson, 1979, Doidge & Satchell, 1982). The NANCis system has also been demonstrated in several other species (Diamond & O'Donnell, 1980; Doidge & Satchell, 1982), but there are no definitive reports to date of a NANCis in sheep airways. In the cat stimulation of the vagus results in an atropine-sensitive bronchoconstriction followed by prolonged bronchodilation, which is resistant to adrenoceptor or adrenergic neural blockade (Diamond & O'Donnell, 1980). This NANC bronchodilation can be reflexly activated by mechanical laryngeal stimulation and is abolished by hexamethonium, suggesting the NANCis is of vagal reflex origin and involves preganglionic neurons (Diamond & O'Donnell, 1980; Szarek et al, 1986; Michoud et al, 1988).

Pre-treatment of guinea pigs with capsaicin, which depletes peptides from sensory nerves, potentiates NANC bronchodilation and the response to exogenous vasoactive intestinal polypeptide (VIP) (Stretton et al, 1989b; Stretton et al, 1990b) and this supports the suggestion that VIP is a putative neurotransmitter for this system (Barnes, 1984; Laitien et al, 1985; Stretton et al, 1990b). Previously Burnstock (1972) proposed adenosine triphosphate as the NANCis neurotransmitter in the gastrointestinal tract, but this is no longer accepted (Diamond & Richardson, 1982). In addition to exogenous VIP having a direct effect on airway smooth muscle, it also inhibits contractions induced by field stimulation, but not by exogenous ACh, suggesting a prejunctional inhibition of ACh release from post-ganglionic cholinergic nerve endings (Sekizawa et al, 1988; Hakoda & Ito, 1990). However, there is a lack of evidence supporting VIP as the NANCis neurotransmitter, not least because of a lack of a specific and potent VIP

antagonist. Strong evidence suggesting nitric oxide (NO) is the endogenous NANC bronchodilator in man, has recently emerged (Belvisi et al, 1991; Belvisi et al, 1992), while in the guinea pig NANCis neurotransmission involves both NO and VIP (Belvisi et al, 1991). Nitric oxide acts by modulating cholinergic bronchoconstriction, but whether this is due to prejunctional modulation of ACh release or some form of antagonism at the smooth muscle target site is not known (Belvisi et al, 1991).

While it has been speculated that alteration in the NANCis system may underlie bronchial hyperreactivity, there is no evidence to support this hypothesis. Ko & Lai (1988) failed to demonstrate a difference in the intensity of NANCis tracheal relaxation in normal and ovalbumin sensitised guinea pigs.

2.1.3. The NANC excitatory system (NANCex):

The NANCex system is generally accepted to involve one or more of the sensory neuropeptides calcitonin gene-related peptide (CGRP), SP or neurokinin A (NKA) as neurotransmitters (Figure 3). Activation of sensory systems by capsaicin results in release of CGRP, NKA and SP and the signs typically associated with sensory activation, including vasodilation and bronchoconstriction (Alving et al, 1991). SP can also be released from ganglion cells by sensory specific stimuli such as, capsaicin, excess potassium (50mM) and bradykinin (MacLean et al, 1990). Rebound increases in neuropeptide Y (NPY), noradrenaline and cortisol can occur after capsaicin treatment, resulting in systemic cardiovascular responses of increased blood pressure and tachycardia.

Non-adrenergic and non-cholinergic bronchoconstriction (NANCex), caused by vagal stimulation and capsaicin pre-treatment releasing neuropeptides from sensory nerve

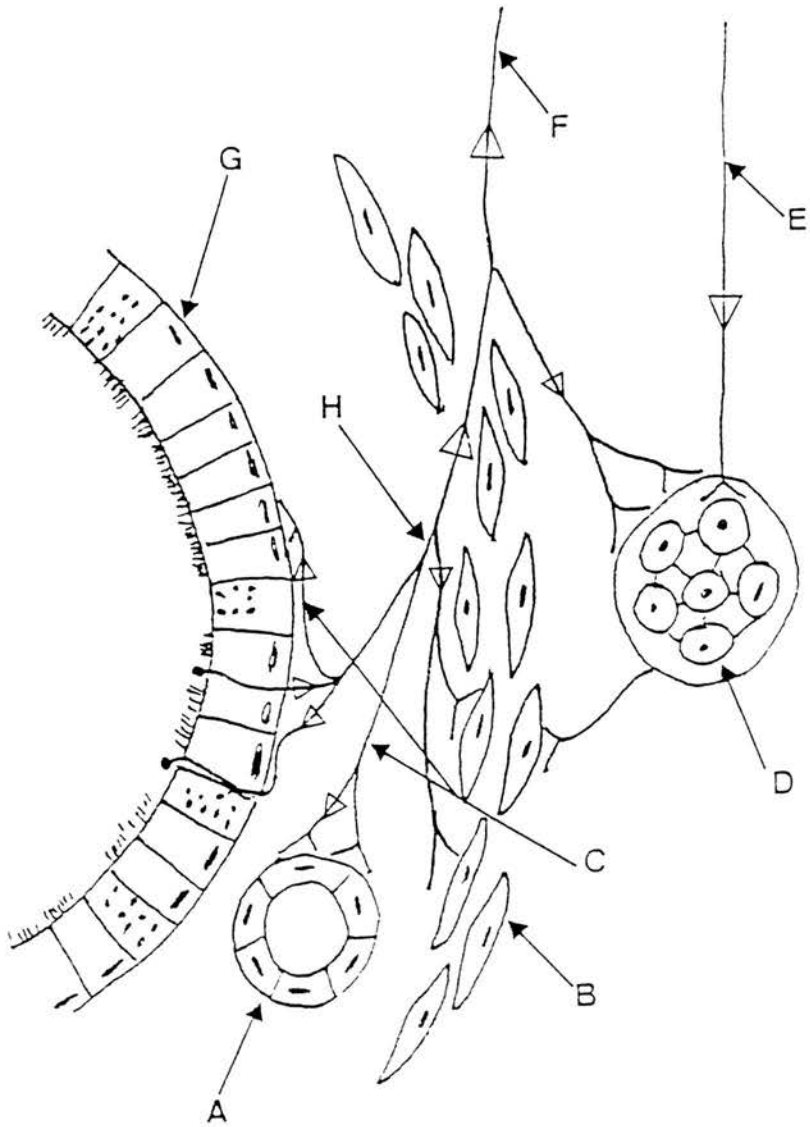


Figure 3. Schematic illustration of the sensory afferent innervation of the airway and the possible involvement of these sensory afferents in central and local-mediated axonal reflexes. Vagal C-fibre afferents terminate between the airway epithelial cells close to the airway lumenal surface. Stimulation of these endings results in local release of peptides from collateral nerve fibres close to smooth muscle, blood vessels and airway mucus glands and local or central modulation of anglionic neurotransmission. A-blood vessel; B-smooth muscle; C- C-fibre afferent endings and collateral axons; D-airway parasympathetic ganglion; E- vagal efferent; F- vagal afferent; G- airway epithelium; H- vagal C-fibre afferent.

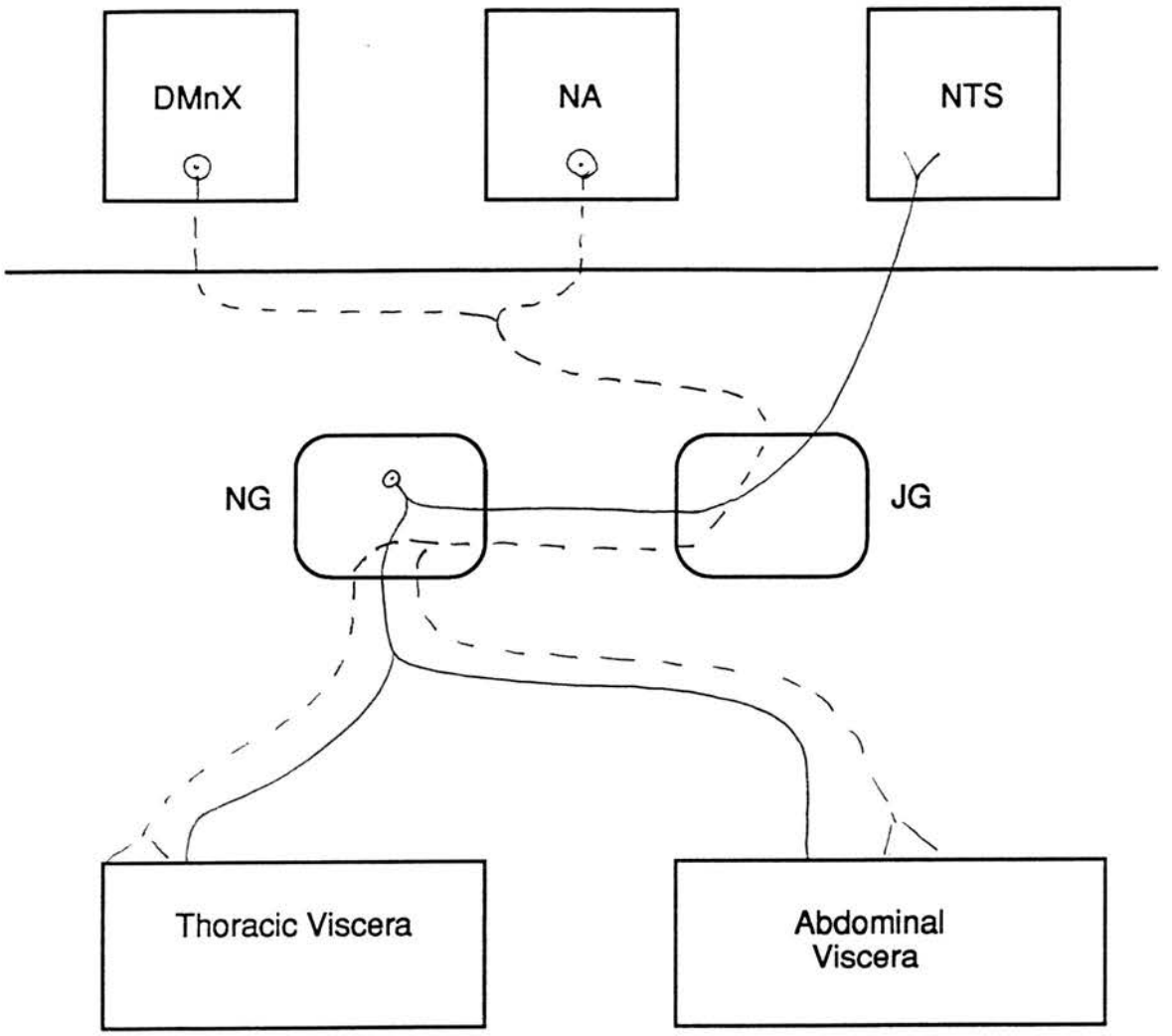


Figure 4. Diagramatic illustratation of the central projections of thoracic and abdominal visceral afferents and the source of motor output from the medulla in the sheep. Visceral afferents travel via the jugular and nodose ganglia to terminate in the nucleus of the solitary tract (nTS) in the medulla. The motor output of the vagus arises in the dorso-motor nucleus of the tenth cranial nerve (DMnX) and the nucleus ambiguus (nA). Motor neuron axons travel predominately the same route as the afferent fibres, through the nodose (NG) and jugular ganglia, (JG) although somefibres may travel by other routes. These neurons project axons into the cervical vagus and synapse on parasympathetic ganglia close to airway smooth muscle.

fibres, can be inhibited or modulated by neuropeptide Y (NPY), opioids and gamma-aminobutyric acid (GABA) (Frossard & Barnes, 1987; Belvisi et al, 1988; Belvisi et al, 1989a,b; Giuliani et al, 1989; Stretton et al, 1989a). NPY, the opioids and GABA do not inhibit bronchoconstriction caused by exogenous SP suggesting blockade of SP release from vagal NANC nerves. The activity of opioids and GABA are mediated primarily through mu-opioid receptors and $GABA_B$ receptors respectively (Belvisi et al, 1988; Belvisi et al, 1989a). Furthermore Johansson et al (1989) demonstrated that morphine also inhibited the cholinergic component of bronchoconstriction induced by electrical field stimulation, but had no effect on NANCis neurotransmission.

Substance P is found in the vagal sensory nodose and jugular ganglia and in the termination of vagal sensory afferents in the nucleus of the tractus solitarius (NTS) (Helke & Eskay, 1985; MacLean et al, 1990) (see Figure 4). SP is also found in spinal dorsal root ganglia and the dorsal horn of the spinal cord and in the axon terminals of primary afferent nerve fibres (Otsuka et al, 1976; Cheshire & Black, 1980). The central termination of capsaicin-sensitive SP vagal afferents is restricted to the lateral part of the NTS, while a sub-population of capsaicin-resistance SP neurones is found in the medial part of the NTS. SP is moved towards the periphery, including the thoracic and abdominal viscera, by axoplasmic transport form the sensory ganglia, and towards the NTS (Maclean & Lewis, 1984).

2.2. Afferent Innervation and Reflex Control of Bronchomotor Tone:

There have been several detailed monographs in recent years outlining the current understanding of the function of vagal afferent innervation in the airways and lung

(Coleridge & Coleridge, 1977a,b, 1984,1986; Paintal, 1977; Sant'Ambrogio, 1982; Widdicombe, 1983; Sant'Ambrogio, 1987). There is general agreement that there are three afferent receptor types, slowly adapting, rapidly adapting and C-fibre receptors, all of which might be involved in the control of bronchomotor tone. These receptors are classified on the basis of activity in the nerve fibres they serve.

2.2.1. Lung Afferent Receptor Sub-Types:

Slowly adapting (SAR) and rapidly adapting (RAR) receptors (see Figure 5 a) are stretch receptors that alter their activity in response to changes in volume and trans-mural pressure within the airways and lungs in phase with respiration (Fahim & Jain, 1979). These stretch receptors are primarily involved in the control of the rate and depth of respiration by a volume-controlled vagal feed-back mechanism (Trenchard, 1977; Clement et al, 1981) and their activation primarily results in extension of the end-expiratory pause (the Hering-Breuer reflex) (Marlot & Duron, 1979). The activity of SARs and RARs is also increased by hypercapnia (Coleridge et al, 1978). Bronchoconstriction also increases stretch receptor activity, presumably by mechanical distortion of the tissue around the receptors (Davenport et al, 1981). However, Sant'Ambrogio (1987) has recently suggested that stretch receptors are involved in the control of bronchomotor tone, but there is no evidence that the activation of SARs results in bronchoconstriction and these are more likely to mediate reflex bronchodilation (Widdicome & Nadel, 1963a,b). An increase in SAR activity does reflexly decrease vagal efferent activity and so acts as a negative feed-back mechanism for bronchoconstriction (Widdicombe & Nadel, 1963b), and they may be important in countering bronchoconstriction in asthmatic patients by initiating hyperinflation manoeuvres (Widdicombe, 1983).

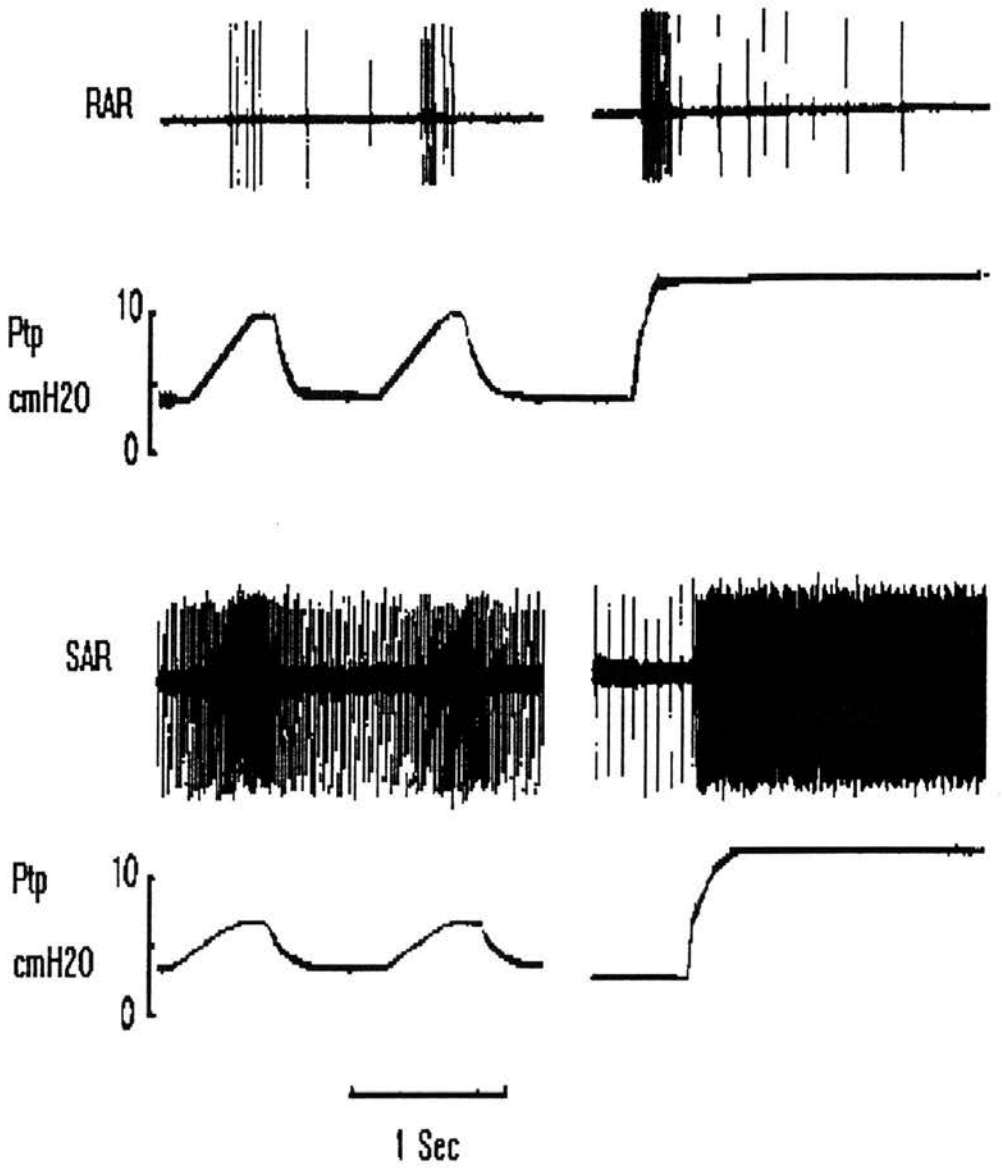


Figure 5 a.. Action potentials recorded from a rapidly adapting receptor (RAR) (upper tracing) and slowly adapting receptor (SAR) (lower tracing) in an adult opposum. The animal was ventilated passively (records to the left) and with a maintained pressure inflation (records to the right). Note the activity of both receptors are modulated by respiration. The RAR exhibits an irregular spiking discharge pattern, which rapidly adapts to a maintained pressure. The SAR has a more regular and frequent discharge pattern, but shows slow adaptation to sustained transpulmonary pressure (Ptp) (modified from Farber et al (1983)).

RAR fibres have similar conduction velocities to SAR fibres (23.3 vs 32.3 m/s; dog) and are activated by hyperinflation, forced deflation and re-inflation (Sampson & Vidruk, 1975). They show a rapid adaptation of their firing response on stimulation and are generally quiescent during quiet breathing (Bergeren & Sampson, 1982). A proportion of RARs are stimulated by histamine and ammonia, but there are interspecies differences in the reponse to other irritants such as ether and cigarette smoke (Bergeren & Sampson, 1982). Consequently the description of RARs as "irritant receptors" is inaccurate (Sampson & Vidruk, 1975). Since the RAR receptor responds to a reduction in lung compliance it is regarded as the principle receptor involved in the initiation of augmented breaths (Davies & Roumy, 1982).

Histamine causes bronchoconstriction by a combination of vagal reflex, involving activation of pulmonary RARs, and by a direct effect on airway smooth muscle (Clement et al, 1981) and this has been supported by vagal cooling studies (Karczewski & Widdicombe, 1969). However, the ventilatory response (increased minute volume and frequency) of RARs to histamine in dogs, but not the bronchoconstrictor response, is due to stimulation of vagal afferent pathways (Bleecker et al, 1976; Cotton et al, 1977). Furthermore, the stimulation of RAR activity by histamine may be secondary to airway smooth muscle contraction (Bergren & Sampson, 1982) making it less likely that RARs are involved in reflex bronchoconstricton. Afferents from pulmonary stretch receptors also depress activity in preganglionic sympathetic neurons with a concomitant reduction in systemic arterial pressure (Gerber & Polosa, 1978).

Reflex bronchoconstriction can also be elicited by airway cooling (Jammes et al, 1986) and while there is evidence that this may be due to activation of C-fibre receptors

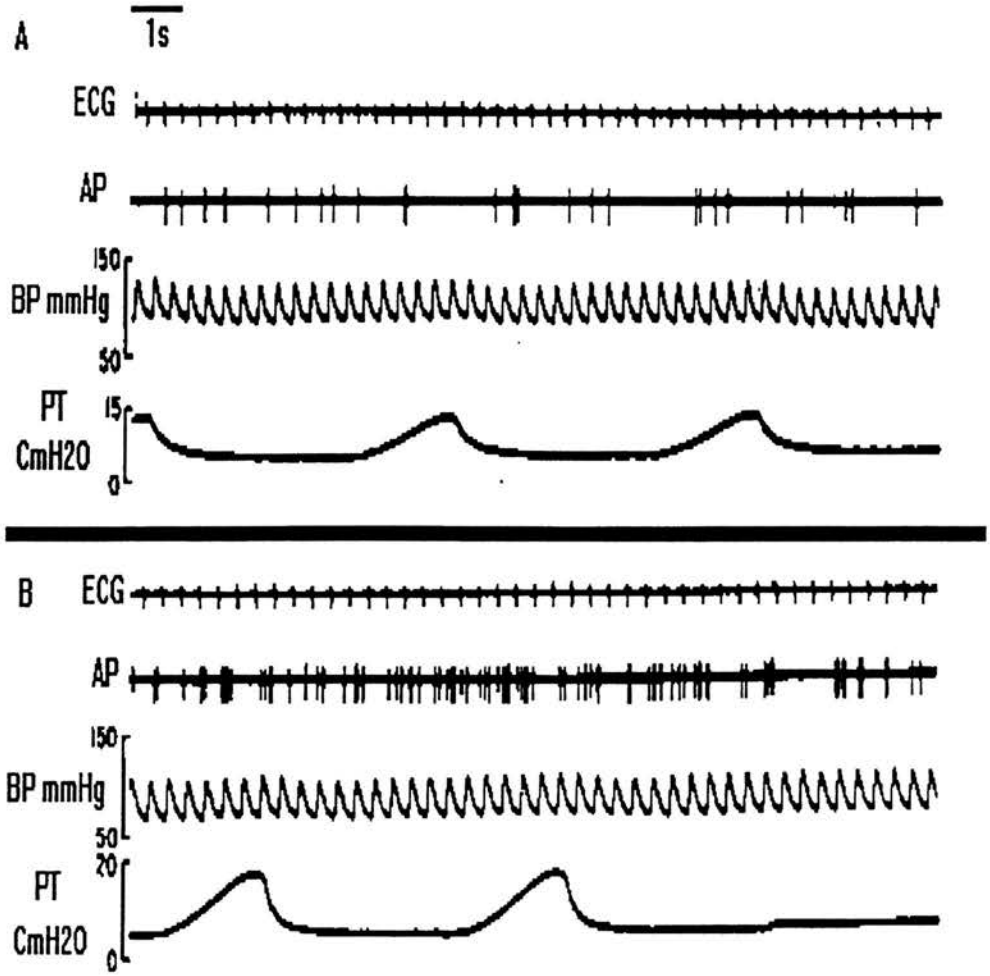


Figure 5b. Recording of the activity of a left vagal filament associated with an airway C-fibre receptor in a mechanically ventilated dog. Upper tracing A: there is an irregular discharge pattern in the C-fibre which is not modulated by respiration. Lower tracing B: Activity in the fibre is increased after inhalation of a 0.4% histamine aerosol. The ventilation pump has been switched off towards the end of the tracing and the response of the C-fibre ending has continued, showing the response is not modulated by respiratory movement. ECG, electrocardiograph; AP, action potential; ABP, arterial blood pressure; Pt, tracheal pressure (modified from Coleridge et al (1978)).

Sant'Ambrogio (1987) has postulated an additional afferent "cold" receptor to explain this phenomenon. This effect is primarily a function of the upper airway and in particular the larynx.

C-fibre receptor afferent nerve fibres (Figure 5b) are unmyelinated and have slower conduction velocities than SAR and RAR fibres (0.8-2.4ms; dog) and are further characterised by a sparse and irregular discharge pattern during normal respiration (Coleridge & Coleridge, 1977a). They are activated by a variety of chemicals including phenylbiguanide, capsaicin and bradykinin and by hypercapnia (Karczewski & Widdicombe, 1969; Kaufman et al, 1980; Delpierre et al, 1981; Sant'Ambrogio, 1982; Green et al, 1984; Coleridge & Coleridge, 1986). They are also activated by hyperinflation (2-3 times VT / 10-30cmH2O) (Kaufman et al, 1982). Their activation by hypercapnia is immediately followed by bronchoconstriction and as they are not activated by mechanical distortion of the airway wall they are the more likely candidate for the afferent component of vagal reflex bronchoconstriction (Delpierre, 1981). Vagal bronchoconstriction has been shown to involve the activation of C-fibre afferents, since bronchodilation caused by bilateral sensory vagotomy coincides with cessation of C-fibre activity (Jammes & Mei, 1979; Russell & Lai-Fook, 1979). Capsaicin, which releases neuropeptides from sensory afferent nerve endings, when injected into the right heart causes bronchoconstriction which is abolished by vagotomy or cooling the vagi to temperatures which block conduction in C-fibre afferent nerve fibres (Coleridge et al, 1982).

Activation of bronchial and pulmonary C-fibres also results in reflex increase in tracheal mucous gland secretion and concurrent tracheal contraction (Davis, B et al, 1982; Shultz et al, 1985). This effect can be demonstrated for both capsaicin and bradykinin.

The initiation of reflex bronchoconstriction can also be achieved by stimulation of the upper airways, including the nasal passages and the larynx, and is not exclusively a function of the intra-pulmonary airways (Boushey et al, 1972; Nadel & Widdicombe, 1962; Sant'Ambrogio, 1987). Laryngeal activation of vagal reflex bronchoconstriction is also accompanied by coughing (Boushey et al, 1972).

2.2.2. The Pulmonary Chemoreflex (Bezold-Jarisch Reflex):

The pulmonary chemoreflex is a complicated response to stimulation of C-fibre afferent receptors in the lung and involves a combination of bradycardia, systemic hypotension, bronchoconstriction and apnoea followed by tachypnoea (Coleridge et al, 1964; Coleridge & Coleridge, 1984; Green et al, 1984). In experimental situations the reflex is usually initiated by injection of capsaicin or phenylbiguanide (Figure 6) into the pulmonary circulation (Karczewski & Widdicombe, 1969; Sant'Ambrogio, 1982; Green et al, 1984; Coleridge & Coleridge, 1986), although it can also be activated by endogenous agents such as bradykinin (Kaufman et al, 1980) and prostaglandins (Coleridge & Coleridge, 1977a, 1986). The exact function of this reflex is not known and it has been suggested that it is not physiologically significant but merely a pharmacological curiosity (Coleridge & Coleridge, 1986).

While it is generally agreed that capsaicin activates the pulmonary chemoreflex (Coleridge et al, 1984) there is also evidence that neuropeptides released from afferent nerve endings within the lung by capsaicin might also be implicated. In rabbits SP increases respiratory rate and elicits augmented breaths when injected into the pulmonary artery, while systemic injection has no effect. Bilateral vagotomy abolishes the response and SP has been shown to stimulate both irritant and C-fibre receptors of

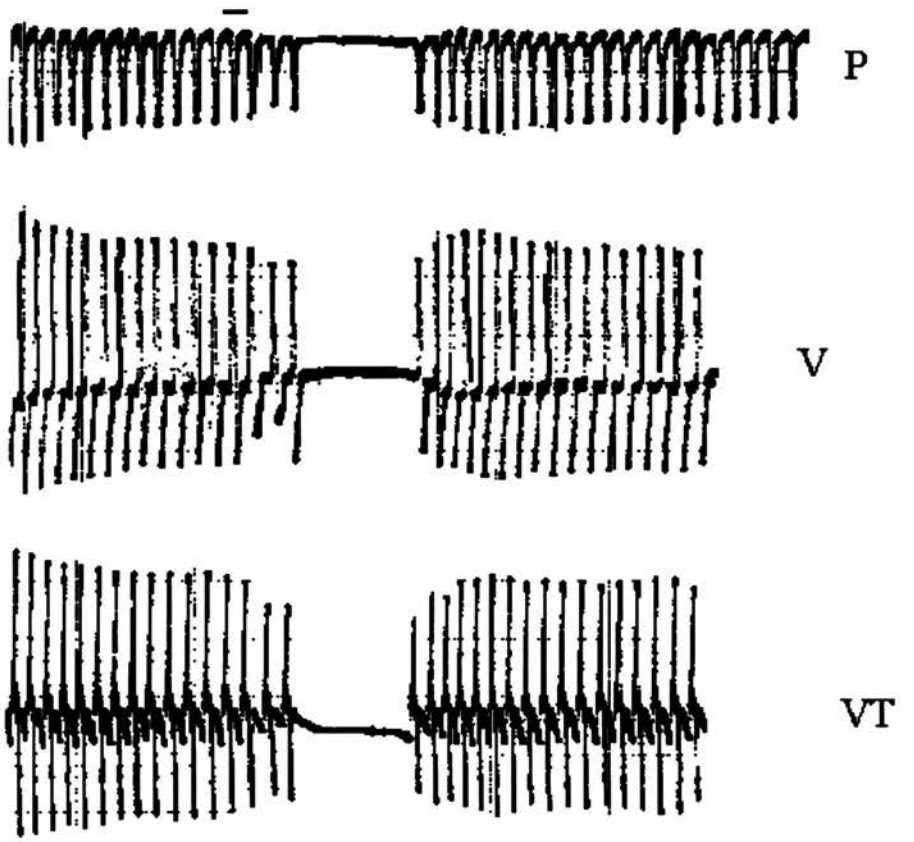


Figure 6. The effect of intravenous phenylbiguanide (PBG) on respiratory parameters in anaesthetised sheep. PBG (50ug/kg) is one of the agents routinely used to initiate the pulmonary chemoreflex. In this tracing PBG has primarily initiated an apnoeic response and systemic hypotension (not shown), without the rapid shallow breathing and bronchosonstriction typically described for this reflex in other species. However, the reflex does show species variation and not every reported feature of the reflex occurs in all species. P - transpulmonary pressure; V-respiratory flow; VT - tidal volume; horizontal bar - injection of PBG.

bronchopulmonary origin (Prabhakar et al, 1987). SP, 5-hydroxytryptamine and bradykinin also have been shown to stimulate A and C-fibre endings in the contractile tissues of the abdominal viscera (Lew & Longhurst, 1986).

Attempts have been made to sub-divide the lung C-fibre receptors on the basis of their circulatory accessibility and response to various chemicals. In the dog there appears to be pulmonary receptors accessible from the pulmonary side of the circulation and activated only by capsaicin, and bronchial receptors accessible from the arterial side and stimulated by capsaicin, phenylbiguanide and bradykinin (Coleridge & Coleridge, 1977a; Coleridge et al, 1978; Kaufman et al, 1980). These pulmonary receptors are synonymous with Paintal's J-receptors (Paintal, 1969). However, there are numerous well-recognised species differences in the response to these chemicals, and it is difficult to state with certainty that two classes of receptors are present in all species (Coleridge & Coleridge, 1986). Nevertheless, while injection of agents into the bronchial circulation will increase C-fibre activity, it is injection into the pulmonary circulation which consistently causes the pulmonary chemoreflex (Coleridge & Coleridge, 1986).

Investigation of vagal afferent activity and the effects of exogenous pharmacological agents can be assessed using vagal blockade techniques (Franz & Iggo, 1968; Derksen et al, 1981; Sant'Ambrogio et al, 1984). Vagal cooling techniques enable reversible blockade of vagal activity and to a certain extent preferential blockade of myelinated and unmyelinated nerve fibres. Differential blockade of the different myelinated nerve fibres from slowly adapting and rapidly adapting receptors is not possible, however it is possible to block most conduction in the myelinated nerve fibres leaving the majority of the unmyelinated C-fibre afferents intact. The range of temperature sensitivity for nerve

fibres has been assessed primarily in the dog and cat and there is no information on the sheep. In general temperatures between 8 and 20°C will block most vagal myelinated nerve fibres (Paintal, 1967) and temperatures below 7°C are required to block unmyelinated fibres. Nevertheless there is probably an overlap in the temperature sensitivity of the different fibre types and it has been shown that conduction in all fibres declines at temperatures below normal body temperature (Franz & Iggo, 1968).

There is conflicting evidence as to the role of afferent receptors in the airways and lung and the control of bronchomotor tone (Sant'Ambrogio, 1987). While all types of receptors are involved to some extent in the reflex control of bronchomotor tone their activation does not necessarily imply they cause bronchoconstriction (Coleridge & Coleridge, 1986). As mentioned previously, the increased activity of stretch receptors found during bronchoconstriction is probably due to the mechanical distortion of the muscle around the receptor rather than an increase in receptor activity initiating smooth muscle contraction (Fischer et al, 1983), making C-fibre afferent receptors the likely candidate for reflex bronchoconstriction, originating in the intra-pulmonary airways.

2.3. Carotid Sinus Baroreceptors:

Carotid sinus baroreceptors also modulate bronchomotor tone. Altering carotid sinus pressure either up or down from its normal resting pressure decreases and increases tracheal tension respectively (Schultz et al, 1987). This reflex change can be abolished by cooling the carotid sinus nerve to 0°C, cutting the laryngeal nerves or pre-treating with atropine, indicating it involves a cholinergic vagal pathway. This change in tracheal tension is comparable to that occuring with activation of C-fibre receptors (Coleridge & Coleridge, 1986).

2.4. Innervation of the Mammalian Lung with Particular Reference to the Sheep:

The innervation of the sheep airways and lung, described to date, is very similar to that described for other species. The central projections of vagal afferents and origin of vagal efferents in terms of the general innervation of the viscera in the sheep have been described in detail. The dorsal motor nucleus of the vagus and the nucleus ambiguus in the medulla are the main sites for the vagal motor neurones, but detail of projection of nerve fibres from these nuclei to the viscera, including the respiratory tract, is not known (Wild et al, 1991). In other species there is a distinct viscerotrophic organisation of efferent output from the motor nuclei to the supra-diaphragmatic and abdominal viscera, but in relation to the heart and lungs viscerotrophic integration might not be as specific as for other organs (Bieger & Hopkins, 1987; Fox & Powley, 1985; Harding & Leek, 1971; Katz & Karten, 1985; Kerr, 1969).

The sensory innervation of the lung is predominantly contained within the afferent vagal projections. Afferent fibres travel in the cervical vagus to the nodose ganglia. Fibres then form the solitary tract, enter the medulla and terminate in the nucleus of the solitary tract (Wild et al, 1991) (Figure 4). The intrinsic cholinergic (motor) innervation of the sheep airways and lung has been mapped in detail by Smith & Taylor (1971), but information on adrenergic, NANC and general peptide innervation is lacking. However, Sheller and Brigham (1982) have demonstrated a functional cholinergic excitatory and adrenergic inhibitory innervation in sheep trachea and bronchi and have suggested that there may be a NANCis system also present. SP, CGRP, and to a lesser extent, VIP, have been demonstrated in sheep airway nerves (Mariassy et al, 1990; this is presented in Appendix I), but overall the neuronal expression of peptides is relatively weak compared to other

species. In contrast there is a dense NPY-containing nerve fibre network in sheep airway smooth muscle (see Appendix I), and as NPY is often co-localised with nor-adrenaline, there may be an equally extensive functional adrenergic nervous system in sheep airways.

The cervical vagus and the cervical sympathetic trunk are fused over much of their length to form the vagosympathetic trunk. The trachea and oesophagus are innervated by the right vagus and its left recurrent branch, while the lower airways and lung are supplied by both vagi (Krahl, 1964). In the cholinergic system (Smith & Taylor, 1971), at the airway level, there are large nerve bundles forming a plexus, containing ganglion cells, in the extrachondral tissues of the posterior border of the trachea. The system generally conforms to that described for other species (Elftman, 1943; Honjin, 1956; Larsell, 1922). Nerve fibres course from the extrachondral plexus between the cartilage rings to form a subchondral plexus. Blood vessels and mucous glands are supplied from both plexuses while the trachealis muscle is innervated from the subchondral plexus. The subchondral plexus fibres then terminate in the submucosa, but some fibres penetrate close to the epithelium and lumenal surface. The bronchi have similar extra- and sub-chondral plexuses. The extrachondral plexus is a combination of nerve bundles of the tracheal extrachondral and the pulmonary plexuses. In the bronchi the mucous glands are poorly innervated and fibres either terminate close to the perichondrium or in the submucosa just beneath the epithelium. At the segmental bronchial level nerve bundles from the extra- and sub-chondral plexuses of the larger airways merge and then branch where the bronchi divide sending fibres into the smooth muscle. Ganglion cells are found at these nerve bundle division sites.

There are marked differences in the cholinergic innervation of the pulmonary vasculature. The pulmonary arteries have a dense innervation incorporating a superficial adventitial layer of large nerve fibres overlying a fine inter-lacing network of fibres adherent to the vessel walls. The pulmonary veins are sparsely innervated, while the bronchial arteries and veins are well innervated. There are also numerous communications between the nerve fibres of the intrapulmonary vessels and the extrachondral bronchial plexus.

2.5. Localization of Neuropeptides in Mammalian Lung:

2.5.1. General Localisation:

With the development of immunohistochemical, radio-immuno assay and autoradiographic techniques, the localization and visualization of neuropeptides, their binding sites and receptors became feasible. The immunohistochemical method of Coons and Kaplan (1950) has been used extensively to visualise antigens present within neuronal structures. The technique has been extensively modified to include the indirect peroxidase-antiperoxidase and immunofluorescence techniques. These modifications greatly improve the specificity of the technique (Pickel, 1979).

SP-like immunoreactivity (SP-Li), and immunoreactivity for a whole range of neuropeptides, has been demonstrated in most mammalian tissues. In addition immunoreactivity for a physalaemin-like peptide, which is structurally different from amphibian physalaemin, has been demonstrated in mammalian respiratory, enteric, genito-urinary systems and spinal cord (Lazarus et al, 1980, 1982). Because of their role as neurotransmitters and potential central mediators of nociception, the localisation of the neurokinins, particularily SP, has been studied in detail in the spinal cord and more

central parts of the nervous system. SP-Li has been demonstrated in primary afferent spinal neurones (Harmar & Keen, 1981; Buck et al, 1982).

In the peripheral nervous system SP-li has been demonstrated in the nodose ganglion and the vagus nerve (Polak & Bloom , 1982; Hayashi et al, 1983; Funakoshi et al, 1989) and in paravertebral sympathetic ganglia and superior sympathetic ganglia (Bohn et al, 1984; Del Fiacco et al, 1984). These nerve fibres in the sympathetic ganglia are collateral branches originating from primary sensory afferents such that activation of these afferents results in release of SP onto sympathetic postganglionic neurones. Saria et al (1985) demonstrated that removal of the stellate ganglia, with or without vagotomy, reduces the amount of SP-Li in guinea pig lung and pulmonary artery, and reduces the brochoconstrictor response to capsaicin, suggesting the airways have C-fibre afferent inputs of both spinal and vagal origin.

For several of the neuropeptides there is co-localization with other neuropeptides and with the classical non-peptide neurotransmitters. SP is co-localised with noradrenaline in sympathetic ganglia (Bohn et al, 1984) and with CGRP in the sensory trigeminal and dorsal root ganglia (Lee et al, 1985) and occasionally with galanin (Ju et al, 1987). However, SP is not present in all CGRP positive cells (Ju et al, 1987). SP is also co-localised with CGRP in nerve fibres in several other structures, including cerebral arteries (Wanaka et al, 1986). NPY is found in association with noradrenaline in adrenergic nerves (Johansson, 1986; Luts & Sundler, 1989).

2.5.2. Localisation in the Respiratory System:

With reference to the respiratory tract and structures associated with respiratory control, several neuropeptides have been demonstrated, including a physaelaemin-like peptide, in

Table 1. A representative sample of immunohistochemistry studies identifying and localising various neuropeptides in the mammalian respiratory tract in various species and in particular their localisation within nerve fibres. SP-substance P; NKA-neurokinin A; CGRP-calcitonin gene-related peptide; VIP-vasoactive intestinal polypeptide; PHI-peptide histine isoleucine; NPY-neuropeptide Y. While there are anatomical and species differences in the localisation of the various peptides in the respiratory system, in general the neurokinins are found in airway smooth muscle, sub-epithelial and in close association with CGRP.

Study	Neuropeptide	Species	Localisation
1. Nilsson et al, 1977	SP	Guinea Pig	sub-epithelial, bronchial smooth muscle
2. Sundler, et al, 1977	SP	Guinea Pig	tracheobronchial smooth muscle, artery and vein walls
3. Ghatei et al, 1982	SP/VIP	Cat/Rat/Guinea Pig	major airways, VIP>SP (cat), SP>VIP (rat,guinea pig)
4. Uchida et al, 1987	NKA	Guinea Pig	bronchial smooth muscle, pulmonary artery and vein walls
5. Palmer et al, 1987	CGRP	Man	cartilagenous airways, nerve fibres and ganglia
6. Martling et al, 1987	SP/NKA/NKB	Man	bronchi (NKA/NKB), pulmonary artery (SP)
7. Sertl et al, 1988	SP	Rat	central airways, epithelium, small vessels in lamina propria
8. Saria et al, 1988	SP/NKA	Guinea Pig	lung homogenates
9. Lundberg et al, 1988	SP/CGRP	Rat/Cat/Man/Guinea Pig	sub-epithelial, arterioles, venous sinusoids, parasympathetic ganglia, tracheal smooth muscle
10. Martling et al, 1988	SP/CGRP	Rat/Cat/Man/Guinea Pig	sub-epithelial, around small blood vessels, pulmonary artery vein, parasympathetic ganglia, tracheobronchial smooth muscle
11. Ollerenshaw et al, 1988	VIP	Man	bronchial smooth muscle, bronchioles, sub-epithelial, close to vessels and glands
12. Martling et al, 1990	SP/CGRP/VIP/PHI/NPY	Pig	sub-epithelial, tracheobronchial smooth muscle (SP/CGRP), exocrine glands, tracheobronchial smooth muscle (VIP/PHI), tracheobronchial smooth muscle (NPY)

Table 1. see legend over

mammalian species (for review see Lazarus et al, 1982) (Ghatei et al, 1982) (Table 1). SP-Li and VIP-Li are present in nerve fibres of the carotid body while enkephalin-like immunoreactivity (Enk-Li) predominates in the glomus cells (Lundberg et al, 1979; Wharton et al, 1980; Smith et al, 1990). A proportion of these SP/CGRP nerves are chemoreceptive C-fibres while the remainder innervate blood vessels (Kummer & Habeck, 1991). This suggests a possible role for neuropeptides in the baroreceptor reflex and the chemoreceptor hypoxia ventilatory response (Smith et al, 1990). In the respiratory tract SP is mainly found in nerve fibres coursing through the smooth muscle and the sub-epithelial layer, while VIP is primarily associated with blood vessels and seromucous glands in the trachea and with smooth muscle in the bronchi (Polak & Bloom, 1982; Said, 1982). The distribution of CGRP is similar to SP, with the nerve fibres travelling in the vagus and being primarily associated with primary sensory afferent nerve fibres (Cadieux et al, 1986; Luts et al, 1990). Double staining techniques demonstrate SP co-localised with CGRP in sensory ganglia and nerve fibres in the airways (Martling et al, 1988). The SP and CGRP fibres originate in the vagal sensory jugular-nodose ganglionic complex, while the CGRP fibres have an additional source in the dorsal root ganglia (Springall et al, 1987; Funakoshi et al, 1989; Luts et al, 1990), although a proportion of SP nerves may originate from airway ganglia (Dey et al, 1991). In addition Lundberg et al (1983) reported 40% of guinea pig sensory afferents originated from thoracic spinal ganglia. The main sites for CGRP receptors are in blood vessel endothelium and vessel and airway smooth muscle, with few present in airway epithelium and mucous glands (Carstairs, 1987; Mak & Barnes, 1988). There is also marked co-localisation of SP with CGRP in airway nerve fibres in guinea pig, cat, rat and man (Martling, 1987; Dey et al, 1990; Luts et al, 1990) and of CGRP with serotonin in hamster lung (Keith & Ekman, 1988). In sheep SP nerve fibres are found in close association with CGRP around mucous glands and blood vessels and to a lesser extent in

airway smooth muscle, while NPY immunoreactive nerves form a dense inter-meshing network in the tracheal smooth muscle (see Appendix 1).

Muscarinic cholinergic receptors also show a close association with SP-containing neurones in the basal ganglia, and with NPY and VIP-containing neurones in the superior cervical ganglion (Ariano & Kenny, 1989; James & Burnstock, 1989). SP nerve fibres show a close association with brainstem motoneurones innervating the extrinsic muscles of the larynx (Holtman Jr, 1988)

2.6. The Neurokinins:

2.6.1. Neurokinin Structure and Chemistry:

The neurokinins (tachykinins) are a closely related family of peptides with a common carboxyl-terminal (C-terminal) amino acid sequence of Phe-X-Gly-Leu-Met-NH2, where X is the aromatic amino acids Phe and Tyr or the branched aliphatic amino acids Val and Ile. Activity of a neurokinin depends on the integrity of the C-terminal sequence and not the amino-terminal (N-terminal). However, the N-terminal is important in regulating (inhibiting) enzymatic degradation (Rogerson et al, 1989), the binding to mast cells and the central behavioural effects of SP. The neurokinin family can be subdivided into the mammalian peptides SP, NKA and NKB, the amphibian peptides, kassinin, eledoisin and physalaemin and the recently identified insect neurokinins, locustatachykinins (Schoofs et al, 1990a,b) and the structures are shown in Table 2.

The first neurokinin discovered was in an impure protein extract of horse intestine and brain which had atropine-resistant spasmogenic and hypotensive activity (von Euler & Gaddum, 1931). The material was given the trivial name substance P (P for preparation)

Neurokinin Peptide Family	(Phe-xxx-Glyc-Leu-Met-NH2)
Mammalian	
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-*Phe*-Phe-*Glyc-Leu-Met-NH2*
Neurokinin A	His-Lys-Thr-Asp-Ser-*Phe*-Val-*Glyc-Leu-Met-NH2*
Neurokinin B	Asp-Met-His-Asp-Phe-*Phe*-Val-*Glyc-Leu-Met-NH2*
Amphibian	
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-*Phe*-Val-*Glyc-Leu-Met-NH2*
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-*Phe-Tyr-Glyc-Leu-Met-NH2*
Molluscan	
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-*Phe*-Ile-*Glyc-Leu-Met-NH2*
Insect	
Locustatachykinin I	Gly-Pro-Ser-Glyc-*Phe-Tyr-Glyc-Val-Arg-NH2*
Locustatachykinin II	Ala-Pro-Leu-Ser-Glyc-*ArgPhe-Tyr-Gly-Val*-NH2

Table 2. The amino acid sequence of the neurokinin family of peptides. Representative examples of mammalian, amphibian, molluscan and insect neurokinins are shown. The family shares a common C-terminal amino acid sequence of Phe-xxx-Glyc-Leu-Met, although the locustatachykinins have a slightly different sequence of Phe-Tyr-Glyc-xxx-xxx. which is similar to the C-terminal sequence of the physalaemin subfamily

and this name has stuck with it ever since. In 1949 Erspamer discovered the closely related neurokinin eledoisin in the salivary glands of the Mediterranean octupus *Eledone Moschata*. It was noted that there were close pharmacological similarities between SP and eledoisin but sufficient differences to suggest they are different compounds. Because eledoisin was easier to collect and purify its amino acid sequence was the first to be determined (Erspamer & Anastasi, 1962). Subsequent work by Erspamer's group isolated and sequenced the peptides physalaemin and phyllomedusin from amphibia (Erspamer et al, 1964; Anastasi & Erspamer, 1971). Indeed Erspamer and co-workers were able to predict the C-terminal sequence of the mammalian neurokinin SP on the basis of their work with invertebrate neurokinins. Due to the rapidity of their effects on intestinal preparations, compared to the more slowly acting bradykinins, the term "tachykinin" was used to group these pharmacologically and structurally related peptides together.

The activity of neurokinins depends on the C-terminal fragment, with the C-terminal heptapeptide of SP being as potent as SP in such diverse assays as guinea pig ileum, salivation in rats, cat dorsal horn neurons and mouse scratch behaviour (Piercey et al, 1982).

2.6.2. Neurokinin Receptors:

Prior to the development of specific neurokinin antagonists the investigation of neurokinin receptor subtypes had depended on assaying the various neurokinins and C-terminal fragment analogs in a variety of in vitro mono-receptor tissue preparations (Piercey & Einspahr, 1980; Piercey et al, 1982). Furthermore, prior to the identification of mammalian neurokinins other than SP, the main evidence for multiple neurokinin receptors was based on the observation of varying degrees of agonist activity for SP and

the non-mammalian neurokinins physalaemin, eledoisin and kassinin. The rank order of potencies for the neurokinins have been used historically to classify receptor sub-types. In the guinea pig ileum physalaemin is more potent than SP, eledoisin and kassinin, while in the rat vas deferens eledoisin and kassinin are markedly more potent than physalaemin and SP (Lee et al, 1982). This ranking of potency suggested the existence of two neurokinin receptors, termed SP-P and SP-E, both of which are activated by SP, but for which the non-mammalian neurokinins have different levels of affinity (Bailey et al, 1986). For the SP-P receptor physalaemin is the most potent and for the SP-E eledoisin is the most potent agonist. This classification was supported, using radio-labelled binding techniques, by the demonstration of a single neurokinin receptor in guinea pig pancreatic acinar cells with a rank order of potency of physalaemin > SP >> eledoisin (Jensen & Gardner, 1979).

Not until the identification of two novel mammalian neurokinins NKA (neurokinin A, substance K) and NKB (neurokinin B, neuromedin K) could neurokinin receptor sub-types be properly classified on the basis of agonist response (Kangawa et al, 1983; Kimura et al, 1983; Maggio et al, 1983). Specific mono-receptor assays for these three neurokinins were also identified and include, the dog carotid artery (SP), the rabbit pulmonary vein (NKA), the rat portal vein (NKB) and several others (Regoli et al, 1987). The dog carotid artery preparation relaxes in the presence of neurokinins, while the other two preparations contract. Using these assay systems it was determined that the endogenous ligands for the SP-P and SP-E receptors are SP and NKA respectively, while an additional receptor class (SP-K) was added to accommodate the specific effects of NKB (Buck et al, 1984). The more recent identification of SP analogs (e.g [Pro2]-SP) which are more potent and selective ligands for neurokinin receptors than their parent neurokinins, and the identification of the structure-activity relationships that confer

receptor selectivity on the different neurokinins, further assists in identifying receptor sub-classes (Naline et al, 1989; Petitet et al, 1991; Cascieri et al, 1992).

Several name changes for the neurokinin receptors have since occurred, include NK-P, NK-A and NK-B (Dion et al, 1987; Regoli et al, 1987a,b; Regoli et al, 1988) and NK-1, NK-2 and NK-3 (SP-P, SP-E and SP-K respectively), with the latter being the current accepted classification (Petitet et al, 1991). This classification has been confirmed in binding studies (Buck & Shatzer, 1988), but pharmacological classification still depends on the development of highly specific neurokinin antagonists for all three receptors. While numerous peptide antagonist analogs of SP and NKA are available, their low affinity and specificity for single receptor classes limits their use in receptor classification (Regoli et al, 1988). However, differences in activity and tissue selectivity with these peptide antagonists indicates there are different receptor sub-classes (Bailey et al, 1986). The recent discovery of novel, highly selective non-peptide antagonists of SP (CP-96,345-1, Pfizer Inc, CT, USA; RP-67580, Rhone-Poulenc Rorer, Vitry-sur-Seine, France) has enabled confirmation, in pharmacological terms, of the existence of neurokinin receptor subtypes (Snider et al, 1991). CP-96,345-1 antagonises the contractile activity of SP in the classical NK-1 monoreceptor preparation and the sialogogic effect in the anaesthetised rat, but does not inhibit the NK-2 and the NK-3 mediated contractile effects of SP (Hakanson et al, 1991; Snider et al, 1991). The fact that CP-96,345-1 is binding to NK-1 receptors has been confirmed using radio-labelled binding techniques in guinea-pig brain (McLean et al, 1991). More recently the existence of neurokinin receptors and the genes encoding for these receptors has been documented and confirmed using molecular biology techniques (Sundelin et al, 1992)

In several tissue systems the biological response to neurokinins can be explained by the

presence of a heterogenous population of receptors, as in the guinea pig ileum (Regoli et al, 1988) and the guinea pig trachea (Gerard, NP 1987) and hamster urinary bladder (Dion et al, 1987a) (the hamster urinary bladder has been reported to contain primarily NK-2 receptors; see Buck & Shatzer, 1988) or alternatively by the lack of selectivity for specific receptor sites (Petitet et al, 1991). Within one organ system there can also be differences in the anatomical localisation of different receptor types (Regoli et al, 1988). NKA preferentially contracts trachealis muscle compared to peripheral lung in guinea pig and overall is more potent than SP, while SP itself is equipotent in its contraction of central airway and peripheral lung tissues (Gerard, 1987). However, Coats and Gerard (1989) have demonstrated, using radio-labelled binding techniques, that a single class of receptor is present in the guinea pig lung, the receptor is NK-1 and both NKA and SP are equipotent in their affinity for this receptor.

Further division of the receptor classes has been proposed on the basis of antagonist activity and species differences. NK-2a and NK-2b receptors are now recognised on the basis of rank orders of antagonist potency, with the NK-2a being typically represented by the rabbit pulmonary artery preparation and the the NK-2b by the hamster trachea preparation (Advenier et al, 1992). Sub-types of NK-1 receptors have been suggested primarily on the basis of species differences in the activity of the specific NK-1 antagonist CP-96,345-1 and the binding affinities for radiolabelled SP (Appell et al, 1992). The non-peptide antagonist CP-96,345-1 is 10 times less potent in antagonising the effects of SP on rat jejunum than on guinea pig ileum (Legat et al, 1992). Furthermore, there may be differences in the NK-1 receptor type depending on its localisation, with, in the case of smooth muscle, both neuronal and smooth muscle receptors being present (Legat et al, 1992).

The studies by Coats and Gerard (1989) were carried out in phosphoramidon and captopril pre-treated animals and while the different biological activities of the neurokinins may be explained by receptor heterogeneity, differences in the rate of metabolism by endogenous peptide enzymes should also be considered in explaining these differences (Martling et al, 1988). Although the degradation of SP in plasma is extremely rapid (<10 s) compared to the NKA half-life of 2 min, this might not be the case in tissue systems, such as the lung and intestine, where the existence of multiple neurokinin receptors are suggested from agonist activity studies.

2.6.3. Neurokinin Antagonists:

While receptors for the different neurokinins have been identified on the basis of agonist activity, the specificity of SP, NKA and NKB for the NK-1, NK-2 and NK-3 receptors respectively is not complete. Consequently differentiation of receptor subtypes depends on the development and use of specific NK-1, NK-2 and NK-3 antagonists (Table 3).

The first generation of neurokinin antagonists are structurally based on modifications of the SP amino acid sequence and in particular the C-terminal hexapeptide, which is required for smooth muscle activity (see Folkers et al, 1981). The substitution of L-amino acids by D-Arg at position 1, D-Pro at position 2 and D-Trp at position 7 and 9 in SP produces a series of SP analogs with reduced agonist activity while introducing antagonistic properties (Engberg et al, 1981; Folkers et al,1981; Bjorkroth et al, 1982). The most potent of this first generation series [D-Pro2,D-Trp7,9)-SP antagonises the contractile effect of SP in guinea pig ileum, taenia coli, oesophagus and rabbit iris sphincter muscle (Folkers, et al, 1981; Leander et al, 1981; Salt et al, 1982; Kamikawa & Shimo, 1984) and inflammatory response in the rabbit eye (Holmdahl et al, 1981). [D-Pro2,D-Trp7,9)-SP does not inhibit NANC-induced contraction of guinea pig urinary

Structure/Name	Tissue/Receptor	Reference
[D-Pro^2,D-Trp^{7,9}]-SP	Rabbit Iris	Holmdahl et al, 1981
[D-Pro^2,D-Phe^7,D-Trp^9]-SP	Guinea Pig Ileum	Hawcock et alo, 1982
[D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP	Guinea Pig Ileum	Rosellet al, 1983
[D-Arg^1,D-Trp^{7,9},Leu^{11}]-SP (Spantide)	Rat Withdrawal Reflex	Wiesebfeld & Durnat,1987
[D-Pro^4,Lys6,D-trp^{7,9,10},Phe^{11}]-SP	Rat Dorsal Skin	Couture & Kerouac,1987
[D-Arg^2,D-Cl^{2}Phe^5,Asn6,D-Trp^{7,9},Nle^{11}]-SP	Guinea Pig Ileum	Ljungqvist et al, 1989
[D-Trp^{6,8},Pyr^4(or Nle^{10})]-NKA$_{4-10}$	Rat Isolated Vas Deferens (NK_2)	Rovero et al, 1990
Ana-Phe-Phe-Gly-Leu-Met-NH_2 (GR 71251)	Guinea Pig Ileum	Ward et al, 1990
[Ala^5,Aib^8,Leu^{10}]-NKA	Guinea Pig Trachealis Muscle (NK_2)	Shanab et al, 1990
D-NicLys^1,Pro^2,3-Pal^3,Pro^{4}D-Cl^{2}Phe^5,Asn^6,D-Trp^7,Phe^8, D-Trp^9,Leu^{10},Nle^{11} (Spantide II)	Guinea Pig Taenia Coli	Folkers et al, 1990
[(2S,3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo-[2.2.2]octan-3-amine (CP 96,345-I)	Guinea Pig Taenia Coli (NK_1)	Hahanson et al, 1991
[(3aR,7aR)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenylethylperhydroisoindol-4-one] (RP 67580)	Guinea Pig Ileum (NK_1)	Garret et al, 1991

Table 3. The evolution of peptide and non-peptide neurokinin antagonists. The tissue in which the activity of the antagonist was assayed, the receptor specificity, if determined, and a reference to its use are given. The peptide antagonists were developed as a result of substitution of amino acids in the parent neurokinin structure, but are fairly non-specific for the different receptor classes. The development of non-peptide neurokinin antagonists has resulted in compounds that have greater affinity for specific receptors.

bladder (Leander, et al, 1981) or the pressor response (Bjorkroth et al, 1982) and has variable effects on SP-induced changes in the central nervous system, providing further evidence for SP receptor heterogeneity (Engberg, 1981; Salt et al, 1982).

However, several of these analogs demonstate partial agonist activity, particularly at high doses (De & Ghosh, 1990), limiting their usefulness as pharmacological tools. [D-Pro2,D-Trp7,9)-SP and [D-Pro2,D-Phe7,D-Trp,9)-SP, in addition to antagonising the contractile effect of SP in guinea pig ileum, can contract it indirectly, either through an SP receptor on postganglionic parasympathetic neurones or by releasing histamine from mast cells (Hawcock et al, 1982; Hakanson et al, 1982; Featherstone, et al, 1986). Several SP analog antagonists are equipotent with SP in stimulating histamine release from rat peritoneal and cutanoeus mast cells (Hakanson et al, 1983; Skofitsch et al, 1983)). [D-Pro4,Lys6,D-trp7,9,10,Phe11]-SP even enhances the SP-induced plasma protein extravasation in rat skin (Couture & Kerouac, 1987).

Modifying [D-Pro2,D-Trp7,9)-SP by including D-Arg and Leu at positions 1 and 11 respectively removes smooth muscle contractile activity (Watson, 1983; Mandahl & Bill, 1984; Rosell et al, 1983). This antagonist is equipotent against both SP and eledoisin in guinea pig ileum, bladder and vas deferens and rat duodenum and vas deferens. It also antagonises SP, trigeminal nerve stimulation, capsaicin, prostaglandin E1, compound 48/80 and histamine induced contraction of the rabbit iris pupillary sphincter muscle (Mandahl & Bill, 1984) but is less potent against physalaemin or against SP in the rat urinary bladder (Rosell et al, 1983) and has no effect on SP or eledoisin induced contraction of hamster bladder (Watson, 1983).

Further modification of SP resulted in the development of spantide ([D-Arg1,D-

Trp7,9,Leu11]-SP) (Folkers et al, 1984). This heralded an new generation of antagonists as spantide has approximately 10 fold greater potency than earlier antagonists and greater specificity for the NK-1 receptor (Buck & Shatzer, 1988). However, spantide still retains the histamine-releasing activity common to the earlier analogs (Lembeck et al, 1986) and stimulates release of ACh in guinea pig ileum, causing smooth muscle contraction (Featherstone et al, 1986). It is also ineffective against SP-induced facilitation of the nociceptive flexor reflex in the rat (Wiesenfeld-Hallin & Durant, 1987). By introducing Asn into position 6 instead of Gln the potency of spantide (spantide I) was dramatically increased 5-10 fold (Ljungqvist et al, 1989), while replacing D-Arg1 and Lys3 with N-nicotinoyllysine (D-Lys(Nic)) and 3-(3-pyridyl)alanine (Pal(3)) reduced the neurotoxicity of spantide (Folkers, et al, 1990). Slight further modification has resulted in the latest undecapeptide SP antagonist, spantide II, which is approximately 100 times more potent than the antagonists of the early 1980s (Folkers, et al, 1990).

Spantide I and spantide II share only the D-Trp amino acids at postions 7 and 9. While spantide II is more potent in blocking the contractile activity of SP in guinea pig taenia coli and the rabbit eye papillary sphincter muscle it is also less potent in releasing histamine from mast cells. However, spantide I and II appear to lack specificity and both will antagonise the contractile effects of NKA in guinea pig taenia coli, while spantide II antagonises (to a lesser extent) NKB and spantide I antagonises the neurokinin-related peptide bombesin (Hakanson, et al, 1990). As SP has agonist activity at NK-1, NK-2 and NK-3 receptors, and as SP antagonists are all modificiations of SP, it is not surprising that SP-derived antagonists lack specificity for neurokinin receptors.

An additional procedure for producing neurokinin antagonist from the parent SP molecule has involved synthesising a variety of tripeptides. This strategy was based on

the assumption that the binding domain of the parent peptide for the receptor involved only a small portion of the whole peptide (Hagiwara et al, 1992). This has resulted in the development of several highly potent tripeptide neurokinin antagonists (eg FR-113,680) that are highly selective for the NK-1 receptor (Morimoto et al, 1992; Murai et al, 1992). FR-113,680 has been shown to antagonise the effects of SP on guinea pig trachea and the atropine-resistant contraction of isolated guinea pig bronchi by electrical field stimulation (Hagiwara et al, 1992; Murai et al, 1992). FR-113,680 is inactive at rat NK-1 receptors which supports the hypothesis of species-specific NK-1 receptor sub-types (Morimoto et al, 1992).

The development of peptide NKA antagonists has employed the same rational as the production of SP antagonists and as with SP amino acid substitution affects the antagonist potency and the specificity. Substitution of D-Trp at positions 6 and 8, Pyr (pyoglutamic acid) at 4 and Nle at 10 in the NKA C-terminal heptapetide NKA(4-10) gives a highly selective, although weak, NK-2 antagonist (Rovero, et al, 1990). Further introduction of D-Trp9 or Phe10, greatly improves potency, but increases NK-1 and NK-3 receptor antagonism respectively, while replacement of D-Trp9 with D-Pro in the nonselective neurokinin antagonist [Arg5,D-Trp7,9, Nle11]-SP(5-11) markedly improves its NK-2 selectivity (McElroy et al, 1992). However, the NKA analog [Ala5,Aib8,Leu10]-NKA contains no D-Trp substitutes and in guinea pig trachea is a highly selective antagonist of NKA, having no apparent effect on SP-induced contraction (Abu Shanab et al, 1990).

Nevertheless, it appears that development of neurokinin antagonists based on simple manipulation of the parent peptide incorporating D-amino acids is unsatisfactory in developing highly potent specific antagonists. More recently introduction of bicyclic

conformational constraints into the neurokinins using such agents as spirolactam has given highly selective NK-1 and NK-2 antagonists (Ward et al, 1990) and increasingly interest is focussing on non-peptide neurokinin antagonists. RP 67580 {(3aR,7aR)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one} is a potent NK-1 antagonist with no activity at NK-2 or NK-3 receptors (Garret, et al, 1991; Carruette et al, 1992). CP-96,345-1 (Pfizer Inc, Groton, CT) {(2S,3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine} is another highly selective quinuclidine non-peptide NK-1 competitive antagonist, a selective NK-1 ligand and can distinguish the NK-1 receptors of different species (Snider et al, 1991; McLean, et al, 1991). Rabbit jejunum requires 10 times the concentration of CP-96,345-1 than guinea pig ileum to block the smooth muscle contractile effect of SP (Legat et al, 1992). Because of these species differences it has been proposed that one type of NK-1 receptor is present in the guinea pig, hamster, gerbil, rabbit, cow, and man while a different NK-1 receptor is present in the rat and mouse (Chassing et al, 1992). The type of receptor present in the sheep has not bee reported. CP 96,345-1 is marginally more potent than spantide I, when assayed in the rabbit pupillary iris sphincter muscle and guinea pig taenia coli muscle preparations, but is 5 to 10 times less potent than spantide II (Hakanson et al, 1991). Both RP-67,580 and CP-96,345-1 have been shown to antagonise contraction of guinea pig ileum, bladder and trachea with SP and the potent NK-1 agonist septide (Carruette et al, 1992), while CP-96,345-1 demonstrates additional effects unrelated to its NK-1 antagonism. In anaesthetised rats it reduces mean blood pressure and heart rate (Donnerer et al, 1992) by a mechanism not involving the NK-1 receptor, but probably by Ca^{2+} channel blockade (Schnidt et al, 1992). Non-peptide antagonists for the NK-2 receptor have also been developed and include SR-48,968 ((S)-N-methyl-N[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) which is a potent competitive antagonist of the NK-2 receptor in rabbits, guinea pigs, man and

hamsters (Advenier et al, 1992).

2.6.4. The Smooth Muscle Contractile Effect of the Neurokinins:

The contractile effect of substance P on gut has been known since its discovery by Von Euler and Gaddum in 1931. However, it was over 40 years before the effect of neurokinins on airway smooth muscle tone was assessed, despite the knowledge that the respiratory and gastrointestinal systems shared a common embryological origin. Impicciatone and Bentaccini (1973) had previously demonstrated that the non-mammalian neurokinin physalaemin and the closely related tetradecapeptide peptide bombesin cause dose-dependent bronchoconstriction in guinea pigs. With the demonstration of SP-like immunoreactivity (SP-Li) in nerves of the respiratory tract of guinea pigs (Nilsson et al, 1977; Sundler et al, 1977) and in particular the close association of SP-Li nerve fibres with smooth muscle, blood vessels and epithelium interest developed in the possible effects of SP on respiratory function (Andersson & Persson, 1977).

The bronchomotor response to SP, the more recently discovered NKA and NKB and the non-mammalian neurokinins varies between species. Concentrating on the mammalian neurokinins, SP contracts guinea pig airway in vitro and causes bronchoconstriction, but has no effect on bronchomotor tone in the cat (Nilsson et al, 1977; Andersson & Persson, 1977; Lundberg & Saria, 1982; Karlsson & Persson, 1983; Karlsson et al, 1984; Stewart et al, 1985; Malo et al, 1986; Schreiber et al, 1988; Gatto et al, 1989; Biggs & Ladenius, 1990; Kroll et al, 1990). The response in guinea pigs can be augmented by pre-treatment with neutral metalloendopeptidase enzyme inhibitors such as L-thiorphan and phosphoramidon (Gerard, 1987; Shore et al, 1988).

SP has been reported to cause wheezing and retro-sternal discomfort in man after subcutaneous injection (Bernstein & Hamill, 1981), but inhalation of SP has little or no effect on bronchomotor tone in normal human subjects (Clarke et al, 1987; Fuller et al, 1987b; Joos et al, 1987; Jamieson et al, 1989). While conflicting results for the response to SP in asthmatic patients have been reported from several studies (Fuller et al, 1987b; Joos et al, 1987; Crimini et al, 1988a, 1988b), these apparent differences in response may be explained by differences in the severity of asthma in the subjects used. NKA does cause significant bronchoconstriction in asthmatic patients and in general is a more potent bronchoconstrictor than SP in man (Joos et al, 1987; Joos et al, 1989a, 1989b). These differences between normals and asthmatics may be due to differing rates of peptide degradation by enzymes in the respiratory tract (Jamieson et al, 1989) and there are greater numbers of SP immunoreactive nerve fibres in the airways of asthmatic patients compared to normals (Ollerenshaw et al, 1991). In one study SP was actually shown to cause mild bronchodilation (Evans et al, 1988). In isolated human airways SP causes a dose-dependent contraction (Palmer et al, 1987), but is less potent than either histamine or acetylcholine (Lundberg et al, 1983).

SP has also been shown to contract isolated airways in the golden hamster (Ishii & Shimo, 1985), rabbit (Tanaka & Grunstein, 1986; Armour et al, 1991), rat (Joos et al, 1988), bovine (Corson et al, 1990), Rhesus monkey (Patterson & Harris, 1990) and ferret (Webber, 1989). SP and NKA are roughly equipotent in contracting ferret trachealis muscle, but in the presence of L-thiorphan NKA is 5 times more potent.

Eledoisin, NKA and kassinin are more potent than SP and physalaemin in constricting

guinea pig airways (Hua et al, 1984; Karlsson et al, 1984; Gerard, 1987; Saria et al, 1987) and rat airways (Joos et al, 1988). While NKB has been reported to be ineffective in guinea pigs and in man (Hua, 1984; Advenier et al, 1987; Naline et al, 1989), several investigators found NKA and NKB to be up to 100 and 10 times more potent than SP respectively in the guinea pig (Advenier et al, 1987; Uchuida et al, 1987; Tschirhart et al, 1989). NKB has been shown to be equipotent with SP in the rat, at least in its effect on dynamic compliance (Joos et al, 1988). In human isolated lower airways (distal bronchi) NKA is several orders of magnitude more potent than SP (Advenier et al, 1987; Martling et al, 1987; Naline et al, 1989; Frossard & Barnes, 1991). Indeed both SP and NKA preferentially contract peripheral rather than central airways suggesting NANC control of airways is more important in the periphery (Frossard & Barnes, 1991). The order of potency suggests the NK-2 receptor predominates in human airways and this has been further supported using highly selective NK-1, NK-2 and NK-3 agonists (Naline et al, 1989). The airway response also correlates with the relative distribution of both peptides in the airways (Martling et al, 1987). SP is more prominent in the pulmonary vasculature than bronchi while the reverse is true for NKA. Furthermore, CGRP, which is often found co-localised with SP in sensory nerves, is more potent than SP in contracting human airways (Palmer et al, 1987) but not in guinea pigs (Gatto et al, 1989). However, in the guinea pig regional differences in the contractile response of airways to both SP and NKA have not been demonstrated, but the content of SP is markedly greater in the distal airways compared to the trachea (Manzini et al, 1989a). In direct contrast SP is a more potent bronchoconstrictor than NKA in pigs (Martling et al, 1990; Haxhiu-Poskurica et al, 1992).

In the lungs of all species SP appears to be restricted to capsaicin-sensitive C-fibre afferents. Capsaicin pre-treatment will release SP from these nerves and desensitise the

individual to the responses normally associated with SP release from airway and lung afferents (Lundberg & Saria, 1982a, 1983; Lundberg et al, 1983).

2.6.5. Neurokinin Metabolism:

The response to neurokinin peptides is affected by enzymatic degradation and variations in the degree of enzyme activity may explain the apparent differences in the intensity of response to different peptides within species and between species (Table 4). Differences in peptide metabolism may also explain the differences in bronchial responsiveness between normal and asthmatic human subjects. Substance P and the other neurokinins can be metabolised by a whole range of enzymes, not all of which are important in modulating the physiological response to these peptides. Early studies demonstrated that SP could be metabolised by various proteolytic enzymes in both crude and relatively pure tissue extracts (Von Euler, 1936; Pernow, 1955; Benuck et al, 1975). Interestingly Boileau and co-workers (1970) failed to demonstrate appreciable metabolism of SP and physalaemin on passage through the pulmonary circulation, despite the large amounts of angiotensin converting enzyme (ACE) in the lung and the association of ACE with SP in the CNS (McGreer et al, 1979).

The most potent enzyme for the metabolism of SP is endopeptidase-24.11 (EC3.4.24.11; NEP), a zinc metalloendopeptidase (Matsas et al, 1984). This enzyme is also important in the metabolism of enkephalins and so is also commonly known as enkephalinase. NEP is functionally and structurally different from the peptidyl-dipeptidase (EC.3.4.15.1) angiotensin converting enzyme (ACE) (Matsas et al, 1984; Skidgel et al, 1984). NEP cleaves SP at positions 6, 7 and 9, while ACE cleaves SP at positions 8 and 9 only (Skidgel et al, 1984). CGRP is also metabolised by NEP and competes with SP for the enzyme (Nyberg et al, 1988) while bradykinin is cleaved at the Pro7 -Phe8 bond

Enzyme Type	Peptides Metabolised	Antagonists	Reference
Dipeptidyl Carboxypeptidase - (Neutral Metallo-Endopeptidase (NEP) - EC 3.4.24.11 -)	Enkephalins Neurokinins (Substance P and Neurokinin A)	Phosphoramidon L-thiorphan	Shore & Drazen, 1989 Borson et al, 1987 & 1989 Matsas et al, 1984
Serine Proteases - Trypsin - Chymotrypsin -	Trypsinogen/Chymotrypsinogen Substance P		Hanson et al, 1980 Pernow, 1955
Angiotensin Converting Enzyme (ACE) - (EC 3.4.15.1 -)	Angiotensin I Substance P	Captopril Enalapril	Subissi et al, 1990 Shore & Drazen, 1989 Strittmatter et al, 1985 CAscieri et al, 1984
Tryptase	Vasoactive Intestinal Polypeptide Peptide Histine Methionine		Tam & Caughey, 1990
Post Proline Cleaving Enzyme -	Substance P		Blumberg et al, 1980
Acetylcholinesterase -	Substance P	Neostigmine etc	Chubb et al, 1980
Rat Brain Homogenate	Substance P		Benuck & Marks, 1975

Table 4. The major classes of peptide cleavage enzymes identified as possible metabolic enzymes of the neurokinins.. The enzyme name, the major families of peptides metabolised, enzyme antagonists identified and references to their use are given. Most of the references refer to the enzymes activity in modulating the airway response to neurokinins. The neurokinins are metabolised primarily by the NEP and ACE classes of endopeptidases.

(Skidgel et al, 1991). This inhibition of SP degradation by CGRP co-released from primary afferents may augment the physiological response to SP (Le Greves et al, 1989).

In the lung NEP is located primarily in the airway tissue and is epithelium-derived, being membrane bound on the surface of all lung cells (Fine et al, 1989; Nadel, 1991), while ACE predominates in the pulmonary vasculature (Johnson et al, 1985). Presumably NEP inactivates peptides entering the respiratory tract or released in close proximity to the respiratory epithelium and ACE can metabolise vasoactive peptides in the pulmonary circulation. NEP interacts with and modifies the numerous and varied respiratory effects of the neurokinins (Borson et al, 1986; Borson et al, 1987; Nadel & Borson, 1987; Umeno et al, 1989) and is important in the physiological response to neurokinins (Lazarus et al, 1987). The effect of NEP can be modified by denuding the airway epithelium, as with respiratory infections (Borson et al, 1989), and on exposure to noxious agents such as cigarette smoke (Dusser et al, 1989).

With particular reference to airway smooth muscle contraction, the bronchomotor response to SP is augmented by pre-treatment with NEP inhibitors, such as L-thiorphan and phosphoramidon, in isolated airways of rabbits, guinea pigs ferrets and in human isolated airways (Nadel & Borson, 1987; Sekizawa et al, 1987; Stimler-Gerard, 1987; Armour et al, 1991; Frossard & Barnes, 1991; Honda et al, 1991) and in anaesthetised sheep (Abraham et al, 1991) and guinea pigs (Shore et al, 1988; Thompson & Sheppard, 1988; Shore et al, 1989a; Martins et al, 1990) and piglets (Haxhiu-Poskurica et al, 1992). The responses to SP and NKA are both augmented by NEP inhibition in guinea pigs (Gerard 1987; Shore & Drazen, 1989b; Martins et al, 1991), sheep (Abrahams et al, 1991) and man (Frossard & Barnes, 1991) with maintenance of the same order of potency, but L-thiorphan has no effect on the response to NKA in piglets (Haxhiu-

Poskurica et al, 1992). The response to NKB is also augmented by L-thiorphan in guinea pig (Shore & Drazen, 1989b)

Several other enzymes have been suggested to control neurokinin metabolism. Contrasting reports on the effect of angiotensin converting enzyme (ACE) inhibitors, such as captopril, on the response to neurokinins have been reported. ACE inhibitors potentiate the bronchomotor response to intravenous SP in anaesthetised guinea pigs and is roughly equipotent with L-thiorphan in this effect (Shore et al, 1988; Shore & Drazen, 1989a,b). They have no effect on SP or NKA infused directly into the guinea pig trachea (Martins et al, 1989; Lotvall et al, 1990), on SP-induced contraction or mucus secretion in isolated ferret trachea (Borson et al, 1987; Sekizawa et al, 1987), NKA and NKB-induced bronchoconstriction in guinea pigs (Shore & Drazen, 1989b) or SP-induced contraction of isolated human bronchus (Honda et al, 1991). However, both captopril and phosphoramidon potentiate the bronchoconstrictor effect of bradykinin in guinea pigs (Ichinose & Barnes, 1990). The combination of L-thiorphan and captopril further augments the bronchoconstriction with SP in guinea pigs (Shore et al, 1989b). The varying effects of captopril may be explained by the occurence of at least two isozymes of ACE in the rat brain (striatal) and lung tissue. Lung ACE is ineffective in metabolising NKA, eledoisin, kassinin and SP 5-11, but cleaves SP and physalaemin (Strittmatter et al, 1985). Furthermore, ACE and bestatin-sensitive aminopeptidase do not metabolise rat brain SP, but are involved in hydrolysis of SP fragments (Oblin et al, 1989). The peptidase tonin which attacks phenylalanyl peptide bonds metabolises both angiotensin II and SP (Chretien et al, 1980) and a post-proline cleaving enzyme that cleaves thyrotropin releasing hormone has been reported to cleave SP at the Pro4-Gln5 bond (Blumberg et al, 1980). Fetal calf acetyl-cholinesterase also metabolises SP (Chubb et al, 1980), but is ineffective in rabbits (Armour et al, 1991). In ferret ileum ACE and acetylcholinesterase

and several other peptidases are also ineffective in metabolising SP (Djokic et al, 1989), but captopril reduces NPY-like immunoreactivity in guinea pig plasma (Dahlof et al, 1991), which has been demonstrated to modulate SP release from NANC nerves in the airway (Belvisi et al, 1990b) . Human lung tryptase, released from mast cells, metabolises the bronchodilating neuropeptides VIP and peptide histidine methionine (PHM) and to a lesser extent CGRP, but has no effect on the neurokinins (Tam & Caughey, 1990). This may augment the airway response to the neurokinins in respiratory disease.

2.6.6. Mode of Action of the Neurokinins:

The biological actions of the neurokinins are mediated via specific receptors and the structure of the three neurokinin receptors NK-1, NK-2 and NK-3 have been identified and the receptor genes have been cloned and sequenced (Sundelin et al, 1992) and their pharmacological profiles determined (Petitet et al, 1991). The neurokinin receptors are of the guanosine-nucleotide-binding-regulatory-protein-coupled type (G-coupled) and all can be activated by the three mammalian neurokinins. While the existence of specific receptor subtypes for the neurokinins is generally accepted, the exact location of these receptors, either on the target tissue or structures innervating the target tissue, has not been completely determined (Coats & Gerard, 1989; Snider et al, 1991). Consequently, the respiratory effects of the neurokinins may involve both direct and indirect mechanisms but are all mediated by specific neurokinin receptors.

2.6.6.1. Direct Mechanisms:

The mechanism of action of SP appears to be largely direct in the guinea and man. However, there are several studies suggesting indirect mechanisms may be involved in

these species. It is well recognised that in guinea pig ileum SP has two separate mechanisms of action. SP stimulates release of ACh from cholinergic nerve endings and this indirect effect of is in addition to its direct effect on ileal smooth muscle (Tietelbaum et al, 1984; Yau et al, 1986; Kowal et al, 1989). The bronchoconstrictor effect of exogenous SP and histamine in the guinea pig can be significantly reduced by pre-treatment with capsaicin, suggesting SP at least requires intact sensory afferents containing peptides for its maximal effect (Goel & Biggs, 1987; Biggs & Ladenius, 1990).

In human airways exogenously administered neurokinins and CGRP appear to act directly and are not modified by cholinergic and adrenergic antagonists, histamine H1 and H2 receptor blockers or tetrodotoxin (Lundberg et al, 1983a,b; Palmer et al, 1987). The majority of human studies, however, have used tissue collected during lung resection of bronchogenic carcinomas in aged patients and the effects of neurokinins in normal young adults and infants is not known. In asthmatic patients the response to SP and NKA is greater than in normal subjects and, more importantly, the response to inhaled NKA and SP can be significantly inhibited by pre-treatment with nedocromil sodium (Crimini et al, 1988a,b; Joos et al, 1989a,b) in a similar manner to that occuring in rats (Joos et al, 1988). The mode of action of nedocromil sodium is not completely understood but may involve a combination of mast cell stabilisation and inhibition of vagal reflex bronchoconstriction (Dixon et al, 1980; Eady, 1986).

The modulation of the response to SP, NKA and NKB by endogenous epithelium-derived metabolic enzymes further complicates the issue. Denuding the trachea of epithelium in guinea pigs and ferrets, or treating with a neutral metallo-endopeptidase inhibitor such as L-thiorphan, potentiates the contractile effect of NKA more than SP, and the mucus

secreting effect of SP more than NKA (Tschirhart et al, 1989; Webber, 1989) suggesting specific NK-2 and NK-1 receptors mediate these responses. Furthermore, we know of several mechanisms that modify the endogenous release of neurokinins from sensory afferent nerves in the lung, but most evidence suggests that these mechanisms are not affected by or modify the response to exogenously administered SP.

NPY suppresses nerve-mediated contraction, presumably caused by neurokinin release from non-cholinergic nerve endings, of guinea pig airways, but has no effect on contraction caused by exogenous SP (Grundemar et al, 1990; Stretton et al, 1990a). Opioids and gamma amino butyric acid (GABA) have also been shown to prejunctionally inhibit SP release from capsaicin-sensitive primary afferent nerves in rat trachea, through activation of the u-opioid and $GABA_B$ receptors respectively (Belvisi et al, 1989a & 1990; Ray et al, 1991a,b) but as with NPY have no effect on exogenously administered SP (Belvisi et al, 1989a). However, Khalil & Helme (1990) demonstrated that opioid receptor antagonists inhibited cutaneous inflammation caused by exogenously administered SP in the rat. There also appears to be a synergism between adenosine and the neurokinins in causing bronchoconstriction in rats which is associated with an increase in circulating histamine levels (Pauwels et al, 1990).

It should also be noted that SP has a direct effect on vascular permeability within the lung, which can result in airway wall oedema and an associated reduction in bronchial calibre. This phenomenon may be significant in the pharmacological response to SP, but is probably more important in the increased resistance to breathing associated with various airways diseases such as asthma (Belvisi et al, 1989b; Lotvall et al, 1990).

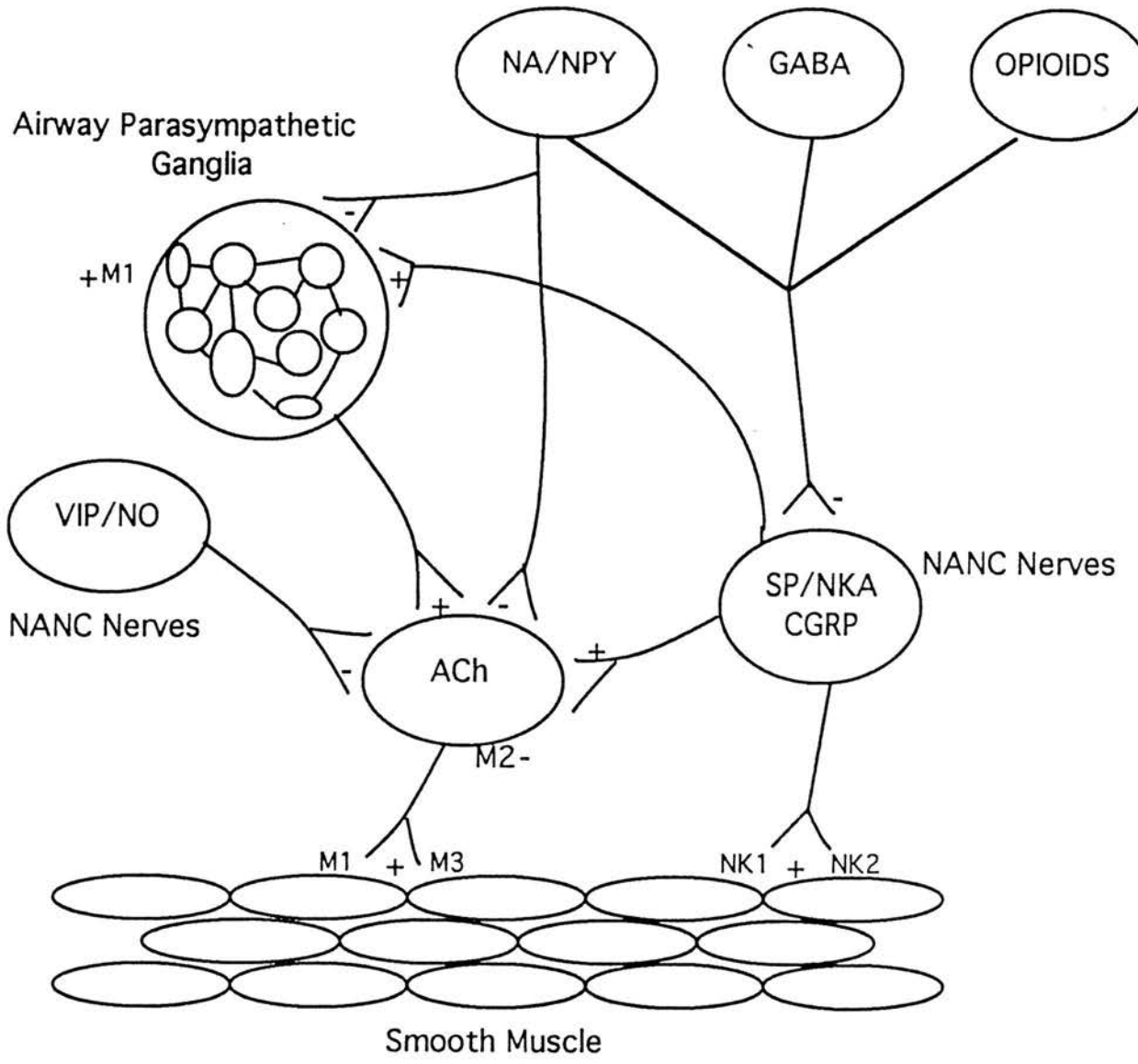


Figure 7. Schematic illustration of the possible mechanisms involved in the neural modulation of cholinergic neurotransmission to airway smooth muscle and the interaction between peptidergic and non-peptidergic neural systems. Smooth muscle tone is modulated by acetylcholine (ACh) released from cholinergic nerve endings activating muscarinic receptors (M1,2 & 3) and neuropeptides substance P (SP), neurokinin A (NKA) & calcitonin gene-related polypeptide (CGRP)) from non-adrenergic non-cholinergic (NANC) nerve endings activating neurokinin receptors (NK1 & 2). Release of peptides from NANC nerves is pre-junctionally inhibited by gamma amino butyric acid (GABA), neuropeptide Y (NPY) and opioids, while released peptide is metabolised by neutral metallo-endopeptidase. NANC peptides prejunctionally stimulate release of ACh, while vasoactive intestinal polypeptide (VIP), nitric oxide (NO), and ACh (M2) inhibit its release. SP, nor-adrenaline (NA), NPY, and ACh (M1) modulate neurotransmission through parasympathetic airway ganglia. + and - refers to stimulation or inhibition of the target neurons, or in the case of the receptor types shown, stimulation or inhibition of smooth muscle contraction.

2.6.6.2. Indirect Mechanisms:

There are numerous studies supporting the hypothesis of an indirect mechanism of action for the neurokinins in mammalian airways. While it is generally accepted that the neurokinins interact with specific receptors, designated NK-1, 2 and 3, the exact location of these receptors in the airways has not been determined (Barnes, 1989). Furthermore, there appear to be definite inter-species variations in the mechanisms of action of the neurokinins in the respiratory system (Joos et al, 1989a; Armour et al, 1991) and there is even some evidence for indirect mechanisms in both man and guinea pigs.

2.6.6.2.1. Modulation of Cholinergic Neurotransmission:

The most common example of an indirect mechanism for neurokinin-induced bronchconstriction involves modulation of cholinergic neurotransmission (Figure 7), either pre-junctionally by stimulating ACh release from cholinergic nerve endings, activating reflex vagal bronchoconstriction, interfering with neurotransmission at the parasympathetic airway ganglia level or even modifying the activity of post-synaptic muscarinic receptors (Grunstein et al, 1984; Tanaka & Grunstein, 1986; Haxhiu-Poskurica et al, 1992).

SP enhances the contractile reponse of tracheal smooth muscle to electrical field stimulation in the rabbit and this is inhibited by atropine, tetrodotoxin and the SP antagonist D-Arg1,D-Pro2,D-Trp7,9,Leu11-SP, but SP does not enhance the response to methacholine itself (Tanaka & Grunstein, 1986). Atropine also inhibits the contractile effect of SP in rabbit airways (Grunstein et al, 1984). These results, however, have been recently questioned by Armour et al (1991) who failed to demonstrate an inhibitory effect of atropine on the contractile response of rabbit isolated trachea and bronchus to SP. In

the rat atropine, but not vagotomy, reduces the bronchoconstriction caused by eledoisin and SP (Joos et al, 1988), while in bovine trachealis muscle atropine abolishes the response to low concentrations of SP, but is less effective when near-maximal concentrations of SP are used (Corson et al, 1990) indicating SP acts both directly and indirectly. Similarily in the pig atropine, albeit at quite a high dose (2mg/kg), significantly reduces tracheal contraction caused by SP and NKA (Haxhiu-Poskurica et al, 1992). This data suggests that in some species there is a cholinergic component to the bronchoconstriction caused by the neurokinins probably involving prejunctional modulation of the release of ACh from cholinergic nerve terminals.

As mentioned earlier, in man, and possibly the guinea pig, the airway response to the neurokinins appears to be direct, involving interaction between the peptides and post-synaptic NK-2 receptors on smooth muscle. However, the bronchoconstriction caused by capsaicin in man can also be reduced by muscarinic blockade, and while this may suggest capsaicin activates vagal reflex bronchoconstriction rather than local release of SP (Fuller et al, 1985), capsaicin may be releasing SP or other neuropeptides from nerve endings which subsequently activate vagal reflex broncho-constriction. In moderately asthmatic patients the muscarinic antagonist ipratropium bromide significantly inhibits the bronchoconstriction caused by inhalation of SP (Crimini et al, 1990) and NKA, in the presence of the potassium channel blocker 4-aminopyridine, has also been shown to prejunctionally potentiate cholinergic neurotransmission to the airways in man (Black et al, 1990).

In the guinea pig SP contracts airway smooth muscle directly. However, Laufer et al (1985) demonstrated SP, NKA and NKB contracted guinea pig ileum by a combination of a direct effect on smooth muscle and indirectly by activating a neuronal receptor

releasing ACh. This neuronal receptor is distinct from the smooth muscle receptor and is preferentially activated by NKB. SP also enhances the airway contractile response to both antigen and ACh (Nagai et al, 1990) (this was not demonstrated in a similar study by Schreiber et al, 1988). This SP-induced enhancement of bronchoconstriction is abolished by vagotomy and is partially inhibited by the cyclooxygenase inhibitor indomethacin (Omini et al, 1989). A similar enhancement by bradykinin has also been noted in the guinea pig. Capsaicin-sensitive sensory nerves are necessary for the toluene diisocyanate-induced increase in bronchial reactivity to carbachol found in guinea pigs (Thompson et al, 1987). This suggests sensory neuropeptides also modulate the activity of post-synaptic muscarinic receptors in guinea pig airway smooth muscle. Atropine, the H1 antihistamine mepyramine, and indomethacin have no effect on SP-induced broncho-constriction in guinea pigs (Stewart et al, 1985; Shore & Drazen, 1989a), but indomethacin inhibits the enhanced response found with repeated challenge with SP, suggesting cyclooxygenase products of arachidonic acid have some role in the response to SP. In contrast, inhibitors of lipoxygenase enzymes enhance SP-induced bronchoconstriction suggesting a lipoxygenase metabolite of arachidonic acid is an endogenous bronchodilator (Stewart et al, 1985).

SP increases ciliary beat frequency by indirect mechanisms in the dog (Wong et al, 1991). The response is modulated, but not abolished, by indomethacin, the ganglion blocker hexamethonium, and the muscarinic antagonist ipratropium bromide, suggesting involvement of a cyclooxygenase-dependent parasympathetic reflex (Wong et al, 1991). The increase in mucus secretion also caused by SP is a direct effect in the guinea pig mediated by the NK-1 receptor (Kuo, 1991), while in the cat the secretion of mucus is entirely abolished by atropine and is not affected by the SP antagonist D-Pro2,D-Trp7,9-SP (Shimura et al, 1991).

2.6.6.2.2. Inflammatory and Cellular Mediators:

i. The Eicosanoids:

The eicosanoids consist of the leukotriene and prostanoid families of compounds which are the metabolites of arachidonic acid (AA) and are produced by the lipoxygenase and cyclo-oxygenase enzymatic pathways respectively (Reaburn, 1990). Arachidonic acid has been shown to exert both contractile and relaxant effects in the airways (Tschirhart et al, 1987) and metabolites of AA may be involved in the bronchomotor response to SP and the activity of peptidergic sensory afferent nerves in the airways. Furthermore, bronchconstrictor prostanoids may act through cholinergic-mediated mechanisms (Beasley et al, 1987). In the spinal cord non-steroidal anti-inflammatory drugs block the increased sensitivity to pain associated with activation of neurokinin receptors (Malmberg & Yaksh, 1992) and this may be further evidence that eicosanoids can affect peptidergic mechanisms. Moreover, a specific ability of PGE_2 to increase the release of SP for sensory afferent neurons, by increasing calcium conductance, has recently been demonstrated (Nicol et al, 1992). Although this does not occur with aracidonic acid, PGF_{2a} or LTB_4, arachidonic acid itself has been shown to cause bronchoconstriction in guinea pigs, and this depends to a large extent on intact sensory afferents, as the response is reduced by capsaicin pre-treatment (Tschirhart et al, 1987; Manzini et al, 1989b). While cyclooxygenase inhibitors, such as indomethacin and aspirin, have been shown to augment the response to SP in some studies, this has not been universally demonstrated (Mizrahi et al, 1982; Regoli et al, 1984; Stewart et al, 1985). Gerard (1987) found the contractile effects of SP and NKA are reduced by cyclooxygenase inhibitors in guinea pig lung parenchymal strips, but not in the central airways. The cyclooxygenase products of arachidonic acid could not be identified in perfusates from both tissues suggesting SP

was stimulating eicosanoid production within smooth muscle cells. The contractile activity of CGRP, which is often co-localised with SP, is not affected by indomethacin.

In intact guinea pigs the increase in pulmonary resistance (RL) and decline in Cdyn bronchoconstriction caused by SP is not affected by indomethacin, but is potentiated by the combined cyclooxygenase and lipoxygenase inhibitors (3-amino-1-(m-trifluoromethyl)phenyl) pyrazoline HCl and eicosatetraynoic acid (Stewart et al, 1985). This suggests that the primary effect of activation of arachidonic acid metabolism in the airways is the production of a lipoxygenase-derived bronchodilator agent rather than a cyclooxygenase-derived spasmogenic eicosanoid. While indomethacin inhibits cyclo-oxygenase activity it also increases lipoxygenase activity (Walker et al, 1980) and a net balance between eicosanoid bronchodilator and bronchoconstrictor agents might occur. The curious enhancement of the airway response to SP caused by repeated exposure to SP reported by Shore & Drazen (1989a) is inhibited by indomethacin, but not the original SP-induced bronchoconstriction.

ii. Histamine:

Several neuropeptides, including SP, VIP, somatostatin (SOM), have been reported to stimulate histamine release from mast cells and this may, in part, underlie the bronchomotor response to these peptides. SP has been shown to release histamine in skin and the flare and wheal response to SP can be blocked by antihistamines (Foreman et al, 1983; Barnes et al, 1986). However, the ability of SP to cause histamine release is restricted to rat peritoneal and human skin mast cells (Shanahan et al, 1985; Repke et al, 1987; Lowman et al, 1988 a,b; McLeod et al, 1990) while the other neurokinins NKA, NKB, eledoisin and physaelamin and bradykinin have no effect. This specificity of SP reflects the heterogeneity of the mast cell population in mammalian species and between

species. On the basis of neutral proteinase content, human mast cells can be subdivided into two groups, T and TC cells (Irani et al, 1986). In the lung the T cell predominates, accounting for more than 90% of mast cells while the TC mast cell predominates in skin. Consequently non-immunogenic stimulation of histamine release from human mast cells by SP, compound 48/40, poly-l-lysine and morphine may be expected to occur more in the skin than in lung, adenoidal, tonsilar, or colonic tissue (Lowman et al, 1988a). However, Louis & Radermecker (1991) recently demonstrated histamine release from human lung mast cells by SP, which was significantly inhibited by nedocromil sodium. This would suggest that histamine might be involved in the bronchomotor response to neuropeptides, but the evidence supporting a definitive role for the mast cell in neurogenic inflammation is still sparse (Casale et al, 1988; Barnes, 1989).

However, inhalation of compound 48/40 by allergic sheep (which in common with SP degranulates skin mast cells by an unidentified activation-secretion coupling pathway (Lowman et al, 1988a) results in both early and late-phase bronchoconstriction (Russi et al, 1984). Furthermore, Pauwels et al (1990) demonstrated a synergism between NKA and adenosine-induced bronchoconstriction, which was associated with a significant increase in histamine levels in bronchoalveolar lavage samples, and suggested the response to NKA and adenosine was due to enhancement of histamine release from mast cells. Intravenous SP has been shown to raise plasma histamine levels in rats (Sertl et al, 1988), and while it was presumed that the histamine came from lung mast cells this was not proven. Although the lung mast cell is possibly not involved in the acute pharmacological response to exogenously administered SP, an interaction between SP, VIP, CGRP and gastrin releasing petide (GRP) and mast cells is probably important in the aetiopathogenesis of respiratory inflammation (Goetzl et al, 1990). Nerve fibres immunoreactive for NKA and CGRP, but not NPY, are found close to lung mast cells,

suggesting a close relationship between sensory nerves and these cells (Nilsson et al, 1990). More recently Crivellato et al (1991) have demonstrated a close anatomical association between mast cells and peptide-containing nerve fibres in rat mesentery. These nerve fibres, which include fibres immunoreactive for SP, VIP, CGRP and SOM, not only run close to mast cells, but also come in direct contact with the mast cell surface. Furthermore, Joos et al (1988) have demonstrated the antihistamine and mast cell stabilising agent ketotifen, but not the classical antihistamine clemastine, reduced the bronchoconstriction caused by eledoisin in the rat. The activation of other resident cell types in the lung, such as macrophages, does not appear to be involved in the responses to SP (Pujol et al, 1989)

It is interesting to note that histamine, along with bradykinin, the nicotinic agonist dimethylphenyl piperazinium and vagal nerve stimulation, stimulates release of neurokinins from sensory nerve endings in the lung (Saria et al, 1988a), indicating there is an inter-action between SP and histamine.

2.6.7. Additional Effects of Neurokinins on Airway Functions:

2.6.7.1. Mucus Secretion and Airway Cilia Function:

Exogenously applied SP increases cilia beat frequency in dogs and stimulates mucus secretion in the guinea pig and cat (Rogers et al, 1989; Shimura et al, 1991; Kuo et al, 1991; Wong et al, 1991). The increase in ciliary beat frequency involves a cyclooxygenase-dependent parasympathetic reflex as it is inhibited by hexamethonium, the muscarinic antagonist ipratropium bromide and indomethacin (Wong et al, 1991). The release of SP from sensory nerve endings by capsaicin also increases ciliary activity (Wong et al, 1990) suggesting SP released during lung injury or inflammation is

important in improving mucociliary clearance. However, in the guinea pig and man the sensory neuropeptides SP, NKA, NKB and CGRP stimulate goblet cell mucus secretion via activation of NK-1 receptors (Rogers et al, 1989; Kuo et al, 1991) and in the guinea pig the response is resistant to atropine and H1 and H2 receptor antagonists (Kuo et al, 1991), while in the cat the response is indirect and cholinergic (Shimura et al, 1991).

2.6.7.2. Plasma Leakage and Plasma Extravasation:

The persistence of bronchoconstriction after inhalation of SP, which can be difficult to reverse by lung hyperinflation, is primarily due to airway wall and alveolar oedema (Lotvall et al, 1990a). SP and other neurokinins and CGRP are potent vasodilators, and in addition the neurokinins cause microvascular leakage, with extravasation of protein and consequent airway wall oedema. This process is important in the aetiopathogenesis of airway and lung inflammation (Lundberg et al, 1984,1988; Rogers et al, 1988; Saria et al, 1988b; Sertl et al, 1988; Belvisi et al, 1989b), but leakage of circulating blood cells does not affect the smooth muscle and vascular effects of SP (Bethel et al, 1988). SP is the most potent peptide causing microvascular leakage (Rogers et al, 1988).

The increased vascular permeability caused by SP appears to be direct as the location of the increase in vascular permeability associated with SP coincides with the highest concentrations of SP (NK-1) receptors (Sertl et al, 1988). The order of potency for the neurokinins (SP > NKA = NKB) in causing microvascular leakage is different from that causing bronchoconstriction in the guinea pig suggesting the NK-1 receptor mediates the vascular response (Rogers et al, 1988). The vascular response to SP is not due to release of histamine from mast cells in the lung (Sertl et al, 1988).

At dose levels below those necessary to cause bronchoconstriction, neuropeptides,

including the neurokinins, VIP, CGRP and NPY, modulate tracheal vascular resistance and may be important in the physiological control of airway systemic arterial blood flow (Salonen et al, 1988).

2.6.8. Neuropeptides as neurotransmitters:

The criteria for identifying peptides as neurotransmitters have proved difficult to fulfill. Particular problems exist in determining if a peptide co-localised with another peptide or non-peptide, is a classical neurotransmitter, like acetylcholine, or a neuromodulator. Makhlouf (1988) has recently re-defined the criteria for peptide neuro-transmitters and most of these criteria have been satisfied for the neurokinins. Peptide synthesis has been demonstrated in neurons and the target tissues innervated by these neurones has been identified in most instances. Specific receptors for the neurokinins have been demonstrated in target cells tissues using pharmacology and molecular biology techniques. However, the modulatory actions of the various neuropeptides co-localised with each other, and with classical non-peptide neurotransmitters, have not been completely separated from their direct actions, and this area needs further investigation.

Fulfilling all these criteria suggest that a peptide is released from nerves and interacts with an effector organ (Dahlstrand, 1990). In this respect most criteria have been effectively satisfied and peptides, including the neurokinins, appear to behave as classical neurotransmitters, but also modulate the activity of other neural elements in the vicinity of their target sites.

2.6.9. Neuropeptides and Respiratory Disease:

The characteristic activities of exogenous SP and capsaicin in the lung have suggested a role for sensory neuropeptides in inflammatory disease, in the lung and elsewhere in the body (Figure 8). SP and the other neurokinins are believed to be the mediators of the so-called "neurogenic inflammatory response" (Barnes, 1986; Yonehara et al, 1990). The concept of neurogenic inflammation can be used to explain certain aspects of the pathophysiology of asthma and other inflammatory airway diseases, various forms of dermatitis, and immunologic and non-immunologic types of arthritis.

The released neuropeptides, as discussed earlier, would affect bronchomotor tone, increase mucus secretion, improve muco-ciliary clearance of this excess mucus and dilate the pulmonary vasculature and the micro-capillary bed resulting in microvascular leakage, plasma extravasation and oedema. In vivo sensory neuropeptides may be released from collateral sensory afferent nerve endings, as part of a local axonal reflex activated by mechanical or chemical stimulation of the airway surface (Lundberg & Saria, 1983a; Lundberg & Saria, 1983; Lundberg et al, 1984; Barnes, 1986; Joos et al, 1989). The denuding of airway surface epithelium during inflammation may expose sensory C-fibre endings to inflammatory mediators such as bradykinin and histamine resulting in reflex bronchoconstriction and the reflex release of peptides from collateral nerve axons (Saria et al, 1988a; Barnes, 1988).

It is because of the description of this elegant model of airway inflammation, and its possible role in the aetiopathogenesis of airway disease, that the expansion in research in this field has occurred in recent years. The search for a clearer understanding of

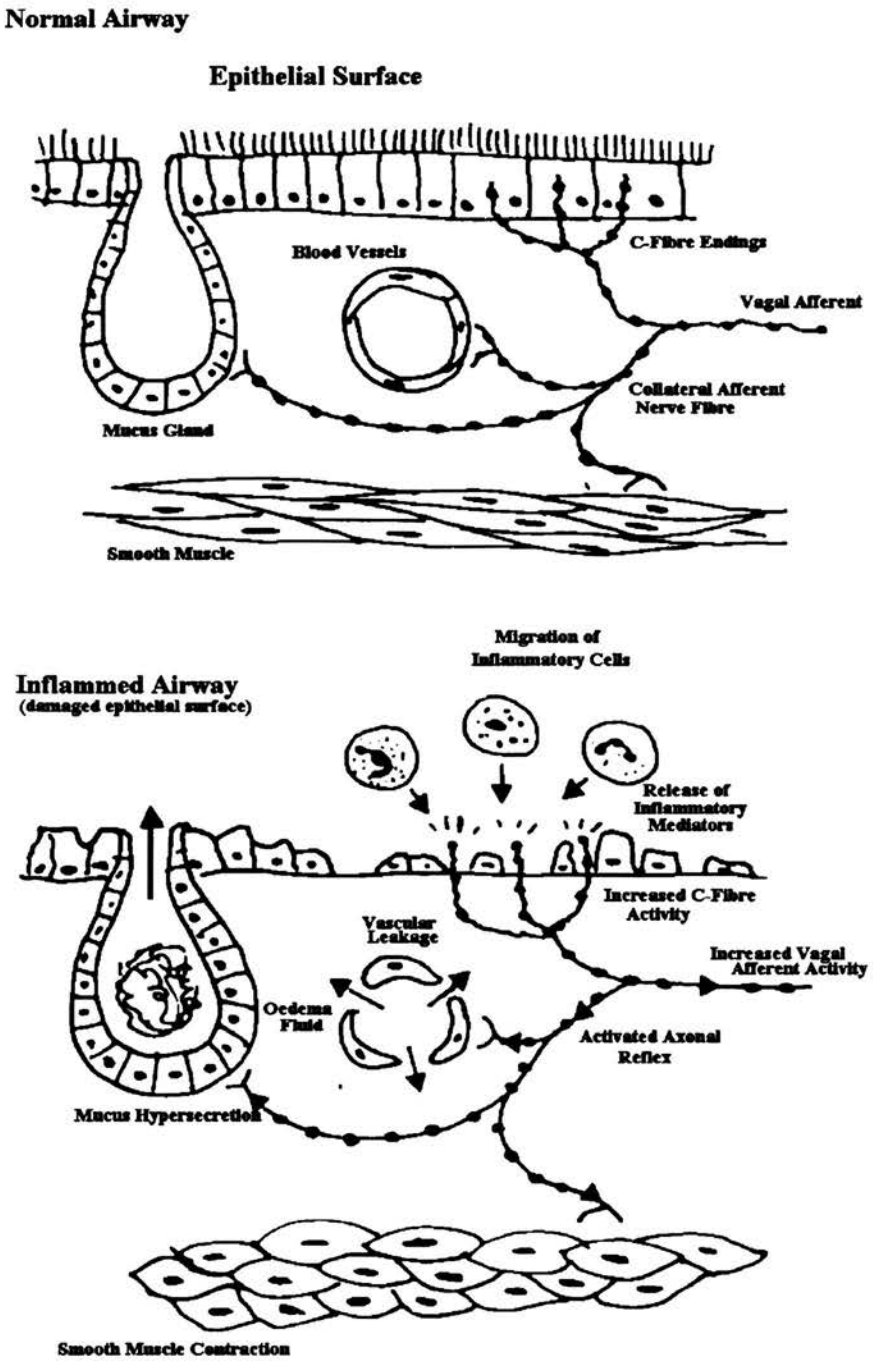


Figure 8. The theory of axon reflex mechanisms of bronchoreactive diseases, such as asthma, as outlined by Barnes (Lancet 1: 242-245, 1986). Damage to the airway epithelium results in exposure of intra-epithelial C-fibre afferent endings. These endings are activated by mediators released from inflammatory cells, resulting in increased vagal afferent activity and stimulation of collateral C-fibre axons. The latter results in localised release of neuropeptides causing contraction of smooth muscle, increased mucus secretion and vascular leakage.

"neurogenic inflammation" and its relationship to airway disease continues to inspire greater research in this area and with the developement of highly specific peptide agonists and antagonists, new directions in respiratory pharmcology are likely to occur in the near future.

CHAPTER 2. GENERAL METHODOLOGY

2.1. Cardiopulmonary Measurement in the Anaesthetised Sheep:

The general methodology for measuring cardiopulmonary parameters in normal sheep and the methodology for investigating the contractility of the isolated sheep trachealis muscle are described in this section. An additional study investigating neurokinins in sheep with an airway allergy to Ascaris suum antigen used a computerised technique for measuring respiratory parameters, and the methodology is described in detail in chapter 4. A further series of experiments investigated the effects of vagal cooling on the bronchomotor response to substance P, and the methodology is described in detail in chapter 7.

2.1.1. Animals:

Experiments were carried out in female cross-Suffolk sheep purchased from commercial sources. The sheep were aged between 4 months and approximately 4 years with a body weight range of 25 to 60 kg. The sheep were housed in purpose-built animal accomodation and maintained on ad lib hay and water. The sheep were housed for at least 7 days prior to experiments.

2.1.2. Anaesthesia:

The sheep were restrained in lateral recumbency and anaesthetised with pentobarbitone (Sagatal, Rhone Merieux Ltd, UK) 25 mg/Kg body weight, administered intravenously through the right tarsal vein. An in-dwelling teflon over-the-needle catheter (Jelco 20g, Critikon Inc, USA) was placed in the right cephalic vein and secured in place and anaesthesia was maintained with a constant infusion of 0.54% pentobarbitone in sterile 0.15M NaCl (Baxter Ltd, UK) adjusted to deliver 2

mg/Kg/h of pentobarbitone. The sheep were intubated with a 10 mm cuffed endotracheal tube (Trimline 10.0 Oral, Leyland, UK) which was securely tied in place, and put in the prone position with the head supported in a sling (Figure 9). Anaesthetic depth was assessed using standard ocular anaesthesia criteria, including palpebral and corneal reflexes, pupil size and the deviation of the eye from the central position (Hossain et al, 1988). Attempts were made to maintain animals between light and deep planes of anaesthesia, with weak palpebral and corneal reflexes, partially dilated pupils and the eyes fixed centrally. However, prolonged anaesthesia was found to cause several problems with the preparation, particularily with the breathing pattern and the appearance of rhythmic apneoa-tachypnoea episodes. Attempts were made to avoid such problems, by limiting the duration of experimental procedures.

2.1.3. Respiratory Measurements:

2.1.3.1. Measurement Equipment:

Respiratory parameters, including flow (V), tidal volume (VT), transpulmonary pressure (P), pulmonary resistance (RL) and dynamic compliance (Cdyn) were measured using a standard combined pneumotachograph and oesophageal balloon technique. Transpulmonary pressure was derived from the pressure difference between the oesophagus (representing pleural pressure) and the airway opening. An oesophageal balloon was constructed from a condom sealed over the distal 10cm of a section of rigid polyethylene tubing (internal diameter 4mm) which had a series of 15-20 spirally arranged holes cut in it (Figure 10). The balloon catheter was positioned in the caudal third of the oesophagus (Figure 1). Position was confirmed by first passing the balloon into the rumen, where a positive pressure deflection is found on inspiration, and then slowly withdrawing the balloon until a pressure signal of opposite sign was obtained. The balloon catheter was then taped to the endotracheal tube to prevent movement. A similar section of tubing was placed at the

Figure 9. The anaesthetised sheep preparation for the measurement of pulmonary mechanics. The sheep is positioned in sternal recumbency with the head supported in a sling. A-endotracheal tube; B-airway catheter; C-pneumotachograph; D-oesophageal balloon catheter; E-intravenous indwelling catheter and anaesthetic constant infusion line.

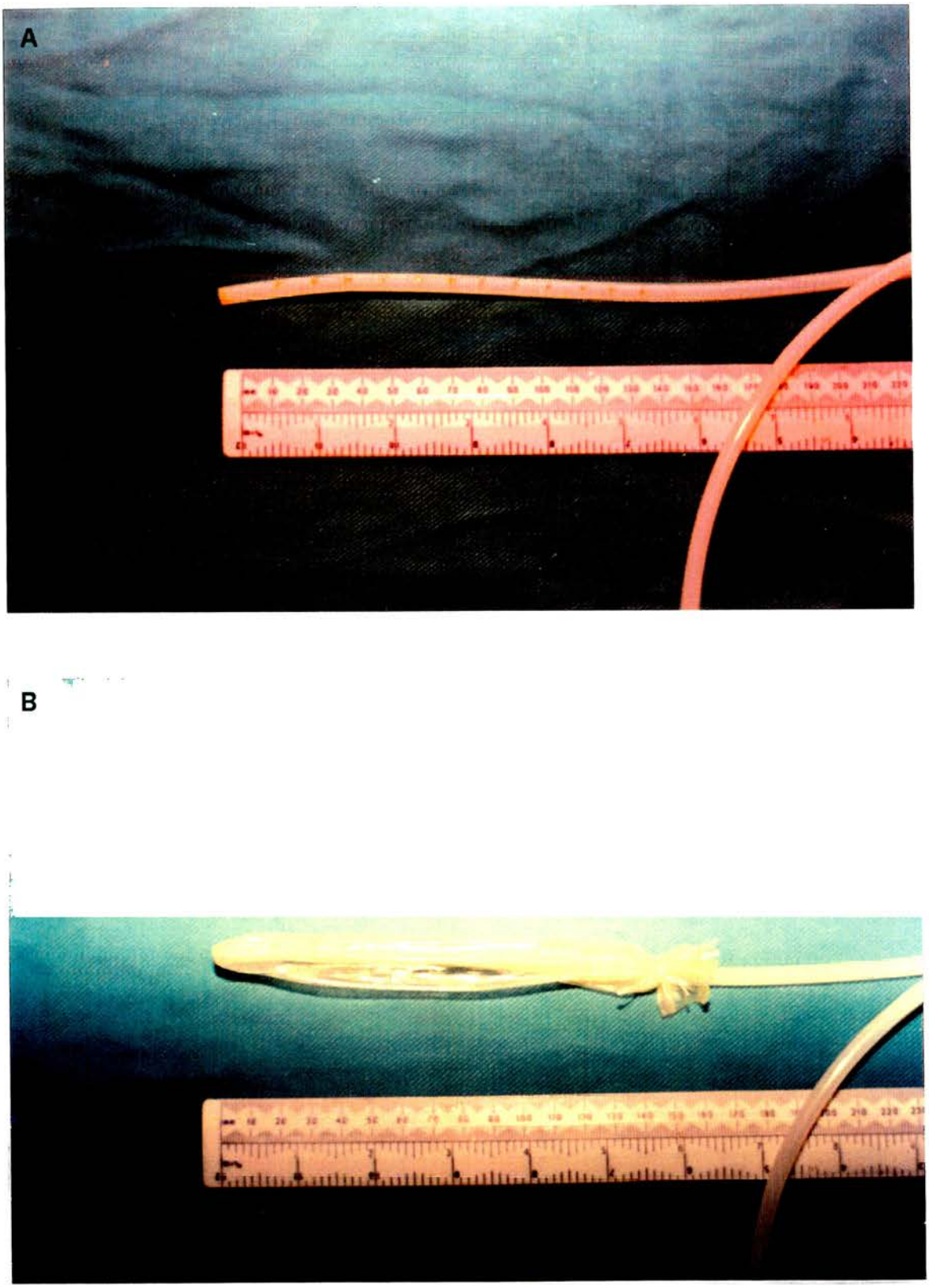


Figure 10. The oesophageal balloon catheter system. The catheter (A) consists of a length of semi-rigid polyethylene tubing with an internal diameter of mm. The distal 10 cm of the tube has 15-20 spirally arranged holes. The balloon is a latex rubber condom and is sealed over the end of the tubing (B).

entrance to the endotracheal tube through the side port of a three-way connector. Both catheters were attached to the input ports of a differential pressure transducer (pressure range; +/-12 cmH_2O (UP2, Pioden Controls, Canterbury, UK).

Airflow at the endotracheal tube opening was measured using a pneumotachograph flow head, with a flow range of 0-100 litres/min (F100L, Mercury Electronics, Glasgow, UK), connected to a second differential presure transducer (pressure range; +/-4 cmH2O). The pneumotachograph flow head was not heated.

Signals from both pressure and flow transducers were amplified (3552 Pressure Pre-amplifier, Devices, UK) and the flow signal was integrated (3655/3630, Devices, UK) to give tidal volume measurements. Outputs were than recorded on heat sensitive paper (MX19 Physiological Recording System, Devices, UK).

2.1.3.2. Equipment Calibration:

Pressure, flow and tidal volume measurements were calibrated before the start of each experiment. There was good day-to-day consistency in the measurement of parameters and only minor adjustments had to be made. The pressure transducer was calibrated with a water manometer constructed in the Faculty of Veterinary Medicine Engineering Workshop, and expressed in units of cmH_2O. The pneumotachograph head was calibrated with a standard temperature and pressure flow meter (SCR2, Glass Precision Engineering Ltd, UK), calibrated in litres/min, through which air was driven with a pump (Cape X Mk 1B, Frank Austen Ltd, UK). The volume calculated from the integrated flow output from the pneumotachograph was calibrated with a large volume syringe (SC1, Mercury Electronics Ltd, UK).

To enable respiratory resistance (RL) and dynamic compliance (Cdyn) to be calculated from pressure, flow and volume measurements, outputs from both

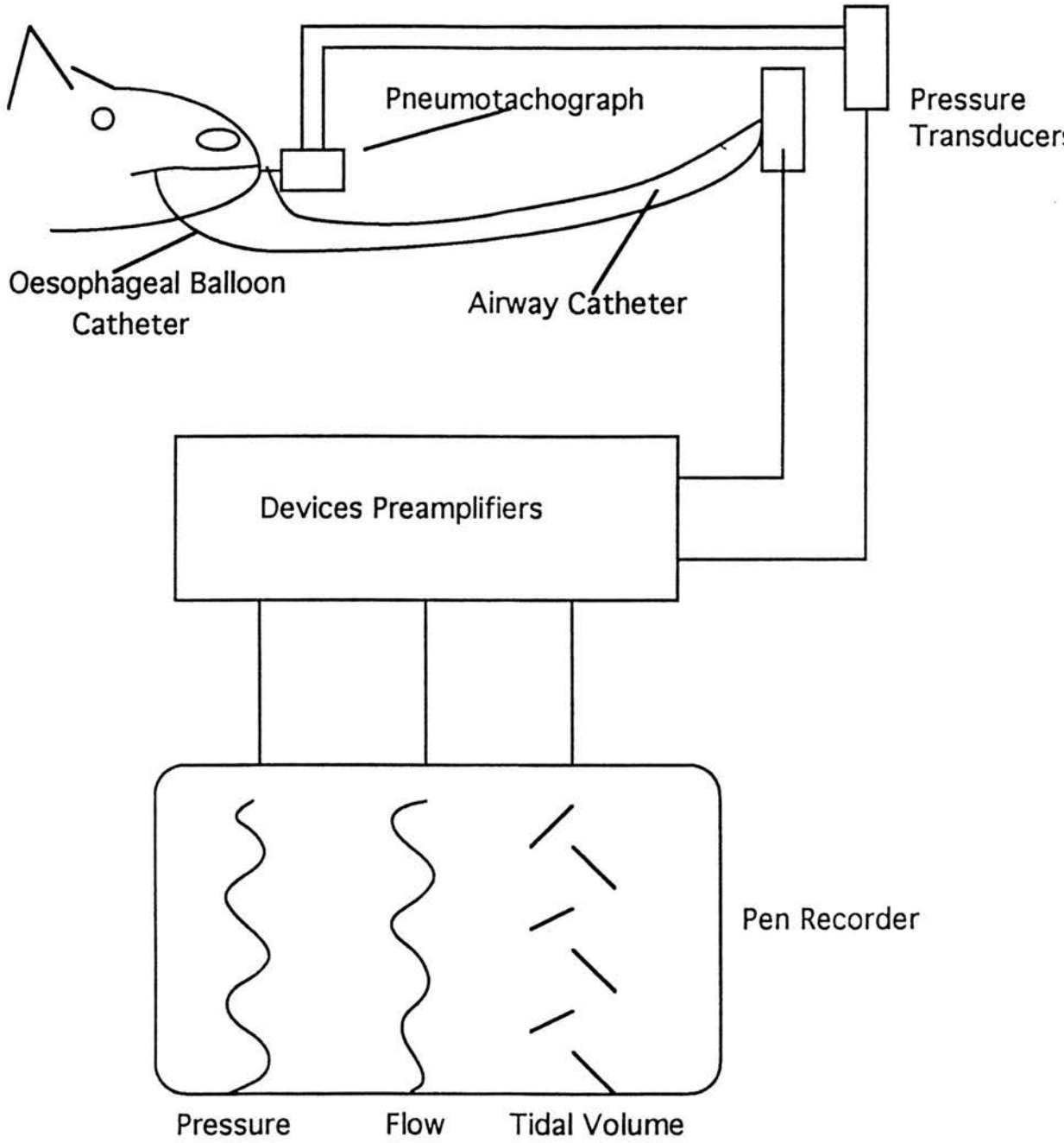


Figure 11. Diagramatic illustration of the method for measuring respiratory parameters in anaesthetised sheep. Oesophageal balloon, airway and pneumotachograph pressures were measured with two matched differential pressure transducers. The transducer outputs were amplified using a series of Devices preamplifiers, and the flow signal was integrated to give tidal volume. Flow, tidal volume and transpulmonary pressure were recorded on heat sensitive paper, from which pulmonary reistance and dynamic compliance were calculated graphically.

transducers were phase matched up to 6Hz. Phase matching was carried out according to the method of Macklem (1975), using a sealed plastic container, a sinusoidal pump delivering a volume of 14ml/cycle and a two channel storage oscilloscope (5111, Tektronix Inc, USA) fitted with a dual trace amplifier (5A18N, Tecktronix Inc, USA) and time base amplifier (5B10N,Tecktronix Inc, USA) (Figure 12). The sinusoidal pump was constructed in the Faculty of Veterinary Medicine Engineering Workshop.

The two ports of the pneumotachograph and the oesophageal balloon and airway catheter were separately phase matched. With the pneumotachograph closed at one end, a sinusoidal pressure signal, generated by volume displacement with the sinusoidal pump, was introduced into the other opening. The output from the pneumotachographs transducer was passed through one channel of the oscilloscope. With equally balanced ports and no phase shift in the signals, no flow signal should be present. Lengthening the tube to the leading port or narrowing the tube using a clamp would adjust its time constant bringing the ports back into phase. With the F100L pneumotachograph phase matching was achieved simply by using equal lengths of the same tubing connected to both ports. Using similar methods the oesophageal balloon and airway catheter were also phase matched.

The output signals from the pneumotachograph and oesophageal balloon catheter were then phase matched. With one side of the pneumotachograph sealed, the other side and the balloon catheter was exposed to a sinusoidal pressure wave in a sealed container. Transducer output from the pneumotachograph and the oesophageal balloon were displayed on two channels of the oscilloscope. Phase matching is indicated by a linear relationship, which does not change with frequency while phase shift is represented by an open loop (Lissajous Pattern) (Figure 13). Narrowing the tubing from the leading system again corrects the phase shift and closes the loop on the oscilloscope. By using transducers of the same manufacture (UP Type, Pioden

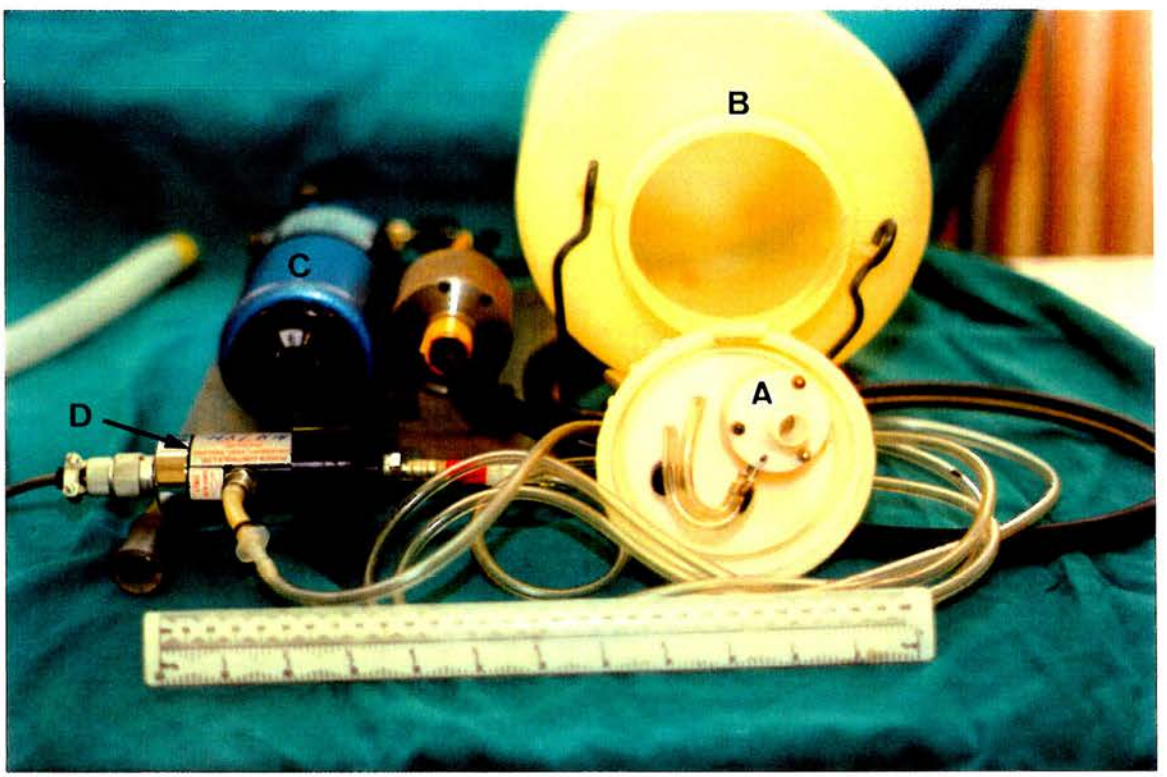
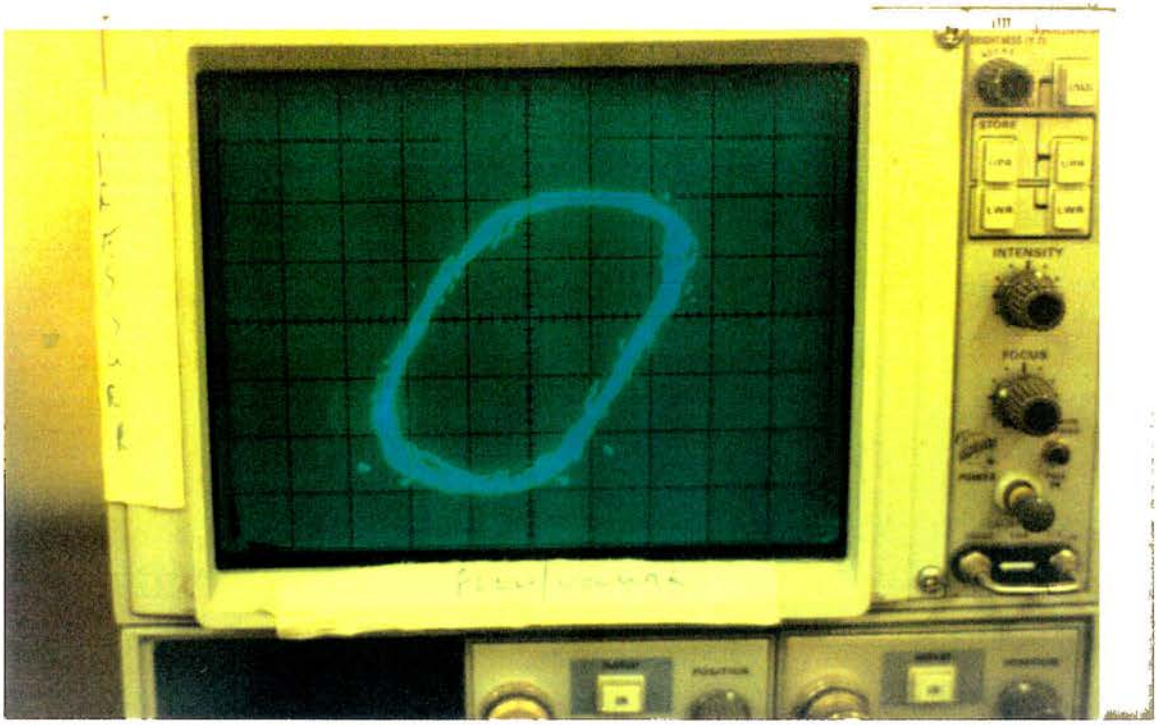


Figure 12. Equipment required for phase matching. The output signals of the differential pressure transducers are phase matched by exposing the measuring equipment to a sinusoidal pressure wave form. The two ports of the pneumotachograph are balanced first, followed by the oesophageal and airway catheters. The pneumotachograph and the pressure catheters are then phase matched with each other to allow measurement of dynamic compliance and pulmonary resistance.
A-pneumotachograph with one port exposed to the sinusoidal pressure wave; B-pressure container; C-sinusoidal pump; D-UP type differential pressure transducer.

A



B

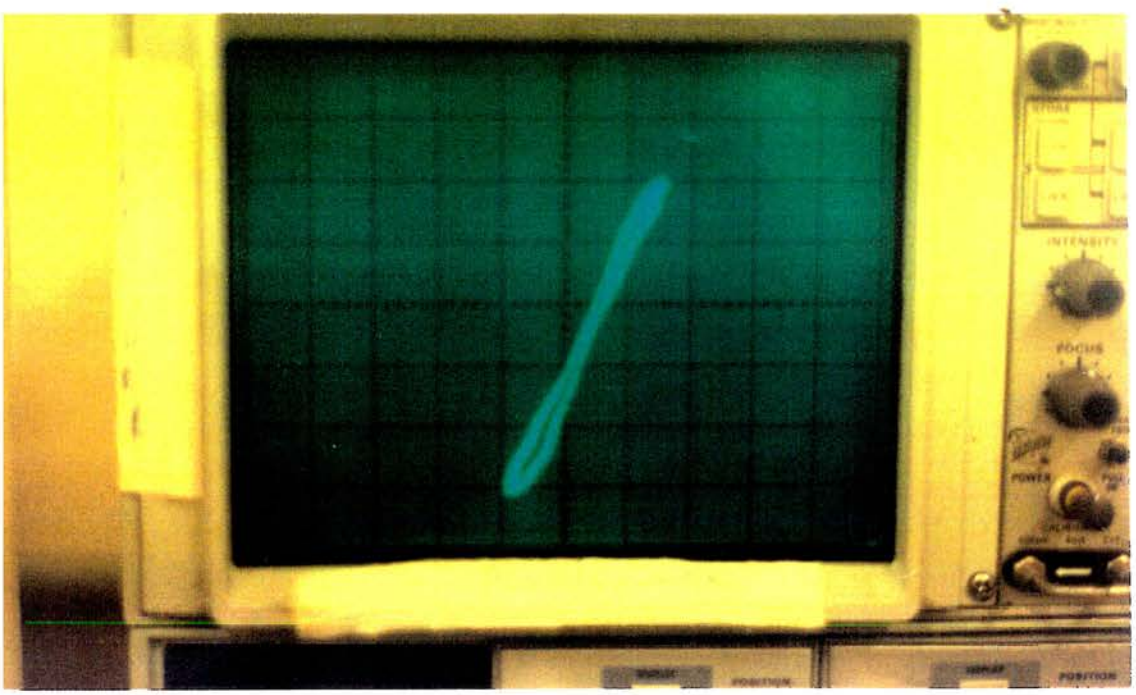


Figure 13. Phase matching and Lissajous patterns. Oscilloscope tracings of the Lissajous patterns obtained from the phase matching of the pneumotachograph and oesophageal balloon catheter. A closed pattern indicates the signals are in phase and can be achieved by changing the diameter or lengthening the tubing to the differential pressure transducer The Lissajous patterns are displayed on a two channel storage oscilloscope. A-open Lissajous pattern when the two pieces of equipment are out of phase. B-closure of the Lissajous pattern after altering the time constant of the leading equipment.

Controls Ltd, UK) and tubing of similar length and internal diameter phase matching of the two systems was achieved. The phase match of the systems were periodically checked over the duration of the project and adjusted if necessary.

2.1.3.3. Respiratory Mechanics:

There are three mechanical properties of the respiratory system that govern the flow of gas in and out of the system in response to a change in pressure (Young, 1990). The pressure required to overcome the resistance of the respiratory system to airflow consists of the flow resistive pressure, the pressure to overcome elastance and the inertial pressure (Figure 14). The total resistance to flow is therfore a function of the airway resistance, the tissue compliance (reciprocal of elastance) and the tissue inertance.

$$P = P_{el} + P_{rs} + P_{in}$$

Figure 14. Airflow pressure formula. The flow of gas in and out of the respiratory system is governed by the pressure drop within the system, which is a function of the forces opposing flow. These forces include the elastic (el), resistive (rs) and inertial forces (in) of the system, and the total pressure required to overcome the resistance to airflow is therefore the sum of the pressure components of these three factors.

The summed elastic and inertial pressure is called the reactance (Rodarte & Rehder, 1986). The inertance is the force opposing flow acceleration, and during breathing at normal frequencies is negligible and can be ignored. However, as the respiratory frequency increases the pressure loss due to inertance increases, decreasing the reactance and hence the value of compliance that can be derived from the elastic pressure. At respiratory rates above 1 Hz inertance becomes significant, but only at

resonant frequency (typically 2-6Hz for larger mammals) does the inertial and elastic pressures cancel each other making measurement of compliance impossible.

The resistance is primarily a function of the frictional forces between the airway walls and the gas moving in the airways, although tissue resistance is also present (DuBois, 1966) and the resistive pressure is in phase with flow (Rodarte & Rehder, 1986). Resistance can be calculated by a variety of means (see later), but in general is taken as the ratio between pressure and flow at points of isovolume when the elastic pressure is zero. The compliance is a measurement of the elasticity or rigidity of airway and lung tissue and the elastic pressure component is in phase with volume (Rodarte & Rehder, 1986; Young, 1990). At points of zero flow, flow-resistive pressure is zero and so pressure is a combination of elastic and inertial pressure. Consequently, compliance is measured when there is no gas flow in the airways. The static value of compliance is a measure of the pressure required to maintain a volume of air in the lungs and can be affected by non-airway factors such as pulmonary vascular volume and resistance and abdominal compression of the thoracic diaphragm. Dynamic compliance is the relationship of the change in volume and pressure between points of zero flow (Mead & Whittenberger, 1953) and during steady breathing can be taken as an indicator of resistance to airflow and patency in the more peripheral airways (Figure 15).

$$P = (1/C)V + RV' + IV''$$

Figure 15. Formula illustrating the relationship between pressure (P), compliance (C), resistance (R) and inertance (I). The reciprocal of compliance (1/C) is elastance and relates pressure to volume, while resistance and inertance relate pressure to flow (V') and flow acceleration (V'') respectively.

While the inertia of the respiratory system is negligible during quiet breathing (Figure 16), the introduction of an endotracheal tube into the airway can affect measured values of resistance and compliance (Sullivan et al, 1976) (Figure 17). The larger the tube caliber the lower the linear and non-linear resistance and hence the lower the error.

$$X = j(wI - 1/wC) \quad (1)$$

$$wI = 1/wC \quad (2)$$

Figure 16. Formula for the reactance (X) of the respiratory system is shown (1) and is defined as the sum of the compliance and inertance, which together are often referred to as the imaginary part of the impedance (Z). j is a complex number ($J^2 = -1$) and w is 2 pi times the respiratory frequency. The magnitude of I and C will be determined by the respiratory frequency and as the frequency increases the importance of I in determining the magnitude of X increases. However, at a respiratory frequency defined by formula (2) the inertial and elastic pressure cancel and the reactance is zero. At this resonant frequency total pressure is equal to flow resistive pressure.

The introduction of an endotracheal tube has certain advantages, not least maintaining airway patency if emergency resusitation is required during an experiment. With respect to measurement of airway mechanics an endotracheal tube eliminates the highly variable upper airway resistance component of total resistance, allowing closer examination of the intrathoracic airways. The upper airways contribution, including the mouth, glottis and extra-thoracic trachea, accounts for a significant component of the pressure loss associated with flow (Ingram & Pedley, 1986) and as the glottis is the major source of upper airway resistance and changes in glottic patency the major source of airway resistance variability, it is sensible to by-pass this area (Higenbottom, 1980).

$$I = \rho L/\pi r^2$$

Figure 17. The inertial coefficent for an endotracheal tube is shown. I = inertia; L = endotracheal tube length; ρ = mass density. Lengthening the tube increases inertia, while widening decreases inertia.

Change in airflow resistance can alter the pressure-flow characteristics of the respiratory system. An increase in transpulmonary pressure invariably denotes a greater increase in expiratory resistance than inspiratory resistance (Campbell, 1958). During quiet breathing the inspiratory resistance can be 15-20% less than the expiratory resistance (Ferris et al, 1964). This is due to recruitment of the respiratory muscle to overcome air-trapping caused by premature closing of the central airways during early expiration. With very large increases in transpulmonary pressure expiratory flow may cease altogether (Figure 18) effectively giving infinite resistance, while there may only be a marginal change in inspiratory resistance.

2.1.3.4. Oesophageal Pressure Measurement:

The most commonly used method for measuring pleural surface pressure is by estimation from oesophageal pressure using an oesophageal balloon, constructed from a latex membrane sealed over a length of tubing, connected to a pressure transducer (Milic-Emili, 1984). Changes in intrathoracic pressure cause similar changes in the oesophagus (Milic-Emili & Petit, 1959) (Figure 19). The pressure recorded by the oesophageal balloon tends to be more positive than true pleural pressure and any difference in the absolute pressure between the oesophageal balloon and the pleural space is due to oesophageal wall elastance and the volume of air in the oesophageal

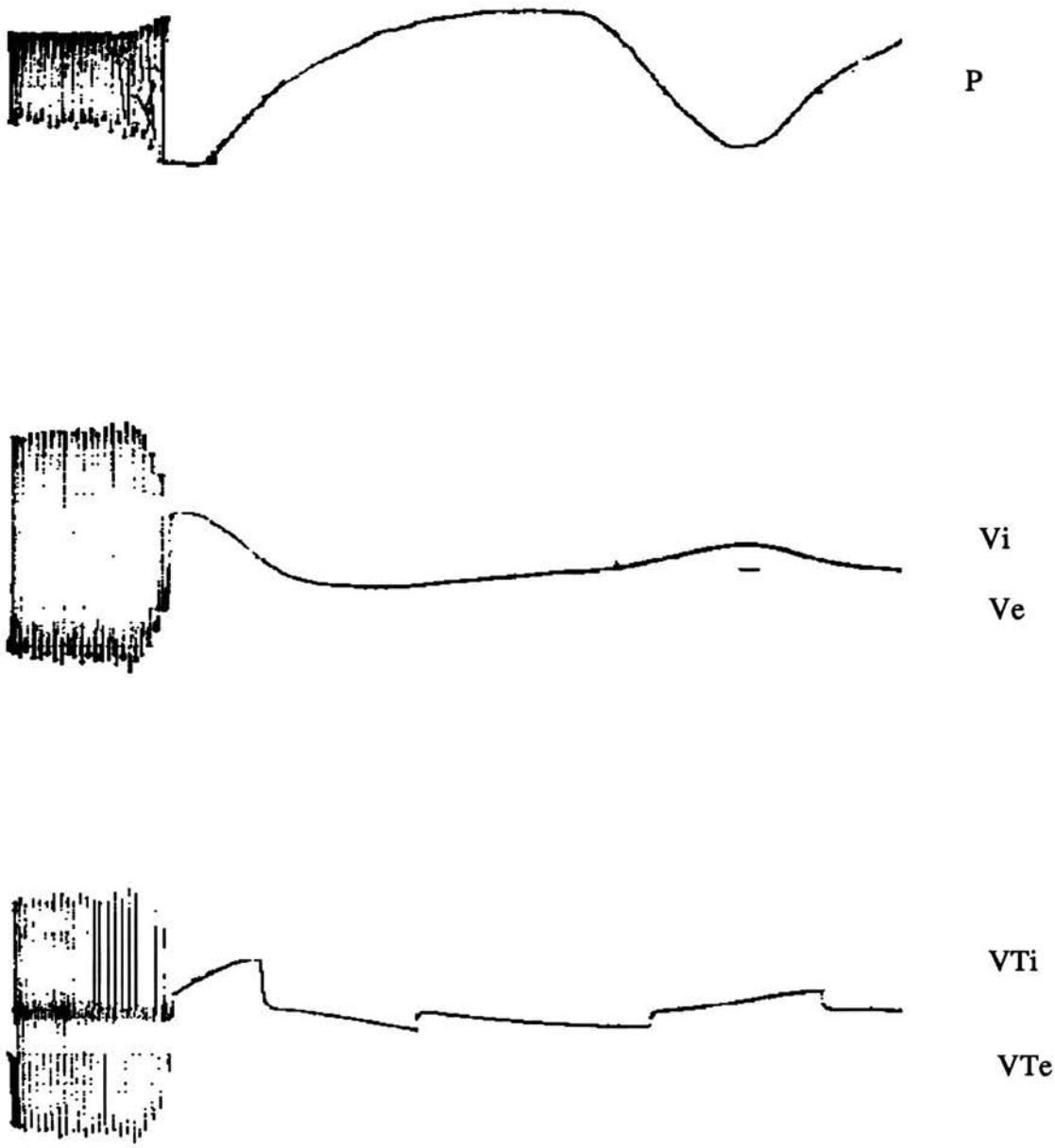


Figure 18. Respiratory tracing from a normal sheep after intravenous administration of substance P. This tracing illustrates the different effects of profound bronchoconstriction on inspiratory and expiratory respiratory mechanics. While there is a marked reduction in inspiratory flow (Vi) and tidal volume (VTi) the effect is greater on expiratory flow (Ve) and volume (VTe) such that there is effective cessation of expiratory airflow. With the large increase in transpulmonary pressure (P) the calculation of expiratory dynamic compliance and resistance is difficult, with values approaching zero and infinity respectively.

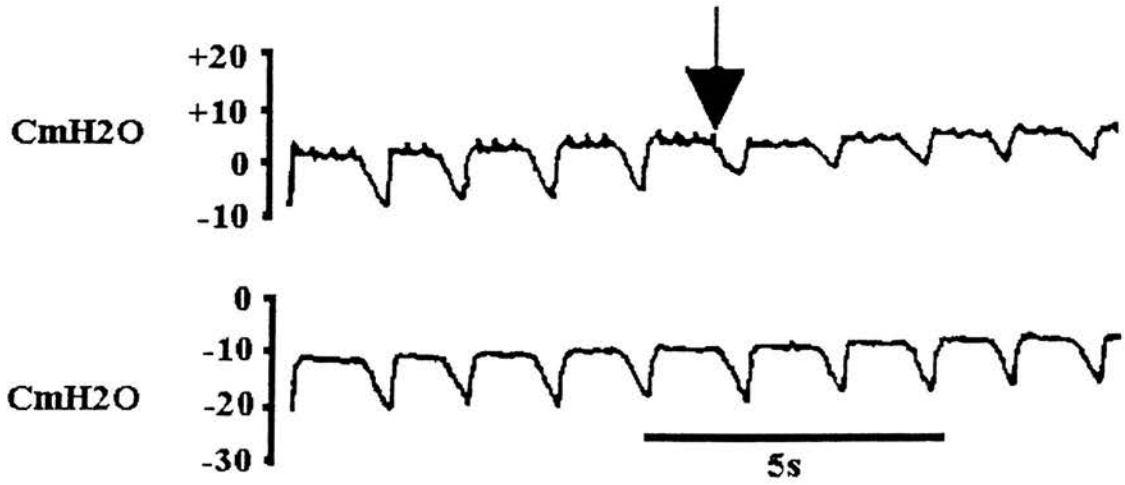


Figure 19. Simultaneous tracings of oesophageal (upper) and pleural (lower) pressure in an anaesthetised dog. It can be seen that the oesophageal pressure is more positive than pleural pressure, but adequately parallels the changes in pleural pressure. When the volume/pressure characteristics of the oesophageal balloon catheter system are changed, there is an alteration in the magnitude of the oesophageal pressure measured. This change is caused by altering the volume of air in the balloon, which not only changes the volume variation/pressure variation of the measuring equipment but also the oesophageal wall elastance. Maintaining balloon volume at 0.1-0.15 ml/kg body weight reduces this source of error. (Modified from Milic-Emili & Petit, 1959).

balloon catheter system (Milic-Emili & Petit, 1959). Furthermore, there seem to be individual variations in the manner in which oesophageal pressure reflects pleural pressure and the ability of the oesophageal pressure change to parallel the change in pleural presure (Cherniack et al, 1955). In order to transmit pressure to the transducer the balloon must contain air, but this should be limited to a volume that does not stretch the balloon membrane. Balloon volumes close to 0.1 ml/kg body weight do not affect oesophageal elastance and are ideal (Petit & Milic-Emili, 1958). Excessive volumes will affect oesophageal wall mechanics which will further affect balloon pressure. (Petit & Milic-Emili, 1958).

While the oesophageal balloon technique may be valid there are potential problems in measuring oesophageal pressure in sheep. The ruminant oesophagus is a very distensible and thin walled structure and contains striated muscle along its entire length (Dyce et al, 1987). Its distensibility is necessary to allow eructation and regurgitation of stomach gas and food contents. The oesophagus obtains its motor innervation from the vagus. The pharyngeal branch of the vagus continues as the oesophageal nerve and with the recurrent laryngeal nerve primarily innervates the cranial portion of the oesophagus. The caudal oesophagus is innervated by the dorsal oesophageal branch of the vagus nerve.

The inherent elastance of the oesophagus has been shown to affect the pressure measured in dogs (Cherniack et al, 1955) and in sheep contraction of the caudal thoracic oesophagus has been demonstrated in both light and deep planes of anaesthesia (Hossain et al, 1988). Gastro-oesophageal reflux can also occur during anaesthesia. Furthermore, there is a high pressure zone close to the gastro-oesophageal junction in sheep oesophagus, and while the pressure in this zone is not significantly affected by anaesthesia, in deep anaesthesia there can be fluctuations in the basal tone in this region (Hossain et al, 1988). Consequently there can be

alterations in the oesophageal pressure measurement at any time in anaesthetised sheep, without a change in tidal volume or respiratory flow, resulting in marked variation in the values of RL and Cdyn obtained (Figure 20). To overcome this problem it is necessary to measure the bronchomotor response to exogenous agents in terms of the percentage change in RL and Cdyn compared to the values immediately prior to administration, rather than absolute values.

With respect to investigating the bronchomotor effects of neurokinins in anaesthetised sheep, the potential effect of the neurokinins on oesophageal tone need to be considered. Substance P immunoreactive nerve fibres have been identified in the oesophagus of several species and exogenously administered SP contracts oesophageal smooth muscle and potentiates the response to electrical field stimulation (Leander et al, 1982). Furthermore, both VIP and CGRP have been shown to relax the lower oesophageal sphincter (Rattan et al, 1988; Yamashita et al, 1992). Therefore, using oesophageal pressure measurement as an indicator of pleural pressure may be complicated when administering tachykinins. This particular problem was noted in several animals in this study, where there appeared to be relaxation of the distal portion of the oesophagus with gastro-oesophageal reflux of stomach contents (Figure 21). With right cephalic vein administration of SP oesophageal relaxation tended to occur after peak changes in resistance had occured and so did not adversely affect measurements. However, with administration into the left side of the heart oesophageal effects occured more rapidly. This will be discussed in more detail in chapter 3.

Position of the animal is important. In man the pressure measured by an oesophageal balloon is elevated in the supine position, due to compression of the oesophagus by mediastinal contents, and a better estimate of pleural pressure is achieved in the sitting or prone positions (Ferris et al, 1959; Knowles et al, 1959). In the present study

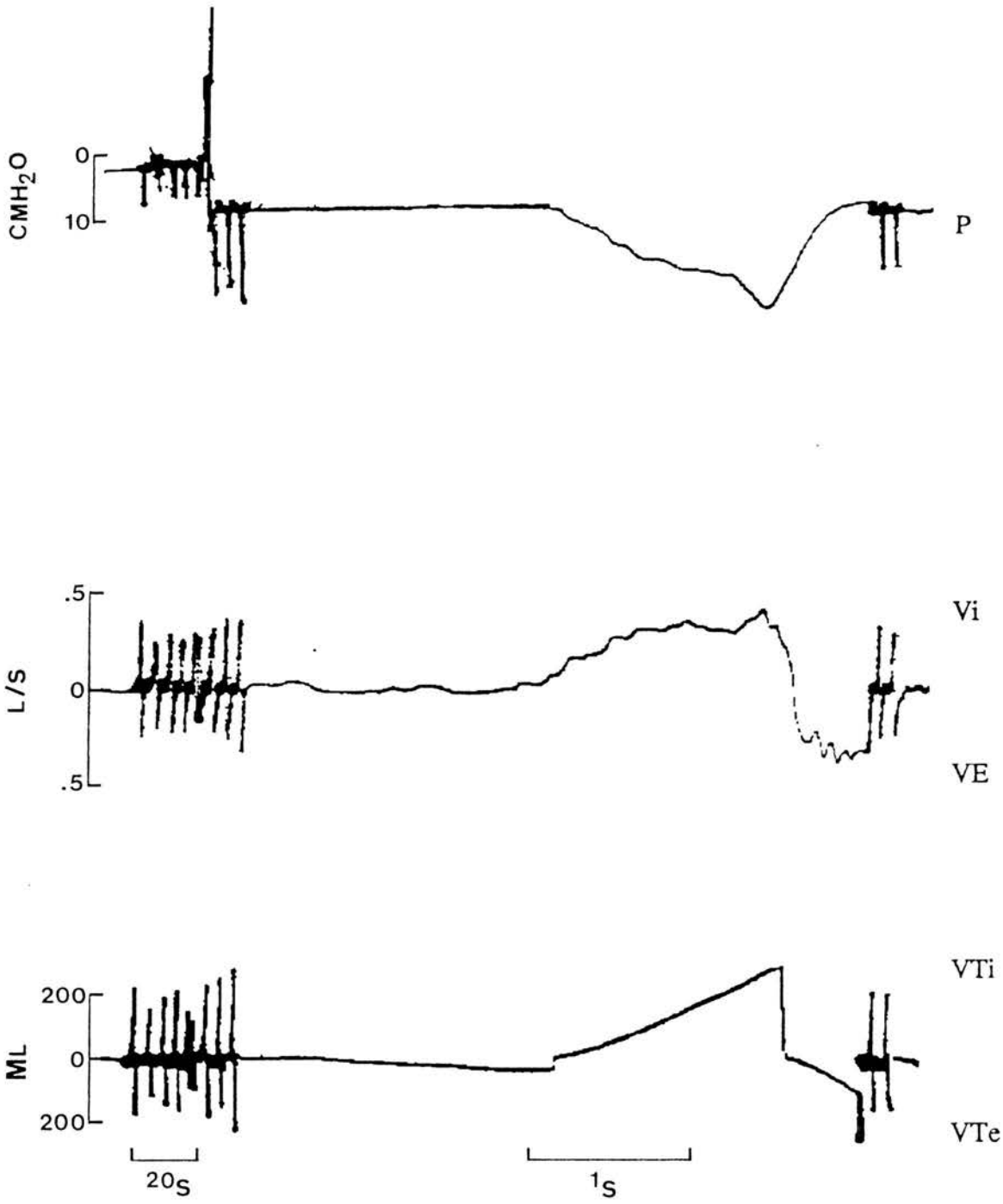


Figure 20. Spontaneous alteration in transpulmonary pressure (P) recorded with an oesophageal balloon in an anaesthetised sheep. There is a large negative pressure spike at the beginning of the tracing associated with gastro-oesophageal reflux and a subsequent change in the pressure baseline and an increase in the pressure recorded. There are minimal changes in inspiratory and expiratory flow and volume values (Vi, Ve, VTi and VTe respectively). The animal resumed steady respiration shortly after this episode, but the pressure did not return to the original baseline.

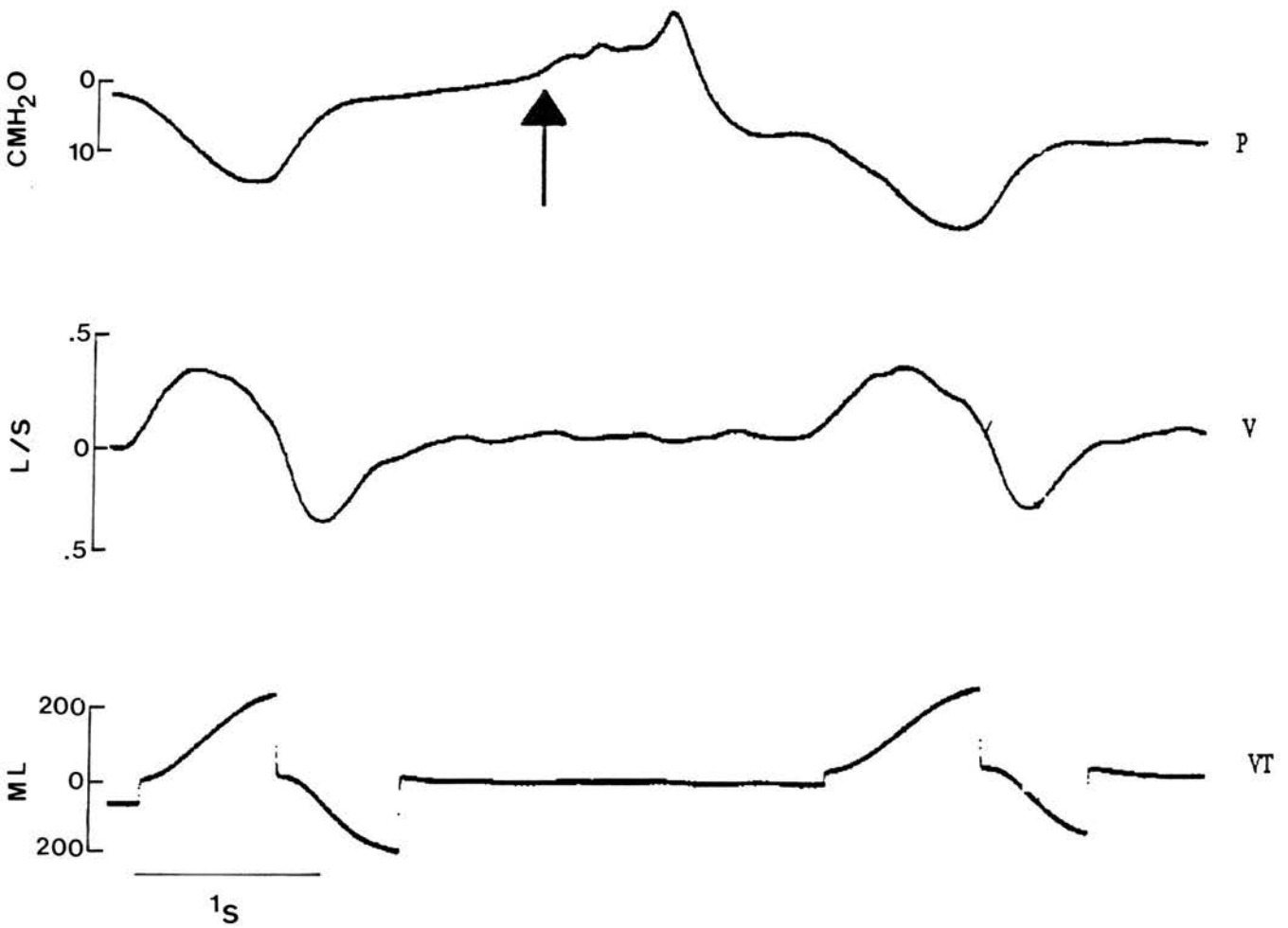


Figure 21. Neurokinin B (NKB) and oesophageal relaxation. This tracing demonstrates the effect of neurokinin B (NKB) on respiratory parameters in anaesthetised sheep. Three breaths are shown. At the beginning of the middle breath (arrow) there is a deflection in the transpulmonary pressure tracing (P) and movement of the pressure baseline consistent with oesophageal relaxation and/or opening of the gastro-oesophageal junction. The following breath had the lowest flow (V) and tidal volume (VT) of this run and was probably the breath at which maximal bronchoconstriction had occured. This NKB effect on oesophageal pressure was reasonably consistent in the four sheep examined and interfered with the accurate assessment of the bronchomotor effects of NKB.

sheep were placed in the prone (sternal) position during measurement of oesophageal pressure and it was noted that lateral recumbency elevated the oesophageal pressure recorded. Positioning of the oesophageal balloon is also important. In the middle and caudal parts of the oesophagus the difference between oesophageal pressue and mouth pressure accurately reflects transpulmonary pressure, but this is not the case if the oesophageal catheter is positioned more cranially (Mead & Milic-Emili, 1964).

2.1.3.5. Respiratory Parameters:

All inspiratory and expiratory respiratory parameters, including peak flow (V; l/s), peak transpulmonary pressure (P; cmH2O), tidal volume (VT; ml), pressure at zero flow and flow at mid inspiratory and mid expiratory tidal points (VTi & VTe) and flow at isovolume points, and the derived parameters pulmonary resistance (RL; cmH2O/l/s) and dynamic compliance (Cdyn; ml/cmH2O), and respiratory rate were measured graphically.

There were several problems with the tidal volume measurements due to technical limitations of the MX19 Physiological Recording System and its ability to integrate flow. The integration hardware of the MX19 involves a limit switch and an integrator. The limit switch operates a relay at pre-selected points of the incoming waveform (eg flow) to pass required segments of the waveform to the integrator. Using the zero limit mode of the limit switch the integrator will integrate the inspiratory flow curve (VI), calculating inspiratory tidal volume, and re-zero the pen as flow approachs zero ready to integrate the expiratory part of the flow curve (VE). However, during quiet breathing, the rate of change of flow with time is greater for inspiration than expiration, such that a greater part of the VE curve is close to the flow rate immediately preceding the end-flow point. The integrator has difficulty in detecting this "low" flow and fails to adequately integrate the entire flow curve. Consequently the inspiratory volume is greater than the expiratory volume. When the rate of change

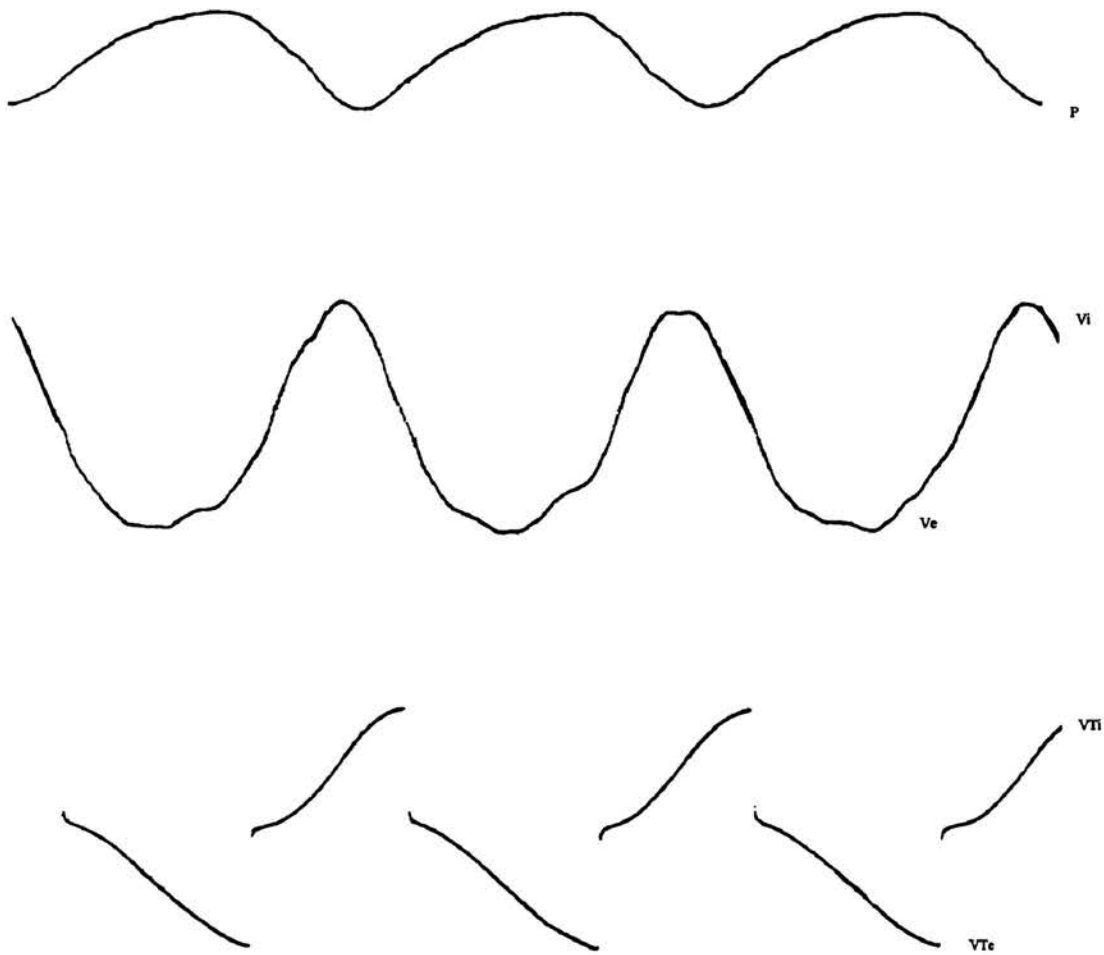


Figure 22. The ability of the Devices integrator to accurately measure the expiratory tidal volume during rapid breathing is shown. When there is no end-expiratory pause and end expiratory flow is high the integrator successfully calculates expiratory volume close to inspiratory volume. P - transpulmonary pressure; Vi and Ve - inspiratory and expiratory flow; VTi and VTe - inspiratory and expiratory tidal volume. The respiratory rate in this tracing was approximately 60 breaths per minute.

of flow is similar for both inspiratory and expiratory curves, such as in tachypnoea when there is no end-expiratory pause, the inspiratory and expiratory volumes are similar (Figure 22). To overcome this problem both the inspiratory and expiratory tidal volume were measured separately and averaged for the entire respiratory cycle.

Cdyn was calculated by dividing the pressure difference at points of zero flow (end-inspiratory and end-expiratory flow points) by tidal volume (Figure 23). Compliance was calculated for the inspiratory and expiratory parts of the respiratory cycle and then averaged to give a Cdyn value for the entire respiratory cycle.

Respiratory resistance was calculated using two methods (Frank, Mead & Ferris, 1957; Wanner & Reinhart, 1978). Using an isovolume method the pressure difference associated with the mid tidal (50%) point for VTi and VTe was divided by the associated difference in flow between these isovolume points (RLiso) (Figure 24). With this method the pressure difference is the flow resistive pressure. In the second method the resistance for inspiration and expiration were calculated separately and averaged to give a resistance value for the entire respiratory cycle. The mid-VTi and VTe points were identified and the corresponding pressure and flow noted. The pressure signal was corrected for that part of pressure due to tissue elasticity by subtracting the pressure associated with compliance (pressure difference at points of zero flow) (Figure 25). The RL value for inspiration and expiration were averaged to give a RL value for the entire respiratory cycle.

The percentage change in pulmonary resistance, calculated by both methods, to intravenous substance P (SP) was assessed by linear regression. For 15 separate measurements there was a close positive correlation between RL and RLiso with an r value of 0.662 ($p<0.01$) and a slope of 0.95 (Figure 26). However, the RL values were more consistent than the RLiso values, with less variance, and had less tendency to

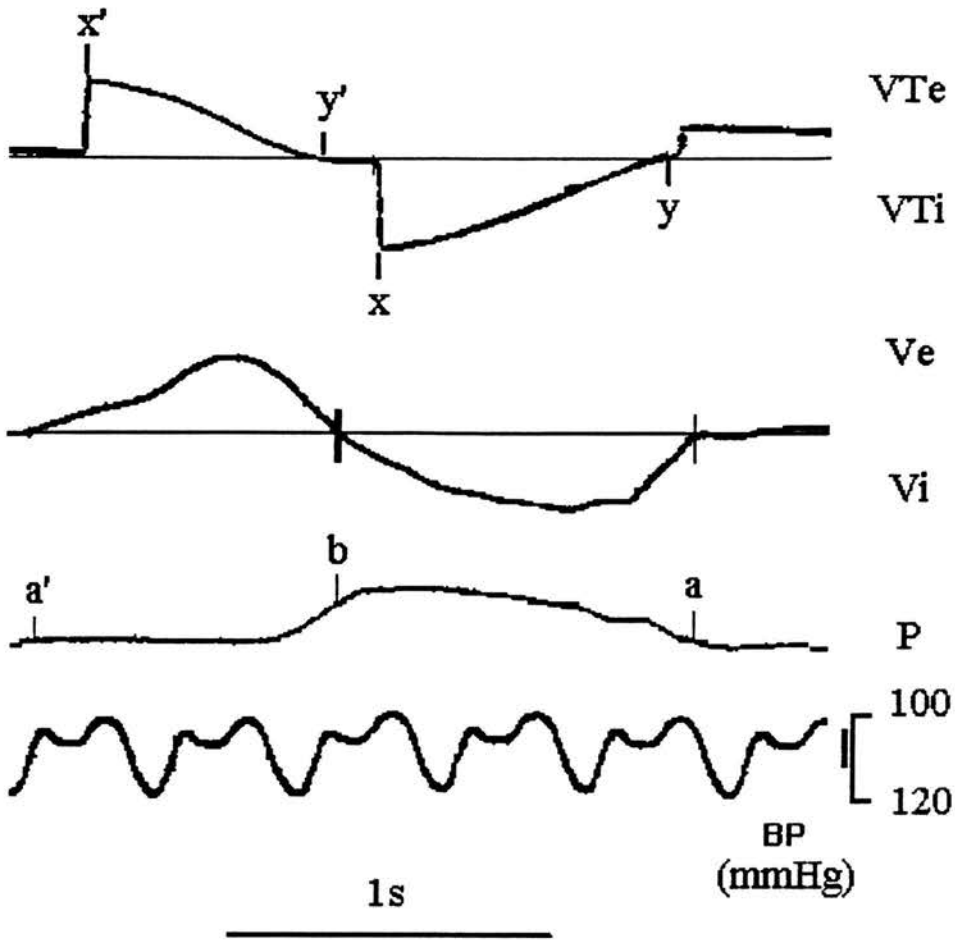


Figure 23. Calculation of dynamic compliance (Cdyn) (ml/cmH_2O). Cdyn is calculated by identifying the points of zero flow and dividing the tidal volume by the difference in transpulmonary pressure at those zero flow points. Cdyn is conventionally calculated for the inspiratory part of the respiratory cycle, but can also be estimated for the expiratory part. Inspiratory Cdyn (x-y)/(b-a); Expiratory Cdyn (x'-y')/(b-a'). An "average" compliance can then be derived for the respiratory cycle. The tracings in this figure and figures 24 and 25 are read from right to left.

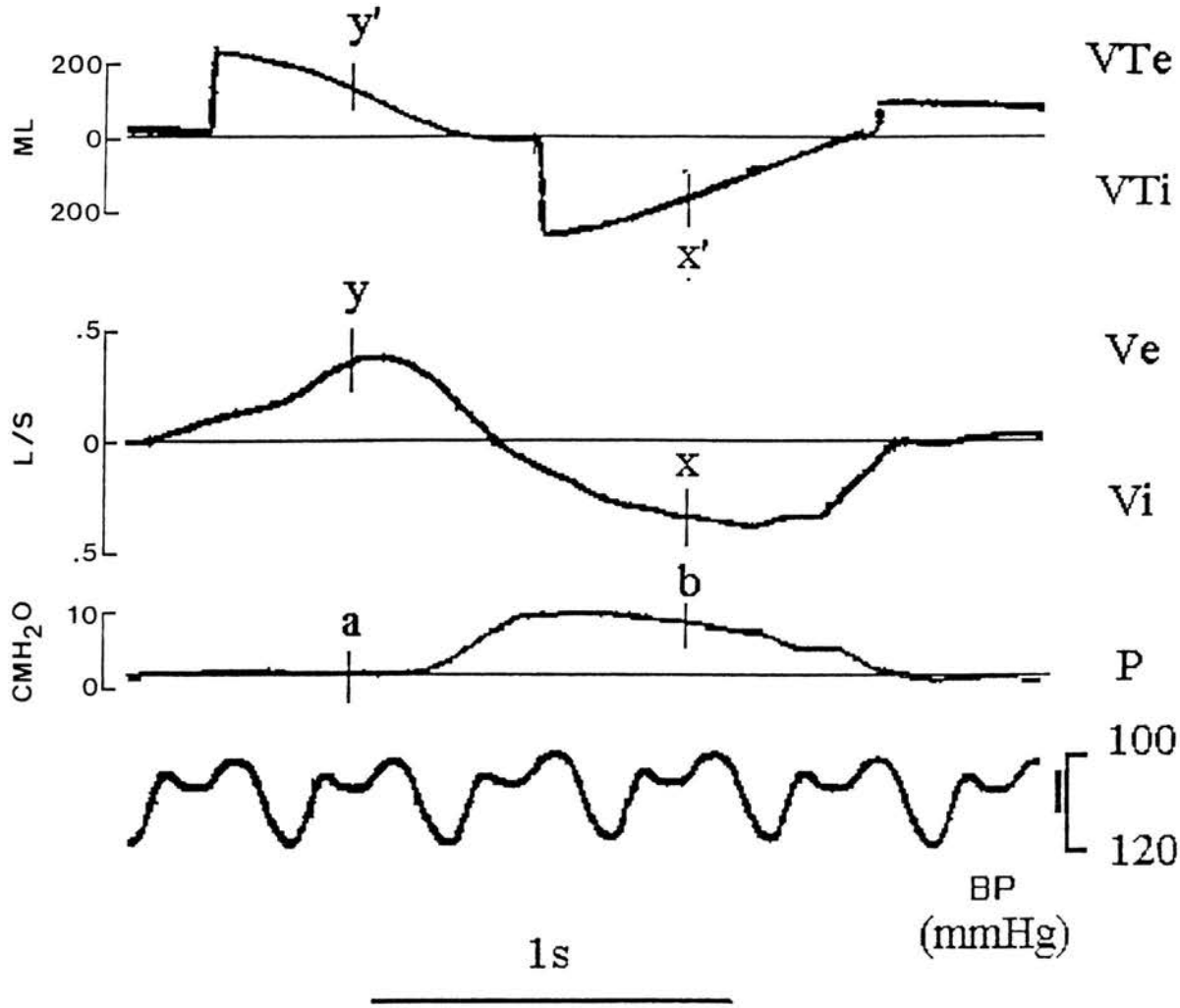


Figure 24. Calculation of pulmonary resistance using the isovolume method (RLiso). The pressure difference (b-a) associated with isovolume points (typically mid-tidal (50%)) for inspiratory (x') and expiratory (y') volume is calculated and divided by the associated difference in flow between these isovolume points (x+y). In theory the mid-tidal points should be the same volume for both inspiration and expiration. In practice, particularly when there is bronchoconstriction, the mid-tidal point for inspiration may be greater than that for expiration. To overcome this problem alternative procedures involve picking an equivalent volume point for both parts of the cycle (eg isovolume point at 25% of VT) or selecting the mid-tidal point independently for inspiration and expiration ignoring what the respective volumes are. VTe, VTi - expiratory and inspiratory tidal volume; Ve,Vi - expiratory and inspiratory flow; P - transpulmonary pressure; BP - blood pressure.

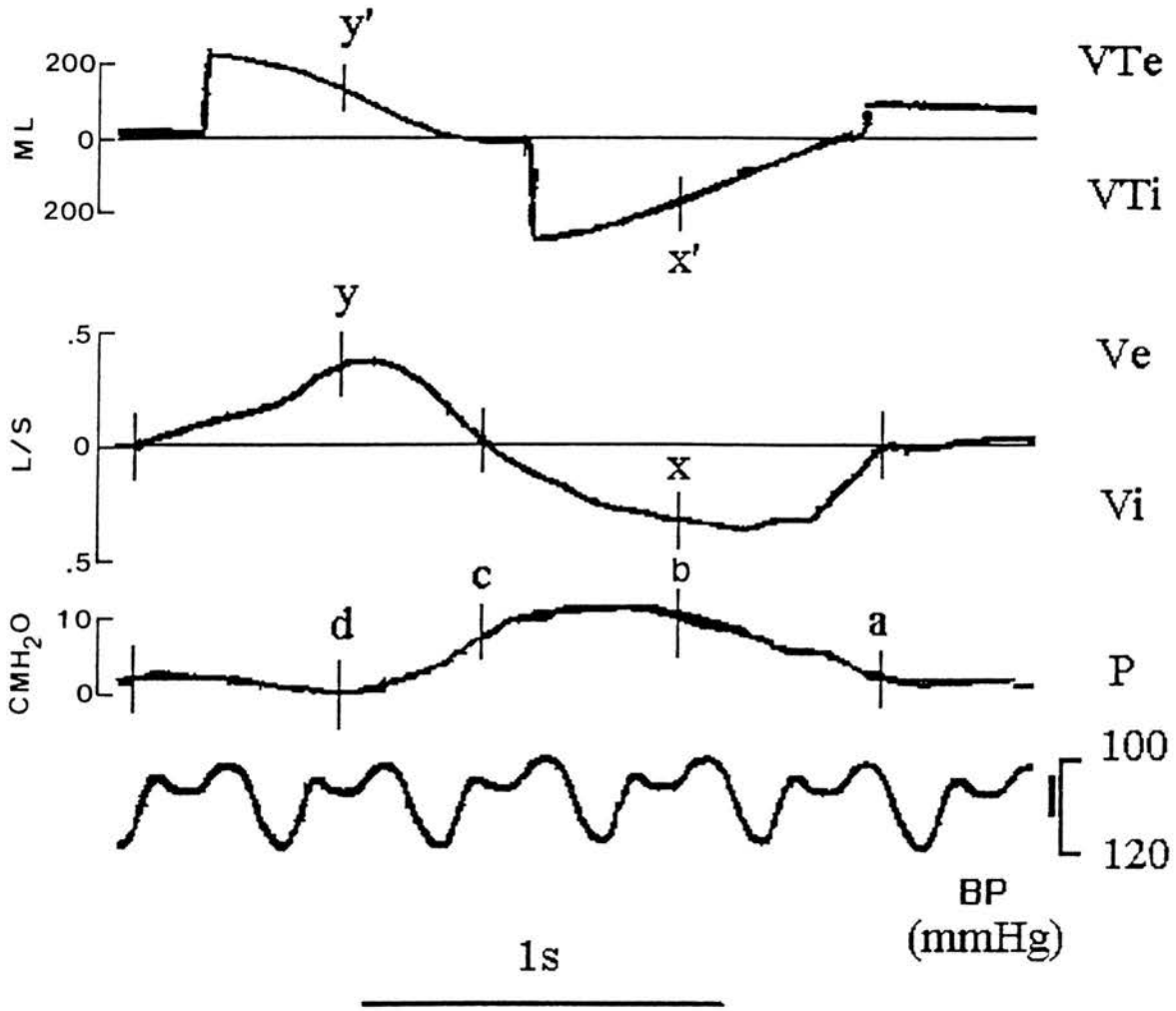


Figure 25. Calculation of pulmonary resistance by averaging the resistance values for the inspiratory and expiratory parts of the respiratory cycle (RL). The mid-tidal points in inspiration (x') and expiration (y') are identified and the associated flows (x and y) measured. The transpulmonary pressure change for these flow points are measured (ab and cd) from which is subtracted the pressure due to tissue elastance (ac). The derived pressures are then divided by their respective flows to give inspiratory (RLin) and expiratory (RLex) resistance ($cmH_2O/l/s$) as follows: RLin = (ab-ac)/x ; RLex = (cd-ac)y. RLin and RLex are added and an average RL for the entire respiratory cycle calculated. VTe, VTi - expiratory and inspiratory tidal volume; Ve, Vi - expiratory and inspiratory flow; P- transpulmonary pressure; BP - blood pressure.

vere towards infinity at the maximal response to SP. The changes in RL and RLiso in response to two doses of SP, administered 20 minutes apart, were assessed in 7 sheep using the Spearman rank-order correlation test. The mean reduction in the calculated resistance values between the two doses were 15 and 49% for RL and RLiso measurements respectively. Furthermore, there was a close correlation between the resistance values for the two doses calculated by the RL method ($r=0.813$; $p<0.05$) but not with the RLiso method ($r=0.098$; $p=0.7$) (Figure 27). Therefore the RL method of calculating pulmonary resistance was considered to be the more accurate and reproducible method.

2.1.4. Arterial Blood Pressure Measurement:

A skin incision was made in the medial aspect of the right thigh and by blunt dissection the femoral artery or an associated branch was identified. The artery was tied off and an arterial catheter was introduced and tied in place. The skin incision was either sutured closed with monofilament nylon or using surgical staples. Patency of the catheter was maintained by periodic flushing with heparinised 0.15M NaCl (250 iu/ml).

Blood pressure was measured with a presure transducer (4-327 I, Transamerica Deval, USA) connected to the MX19 physiological recording system. The pressure signal was used to measure heart rate, diastolic (D), systolic (S) and mean blood pressure (MBP). Mean blood pressure was calculated as follows; $MBP = D + 0.33(S-D)$.

The blood pressure transducer was calibrated with a mercury manometer (mmHg) at the beginning of each day's experiments.

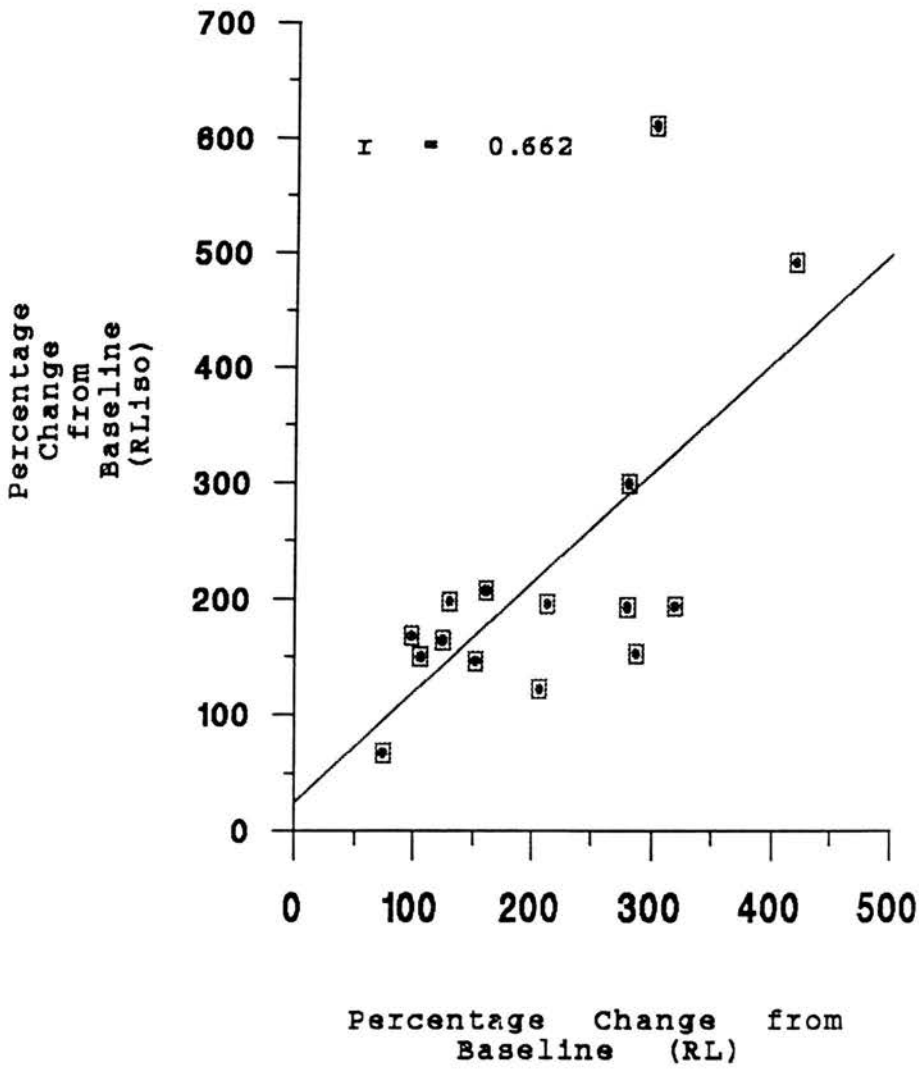


Figure 26. The percentage change in pulmonary resistance calculated by an isovolume method (RLiso) and a mid-tidal method (RL). Fifteen seperate measurements were used from six sheep and the correlation between RL and RLiso was examined using the method of least squares. There is a close positive correlation ($r = 0.662$; $p<0.01$) between the resistance changes determined by the two methods.

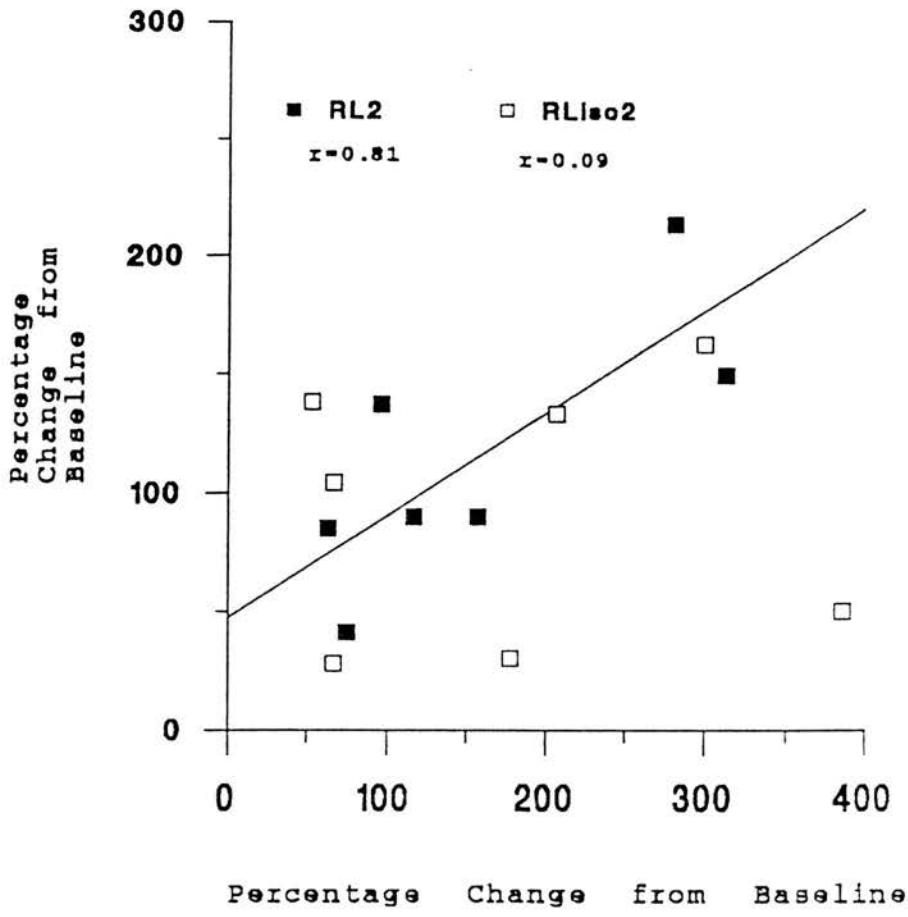


Figure 27. Comparison of the change in pulmonary resistance, calculated by the isovolume (RLiso) and mid-tidal (RL) methods, after the same dose of substance P (SP) was administered intravenously twice, 20 minutes apart, in seven sheep. There was a strong positive correlation ($r = 0.813$) between the resistance value calculated by the RL method at the two dose times, but not when the RLiso method was used ($r = 0.098$) (Spearmann Rank-Order Correlation Test). Furthermore the mean reduction in calculated resistance values for the two doses was 15 and 49% for RL and RLiso respectively.

2.1.5. Intra Cardiac Catheterisation:

The effect of intra-cardiac administration of drugs was assessed in some sheep. After anaesthesia the sheep was placed in dorsal recumbency and a mid-line cervical incision made (Figure 28). The underlying muscle and connective tissue was separated by blunt dissection and the external jugular vein and external carotid artery identified. Estimate of the length of catheters required were obtained from external body measurements and the catheters were pre-filled with heparinised saline. The jugular vein was tied off cranially and a 60cm cardiac catheter, was introduced and positioned in the right atrium. The position of the catheter was determined by continuous monitoring of the jugular pressure. Entrance of the catheter into the right atrium gave a slightly greater pressure fluctuation. The carotid artery was cannulated in a similar manner, but without tying off. Positioning of the carotid catheter in the left ventricle was more difficult to achieve but easier to confirm by monitoring pulse pressure (Figure 29).

2.2. Drug Preparation and administration:

2.2.1. Preparation:

Neuropeptides were supplied in a lyophilised form and were immediately reconstituted with phosphate buffered saline (PBS), and in the case of neurokinin B with 10% ammonium hydroxide, on delivery. The peptides were initially diluted to a concentration of 1 M and stored as 1 ml aliquots in Eppendoff tubes at -40°C. on the day of each experiment the peptides were further diluted to 100 umol/ml and stored on ice.

All other drugs were made up on the day of each experiment and dissolved in PBS immediately prior to administration.

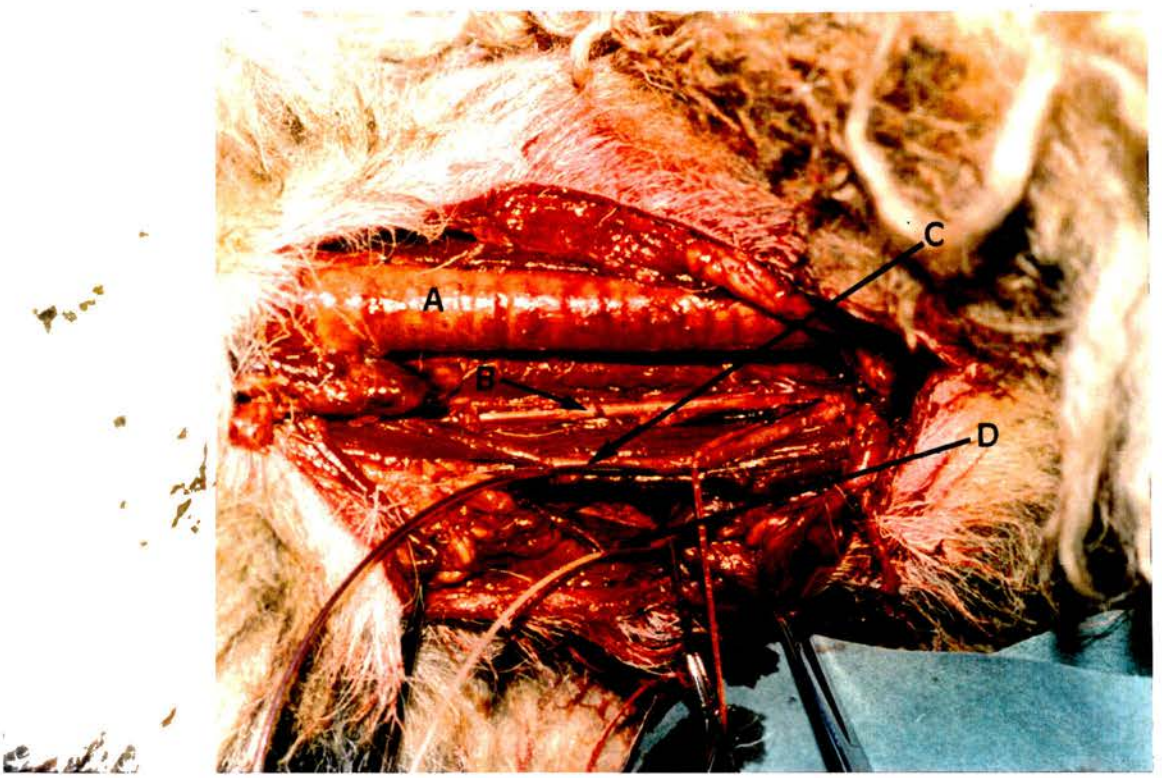


Figure 28. Intracardiac catheterisation. Photograph of the surgical exposure of the external jugular vein and carotid artery and placement of phosphate buffered saline catheters. The vessels have been ligated proximally and the catheters guided into the right and left sides of the heart by monitoring pressure in the catheters (see Figure 29). A-trachea; B-vagus nerve; C-carotid artery; D-jugular vein.

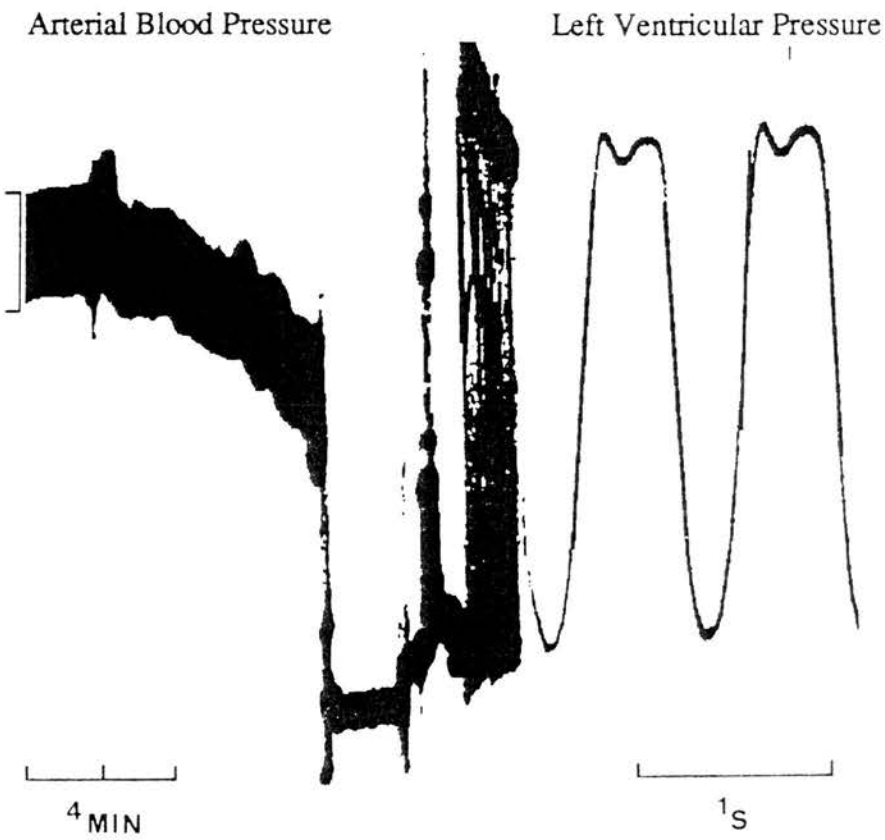


Figure 29. Blood pressure tracing from the right external carotid artery demonstrating introduction of the carotid catheter into the left ventricle. Arterial blood pressure is shown on the left and the scale markings are 100 and 120mmHg. On introducing the catheter into the left ventricle the end-diastolic pressure is close to zero (bottom of tracing) with a large increase in systolic pressure relevant to arterial pressure. The sensitivity for the left ventricular portion of the tracing is half that of the arterial side.

2.2.2. Administration:

Drugs were administered by three routes. In conventional studies of the tachykinin peptides and antagonists administration was via the indwelling catheter in the right fore leg. In studies looking at the effects of direct intra-cardiac injection of peptides administration was via a catheter in the external jugular vein positioned in the right atrium or a catheter in the external carotid artery positioned in the left ventricle. With intracardiac administration the catheters were pre-loaded with the agent under investigation and then flushed as required with phosphate buffered saline. In inhalation studies in sheep with airway allergy to ascaris suum antigen and normal sheep, agents were administered as nebulised aerosols and the details are outline in chapter 4.

2.3. The Isolated Sheep Trachealis Muscle Preparation:

Tracheal samples were obtained from female sheep slaughtered at a local abattoir. The trachea was removed from the sheep within 10 minutes of slaughter and immediately immersed in "ice-cold" (4°C) oxygenated (95%O_2/5%CO_2) Krebs solution (composition mmol/L; $MgSO_4.7H_2O$, 1.176; KH_2PO_4 1.175; KCl, 4.67; NaCl, 118; $NaHCO_3$, 24; glucose, 11.6; $CaCl_2.2H_2O$, 2.52). The tissue was transported to the laboratory in a sealed and insulated ice-box with an approximate journey time of 20 minutes. The material was transferred to fresh Krebs solution at room temperature and continuously oxygenated for 30 minutes. The trachea was opened along its ventral border and sections were taken roughly equivalent to the width of a cartilage ring. The trachealis muscle strip was prepared with 10mm of cartilage attached at each end. One cartilage piece was pierced with a stainless steel clip and tied to the end of a glass rod with cotton thread. The other cartilage piece was

attached to a strain gauge transducer (Washington Transducer Type D, Palmer-Bioscience) in the same manner. The preparation was mounted in a 20ml glass organ bath containing Krebs solution at 37°C and continuously oxygenated. The transducer was connected to a Washington pen recorder to allow measurement of isometric tension (Figure 30). The tension of the strip could be altered using a micromanipulator. The tissue was allowed to equilibrate for 60 minutes, with the tension adjusted to approximately 5g, and the Krebs solution was changed every 15 minutes. Once the tissue had equilibrated the tension was set at approximately 2g for experiments (Eyre, 1969).

Agents to be tested were added directly to the organ bath in volumes not greater than 0.1ml. Agents were either added to the organ bath as single doses (SD), each one followed by wash-out with Krebs solution, or in a cumulative dose fashion at three minute intervals, again followed by a wash-out. The contractile response to 1 uM of methacholine chloride was assessed in each tissue. Three consecutive methacholine responses of similiar magnitude were obtained before a tissue was used to assess the effect of test agents. The tissue response to test agents were expressed as a percentage of the response to 1 uM methacholine.

The effect of substance P (SP) on the trachealis muscle preparation was assessed in the presence of the neutral metalloendopeptidease inhibitor phosphoramidon. Phosphoramidon (2 uM) was added to the organ bath 2 minutes before the peptide and had been found in preliminary experiments to augment the contractile effect of SP and markedly improve the quality of the response.

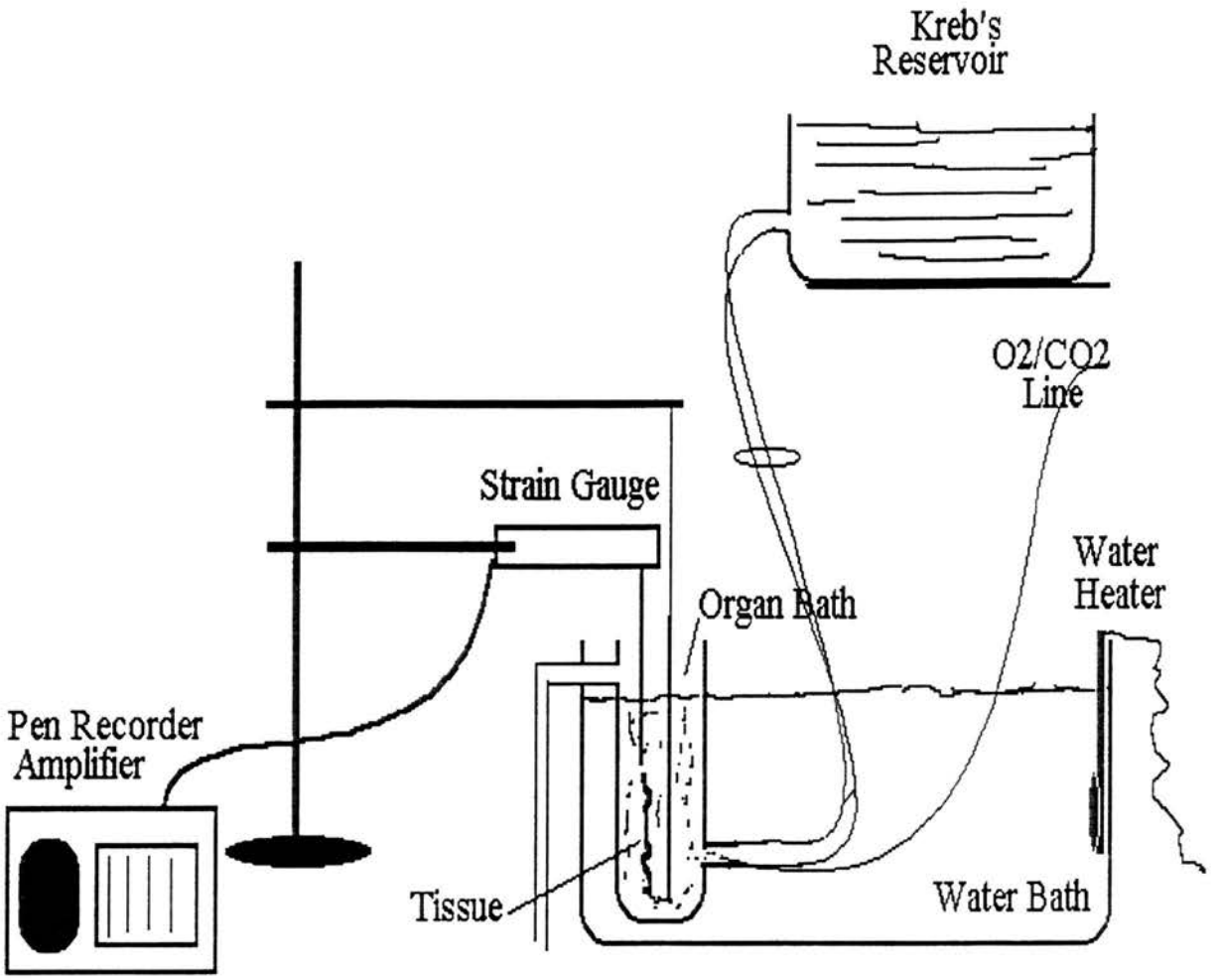


Figure 30. Measurement of the contractile response of the sheep trachealis muscle preparation in vitro . The tissue was mounted in a 20ml organ bath, in a water bath kept at 37°C, attached to a glass rod and a strain gauge pressure transducer. Changes in tension were recorded on a Washington pen recorder amplifier. The Kreb's solution in the organ bath could be changed using a reservoir tank and is aerated with a 95%O_2/5%CO_2 gas mixture.

2.4. Statistical Analysis of Data:

The manner in which the data was analysed is outlined in each experimental chapter. All data are presented as the mean and standard error of the mean (SEM) unless otherwise stated. Statistical analysis involved the use of the one way analysis of variance test (ANOVA) and the paired and unpaired t-tests, where appropriate.

CHAPTER 3. CARDIOPULMONARY RESPONSE TO INTRAVENOUS NEUROKININ NEUROPEPTIDES

3.1. Introduction:

The tachykinin neuropeptides are potent bronchoconstrictor and vasodilator agents and their effects have been demonstrated in vivo and in isolated tissue preparations in a variety of species including cat, rat, guinea pig, man, sheep and several others (Nilsson et al, 1977; Andersson & Persson, 1977; Lundberg & Saria, 1982a; Joos et al, 1987; Abrahams et al, 1991), but there are marked differences in the response to the different members of this family of peptides and variations in the response of different species.

With respect to the mammalian tachykinins NKA is, in general, the more potent bronchoconstrictor (Martling et al, 1987). However, in the sheep and pig SP is more potent than NKA (Martling et al, 1990; Abrahams et al, 1991; Haxhiu-Poskurica et al, 1992). In the guinea pig the bronchoconstrictor order of potency is NKA > NKB > SP, with NKA being up to 40 times more potent than SP (Uchida et al, 1987; Saria et al, 1988a), while in man the order of potency is NKA > SP > NKB (Naline et al, 1989).

In contrast to the order of potency for the tachykinins in contracting airway smooth muscle SP has a more profound effect on diastolic blood pressure in man and cutaneous vascular resistance and vascular permeability in both man and guinea pig (Hua et al, 1984; Fuller et al, 1987; Evans et al, 1988), while in the dog NKA is a more potent dilator of the tracheal vasculature than SP (Salonen et al, 1988). However, direct application of SP to structures in the CNS causes a rise in blood pressure and heart rate (Brattstrom & Seidenbecher, 1992) which may involve

activation of serotonergic neurons (Gradin et al, 1992). In the heart itself SP modulates the activity of vagal and sympathetic nerves and may be important in controlling reflex cardiac effects associated with activation of cardiac afferent nerve endings (Smith et al, 1992).

There may also be variations in the site of action of the tachykinins relevant to vasodilation and broncho-constriction. SP increases vascular permeability in the trachea and to a lesser extent at the hilus, but is ineffective in the peripheral lung of rats (Sertl et al, 1988). In contrast SP and NKA contract smaller peripheral airways more than the central airways (Frossard & Barnes, 1991) and SP is more potent than NKA in guinea pig peripheral airways (Gerard, 1987) and capsaicin-induced responses, including bronchoconstriction and plasma extravasation, are greater in the lower than upper airways (Manzini et al, 1989a).

SP, NKA, and the additional sensory neuropeptide CGRP, are also potent pulmonary arterial vasodilators and may be important in the neurogenic control of pulmonary vascular diameter and vascular smooth muscle tone (Adnot et al, 1991). SP and NKA are roughly equipotent in their ability to relax serotonin pre-contracted human pulmonary arteries (Martling et al, 1987).

The aim of this study was to assess the cardiopulmonary effects of intravenous SP, NKA and NKB in anaesthetised normal sheep.

3.2 Materials and Methods:

Dose-response curves for SP (n=11), NKA (n=9) and NKB (n=4) were obtained in 6 to 12 month old Suffolk cross female sheep, using the methods previously described. In the case of SP and NKA each experiment began with a dose-response curve to intravenous SP followed one hour later with a similar dose-response curve for NKA.

In preliminary studies it was found that the airway response to SP and NKA was not affected by the order in which the two peptides were administered, but as SP is more rapidly metabolised than NKA (Shore & Drazen, 1989) it was felt that SP should be administered prior to NKA. Dose-response curves to NKB and NKA were obtained separately in different sheep. During each dose response curve at least three minutes elapsed between peptide doses. Where there was marked bronchoconstriction at higher dose levels the subsequent dose was not administered until transpulmonary pressure had returned to baseline. After high doses forced lung inflation was occasionally necessary to return transpulmonary pressure levels to baseline. Cdyn and RL were measured 20 to 30s after peptide administration as this was when the most pronounced airway changes occurred. Changes in respiratory parameters usually began to return to baseline levels 40 to 50s after peptide administration. Respiratory rate was also measured during this period. Values of Cdyn, RL and respiratory rate were compared to those occurring immediately prior to each dose.

The respiratory responses to administration of a sub-maximal dose of SP into the cephalic vein, left ventricle and right atrium, were compared in six sheep, in order to determine if circulatory accessibility could affect the response to SP. All sheep were approximately 6-12 moths old, half were close to 6 months (#s 25,26,27) and the others (#s 32,33,34) close to 12 months. This apparently minor age difference may have affected the results (see later). Preparation and placement of intracardiac catheters has been described in chapter 2. However, in one sheep (#25) a catheter could not be introduced into the right atrium. In addition to the effects of SP on respiratory parameters, the time to the change in respiratory parameters and to development of peak bronchoconstriction was also assessed.

Mean blood pressure (MBP) and heart rate (HR) changes were measured for SP (n=7) and NKA (n=4), but not for NKB, 10 to 15 s after injection. Values were compared to

those at the beginning of each dose-response curve as changes in MBP and HR, in contrast to respiratory parameters, did not always return to baseline values between doses.

Preliminary studies suggested that tachyphylaxis could develop to the bronchomotor response to SP. It was found that subsequent responses to the SP doses eliciting a maximal, or close to maximal, bronchoconstriction were markedly attenuated. The time delay required to reverse tachyphylaxis to SP was investigated in one sheep and a separate group of four sheep. Repeated doses of SP were administered at 20 minute intervals four times in the four sheep.

Data are presented as the mean (+/-sem). The significance of any difference between SP, NKA and NKB and between the peptides and baseline measurements were determined using one-way analysis of variance or the t-test where appropriate. $P<0.05$ was taken as significant.

3.3 Results:

The respiratory effects of SP, NKA, and NKB and the cardiovascular effects of SP and NKA were assessed in anaesthetised sheep. Eight sheep were assessed for both SP and NKA and four sheep for SP and NKB. No sheep were assessed for both NKA and NKB. There was no significant difference between baseline values of RL and Cdyn in the sheep, prior to administration of SP, NKA or NKB (Table 5). All three peptides caused a dose-dependent reduction in Cdyn with an order of potency of SP > NKA > NKB (Figure 31). The maximal change in Cdyn with SP (-81% +/-4.8) and NKA (-30% +/-7.1) occured at the highest dose level of 5 umol/kg, while the response to NKB (-17.5% +/-4.78) reached a plateau at 1 umol/Kg. In direct contrast, SP had a marked effect on RL, with a maximal change of +1020% (+/-154), while NKA (+35% +/-16) and NKB (+32% +/-39) had no significant effect on RL. The difference

	Substance P	Neurokinin A	Substance P	Neurokinin B
RL ($cmH_20/l/s$)				
mean	3.80	2.99	6.07	6.17
s.e.m.	0.96	0.57	1.22	1.55
Cdyn (ml/cmH_20)				
mean	35.60	28.60	22.00	20.35
s.e.m.	7.48	4.44	2.90	0.67

Table 5. The table details the data (mean +/- s.e.m.) for baseline pulmonary resistance (RL) and dynamic compliance (Cdyn) in sheep prior to deriving dose response curves for intravenously administered substance P (SP)and neurokinin A (NKA) (n=8) and SP and neurokinin B (NKB) (n=4). There was no significant difference between baseline measurements for SP and NKA or SP and NKB (t-test).

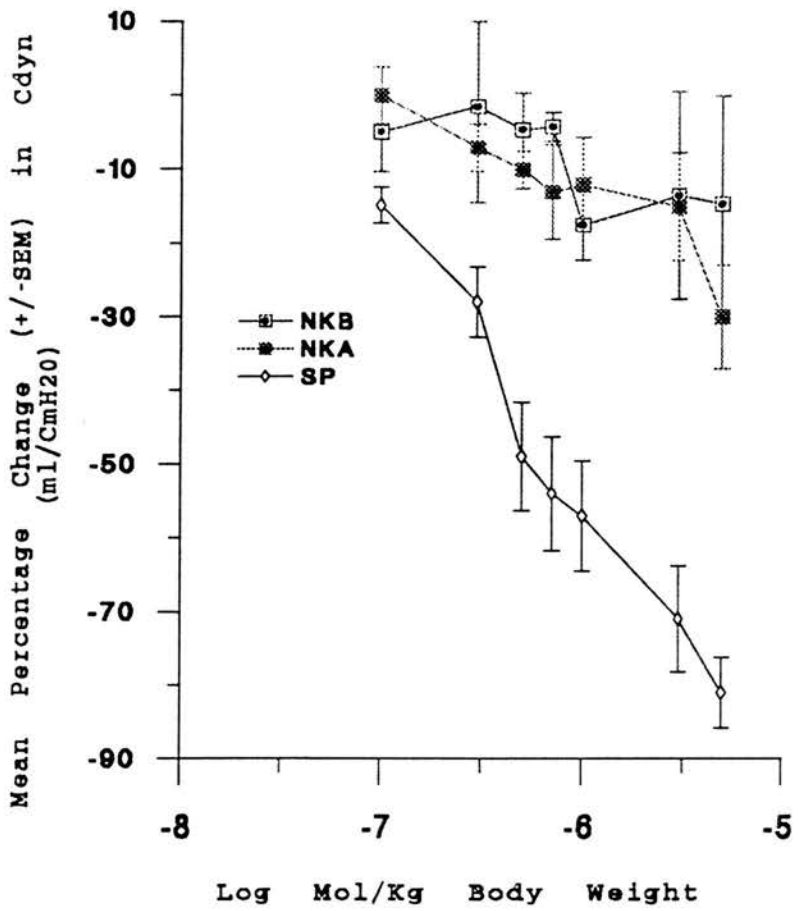


Figure 31. Percentage decrease in dynamic compliance (Cdyn) after intravenous administraion of substance P (SP) (n=11), neurokinin A (NKA) (n=9) and neurokinin B (NKB) (n=4) (0.1-5 μmol/kg).

between SP and NKA and NKB was statistically significantly different at doses of 0.5 umol/Kg or greater ($P<0.05$) (Figure 32).

Measuring the effect of NKB on bronchomotor tone was difficult as NKB appeared to cause oesophageal relaxation. In certain instances there was a drop in transpulmonary pressure just prior to the decline in flow and tidal volume that signals the onset of bronchoconstriction. This change in transpulmonary pressure did not coincide with the changes in flow and volume, and so was not due to suppression of respiratory drive.

There were consistent differences in the response to SP administered by the three different routes. The change in Cdyn and RL and the increase in peak transpulmonary pressure were similar for the cephalic and right atrial routes and were consistently, although not significantly, different from the left ventricular route (Table 6). The changes in VI, VE and VT were roughly similar for the three routes. The time to onset (To) of the respiratory response and the time to maximal bronchoconstriction (Tmax) was shortest for the right atrial route (Figure 33), although the times were not consistent. In two sheep from the younger group of animals (#s 26,27) in which right atrial injection was possible, the response started within 2s of administration. In the older sheep (#s 32,34,35) the bronchomotor response to SP was less than in the younger animals and the onset of response was more rapid for left ventricular (8s +/- 2) than right atrial (11.8s +/-0.57) administration as was the time to maximal bronchoconstriction (17.9s +/-0.17 vs 26.5s +/-2.74) (Table 7).

There was a dose-dependent reduction in respiratory rate with SP and NKA, but not with NKB, and this was significantly different from baseline values at doses of 0.7 umol/Kg or greater ($P<0.05$) (Figure 34). NKA had a more marked effect on respiratory rate than SP but the difference was not significant.

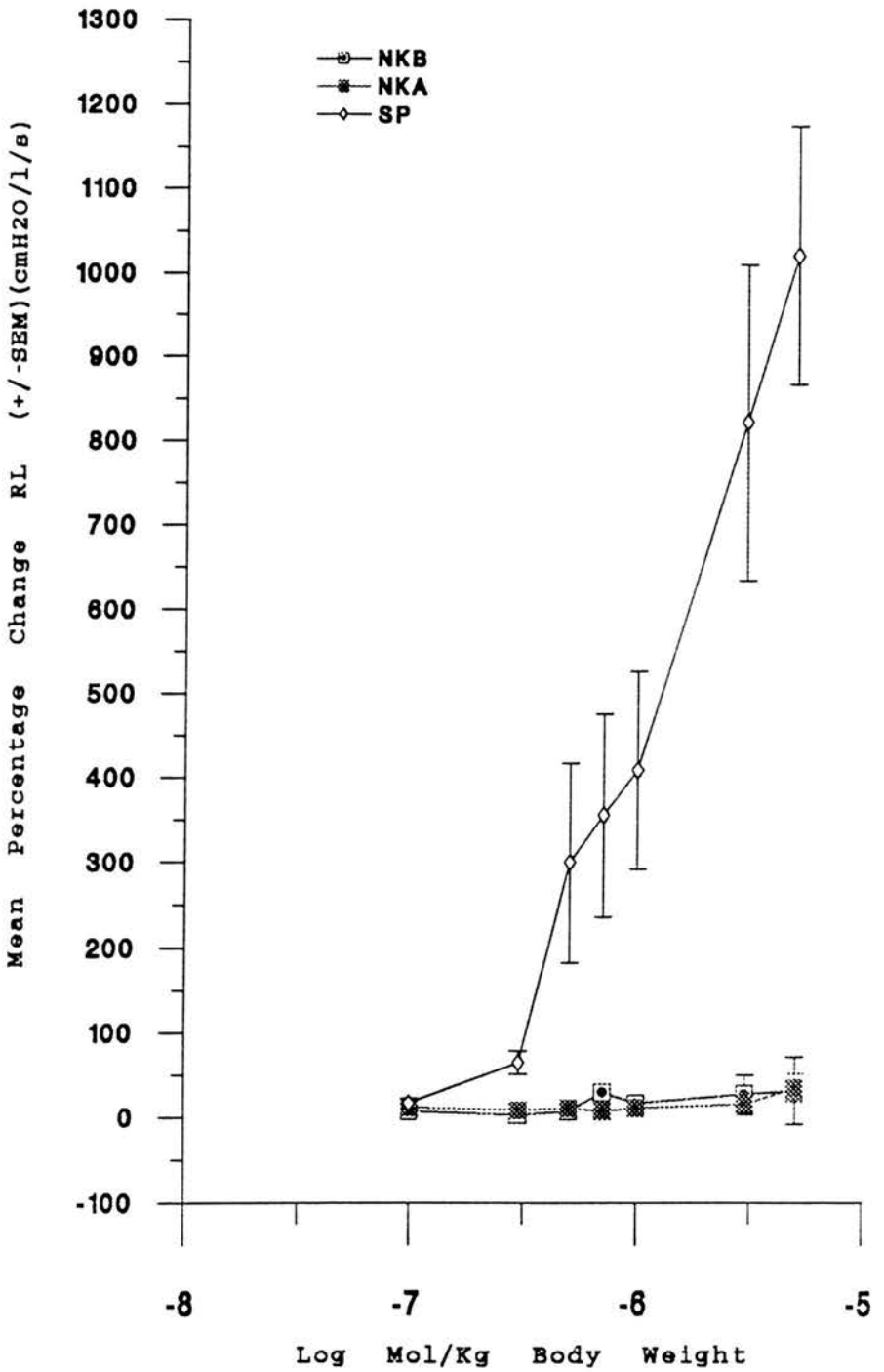


Figure 32. Percentage increase in pulmonary resistance after intravenous administration of substance P (SP) (0.1-5 μmol/kg) (n=11), neurokinin A (NKA) (n=9)and neurokinin B (NKB) (n=4).

	Cephalic Vein	Right Atrium	Left Ventricle
% Change RL			
mean	+ 469	+ 537	+ 285
s.e.m.	204	237	114
% Change Cdyn			
mean	- 59	- 68.8	- 54
s.e.m.	9	10.5	9

Table 6. The percentage change in pulmonary resistance (RL) and dynamic compliance (Cdyn) from baseline measurements after administration of a sub-maximal dose of substance P (SP) into the cephalic vein, right atrium and left ventricle (n=6). The responses to SP were similar for the cephalic and right atrial routes and were consistently, particularily with respect to the change in RL, greater (although not statistically significantly different) than the response with left ventricular injection.

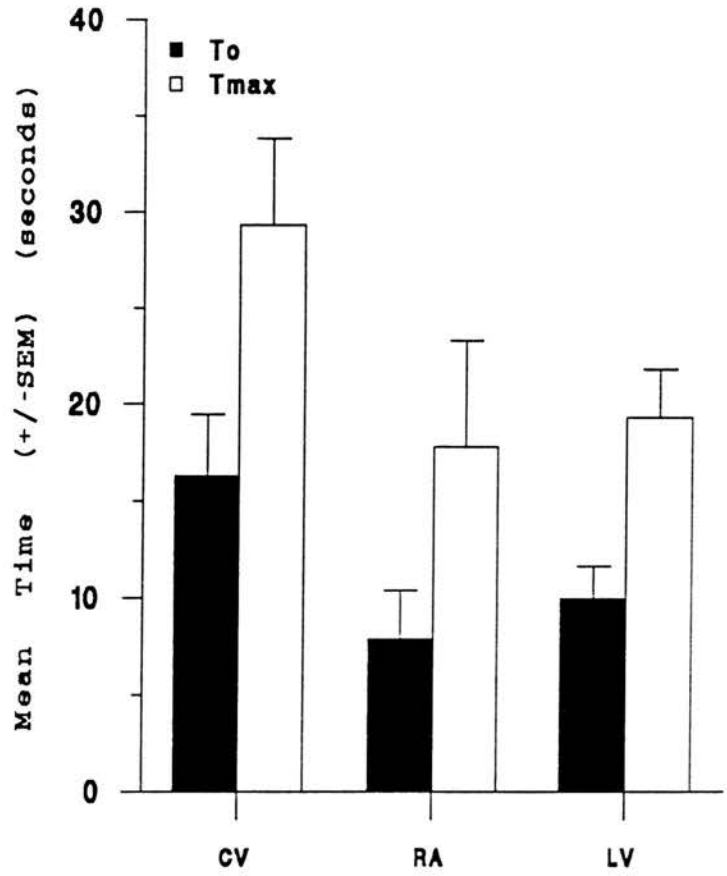


Figure 33. The time (seconds) to the onset (To) of the respiratory response and the time to maximal bronchoconstriction (Tmax) after administration of substance P (SP) via the right cephalic vein (CV), the right atrium (RA) and the left ventricle (LV) in normal sheep (n=6). While the response times were faster with right atrial injection, and approached significance (t-test; p=0.07) the times were not consistent and there were differences between individuals that could be explained on the basis of age differences within the group (see text).

Sheep #	To			Tmax		
	CV	RA	LV	CV	RA	LV
< 6 mths						
25	10.0	-	12.0	27.0	-	30.0
26	12.0	2.0	16.0	25.0	6.0	21.0
27	10.0	2.0	8.0	19.0	3.7	11.4
<12 mths						
32	14.0	6.0	10.4	23.2	24.3	17.6
33	28.0	15.0	12.0	32.0	32.0	18.0
34	24.0	10.0	6.0	50.0	23.3	18.2

Table 7. Comparison of the differences in the time to onset (To) and the time to maximal bronchconstriction (Tmax), in seconds, in two different age groups of sheep. In contrast to the younger sheep (top group), the older sheep (bottom group) the response times were more rapid for left ventricular (LV) injection than right atrial (RA) and right cephalic vein (CV) injection

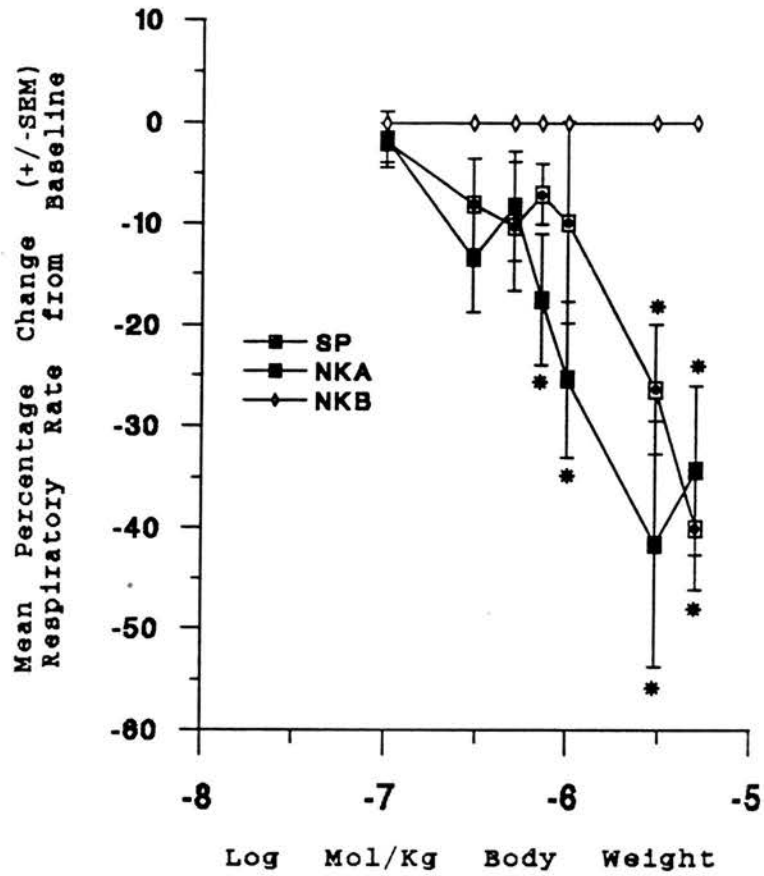


Figure 34. The effect of substance P (0.1-5 μmol/kg) (SP; n=10), neurokinin A (NKA; n=7) and neurokinin B (NKB; n=4) on respiratory rate in anaesthetised sheep. Both SP and NKA had similar effects on respiratory rate which were statistically significantly different from baseline (ANOVA; ** $P<0.01$). NKB had no effect on basal respiratory rate.

SP caused a dose-dependent reduction in MBP while NKA caused a marginal increase (Figure 35). With both peptides, changes in MBP were due to changes in both systolic and diastolic pressures (Figure 36). The change in MBP with SP was significantly different from baseline at doses of 1 umol/Kg or greater.

In the tachyphylaxis study the effect of a near to maximal dose of SP (0.3umol/kg) was assessed sequentially at zero, 5, 25, 30, and 50 minutes (two five minutes and two 20 minute intervals) in one sheep (#19) and repeated doses of SP at 20 minute intervals were assessed in four sheep. In sheep #19 there was marked reduction in the response to SP when only five minutes were allowed between doses, but no attenuation with the 20 minute intervals (Table 8). In the four sheep group there was minor reduction (88% of the first dose) in Cdyn with the second dose, but the subsequent 3rd and 4th doses were greater than the first. All the RL readings were greater than the first, but this was not statistically significant (Figure 38). This study suggested the optimal inter-dose interval for sub-maximal doses of SP is approximately 20 minutes.

3.4. Discussion:

This study demonstrates that SP is a potent bronchoconstrictor agent in the sheep and has a marked effect on both central (RL) and peripheral (Cdyn) bronchomotor tone. NKA has some effect on Cdyn and is more potent in this respect than NKB, but both NKA and NKB have little or no effect on RL. These results compare with inhalation studies where SP, in allergic sheep pretreated with the neutral endopeptidase inhibitor thiophan, is a more potent bronchoconstrictor than NKA (Abraham et al, 1991; see chapter 4). However, the bronchoconstrictor effect of SP in allergic sheep is only modest and is far less than that achieved with bradykinin. The effect of NKB, or injected SP and NKA, on bronchomotor tone in allergic sheep has not been reported.

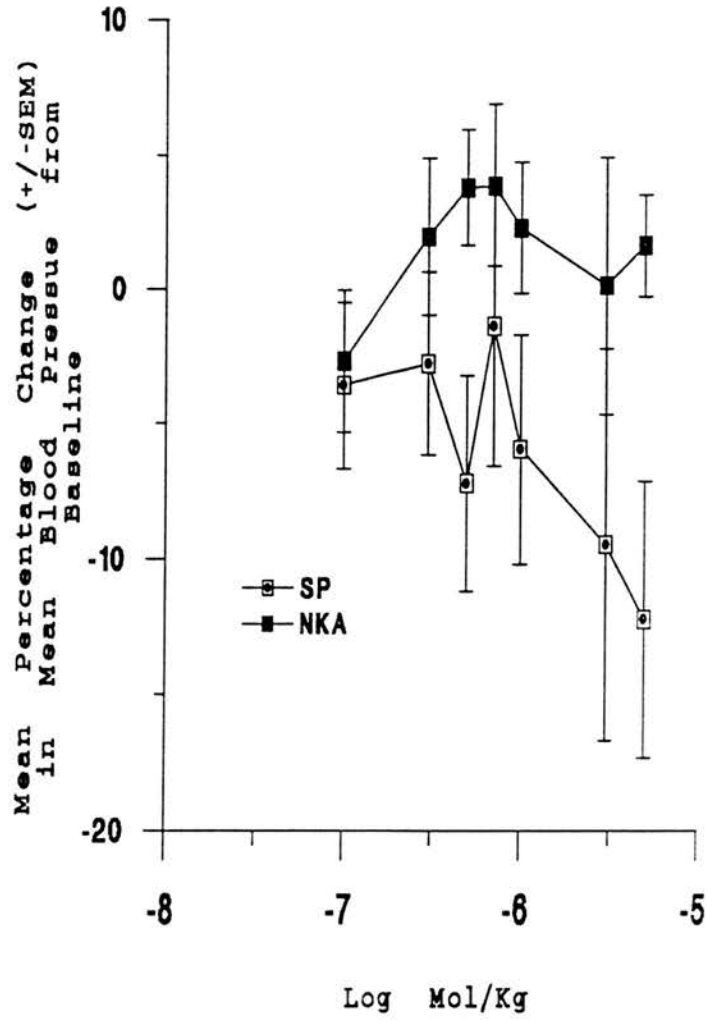


Figure 35. The Effect of substance P (SP) and neurokinin A (NKA; n=4) (0.1-5 μmol/kg) on mean blood pressure (MBP) in anaesthetised sheep. SP caused a dose-dependent reduction in MBP, while NKA caused a marginal increase. Neither peptide caused a statistically significant change from baseline MBP.

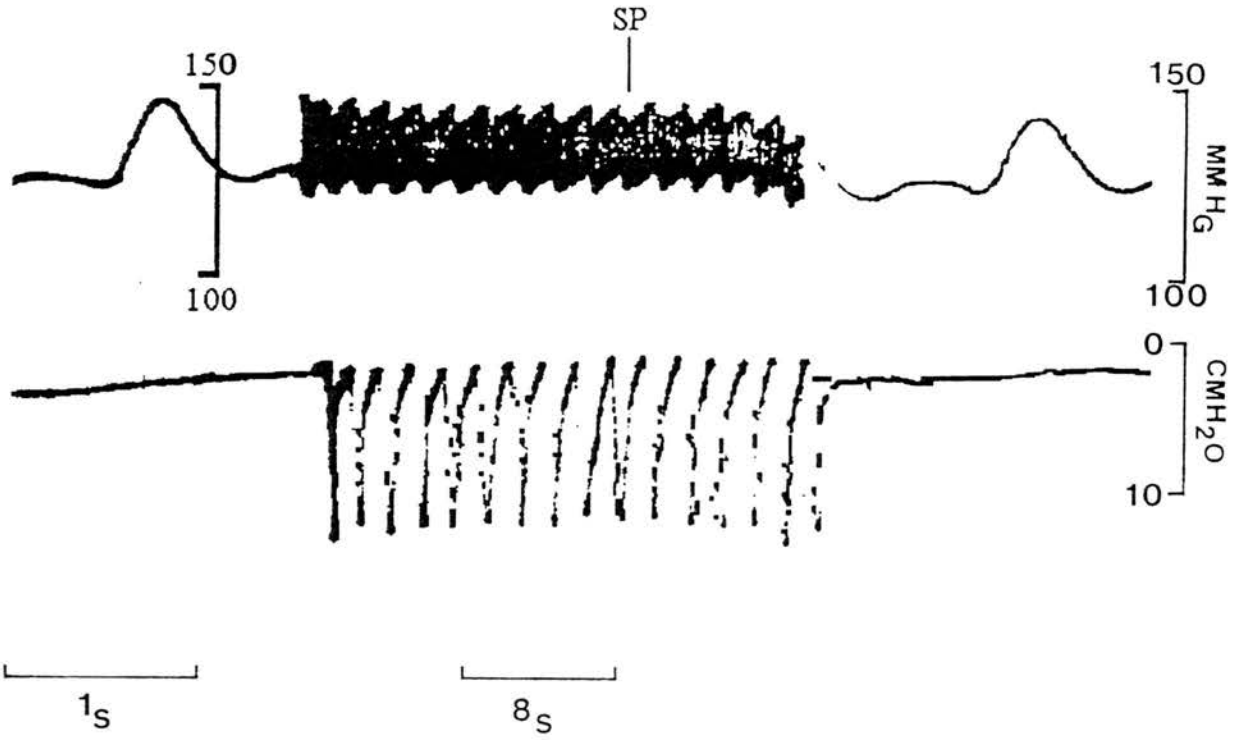


Figure 36. The effect of substance P (SP) on arterial blood pressure (BP, mmHg; upper tracing). SP caused a decrease in both diastolic (D) and systolic (S) blood pressure approximately 15 seconds after injection into the right cephalic vein. The fall in BP was maximal 29 seconds after injection and coincided with the peak of bronchoconstriction (not shown) and the change in S/D was from a pre SP level of 145/120 mmHg to 120/105. After a further 26 seconds BP returned to pre SP levels. Note the respiratory modulation of the BP tracing. Lower tracing is transpulmonary pressure.

Sheep #19 Repeated Substance P Dose

	Time (minutes)				
	0	5	25	30	50
Cdyn	-94	-5	-90	3	-91
RL	485	3	460	8	580

Table 8. The effect of repeated intravenous substance P (SP) (0.3μmol/kg) on dynamic compliance (Cdyn) and pulmonary resistance (RL) in sheep #19. The SP dose was administered twice at 5 minutes intervals and twice at 20 minute intervals. At 5 minute intervals there was complete loss of the response to SP, and this loss was reversed if doses were spaced 20 minutes apart (see also Figure 38).

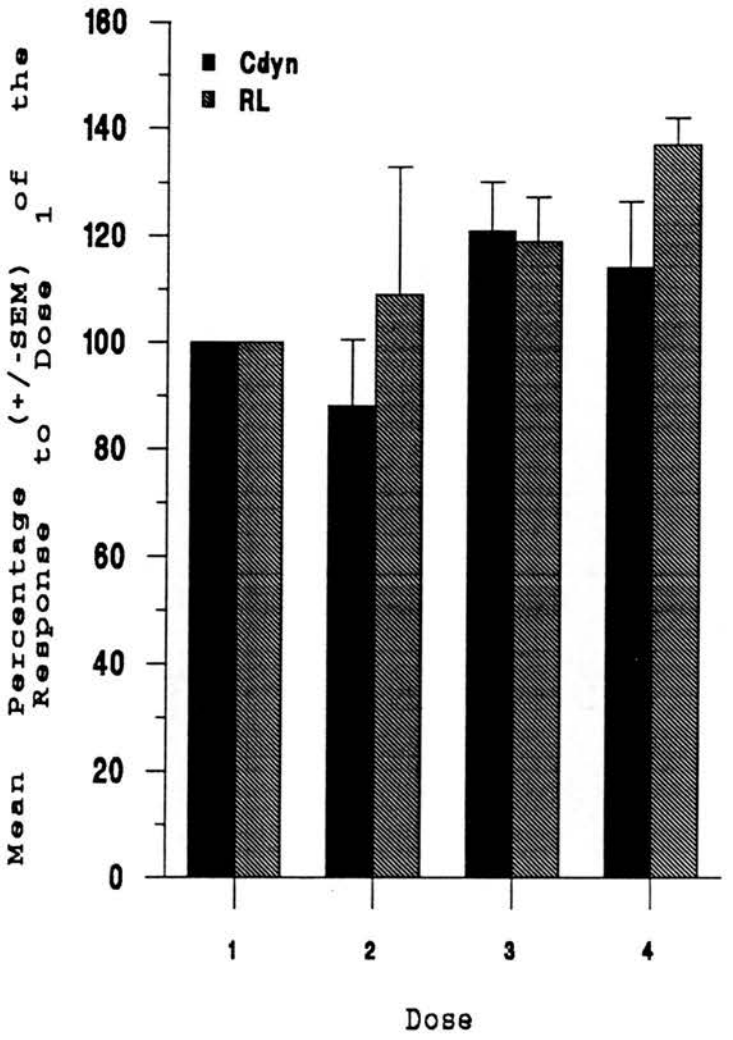


Figure 37. Investigation of the tachyphylaxis to the substance P (SP) bronchomotor response. In preliminary work it had been noted that there was a reduction in the bronchomotor response to SP if doses were administered close to each other (ie < 5 min apart). Therefore the response to repeated doses of SP was assessed in anaesthetised sheeep (n=4). Dose-response curves for SP were obtained and the dose giving approximately 50-80% of the maximal bronchoconstriction was determined. This dose was then administered four times (1,2,3 & 4) at 20 minute intervals. There was no significant alteration in the change in dynamic compliance (Cdyn) and pulmonary resistance (RL) associated with SP, but there was a marginal increase in response over time. It was concluded that the optimal time interval for repeated administration of sub-maximal doses of SP was around 20 minutes.

The bronchoconstrictor order of potency for SP and NKA in sheep contrasts with that reported in most other species, while the low potency of NKB agrees with other studies. NKA is more potent than SP in rats (Joos et al, 1988), in guinea-pigs (Hua et al, 1984; Uchida et al, 1987) and in isolated airway preparations in man (Advenier et al, 1987; Naline et al, 1989; Frossard & Barnes, 1991). In both asthmatic and normal humans inhaled and infused SP has variable effects on bronchomotor tone (Joos et al, 1987; Evans et al, 1988; Crimini et al 1988a,b, 1990), while NKA is an effective bronchoconstrictor agent in asthmatic human subjects (Joos et al, 1987). However, in young and adult pigs SP is a more potent bronchoconstrictor than NKA (Martling et al, 1987; Haxhiu-Poskurica et al, 1992). The apparent reduction in oesophageal pressure with NKB contrasts with the contractile effect of SP demonstrated in guinea-pigs, cats, and pigs (Leander et al, 1982), and the effect of this oesophageal response on the transpulmonary pressure measurements achieved with SP and NKA is not known.

The cardiovascular effects of SP and NKA (NKB was not assessed) could indirectly affect bronchomotor tone through arterial baroreceptor reflexes. Baroreceptor activation results in reflex inhibition of respiration and bronchodilation (Grunstein et al, 1975). The marginal pressor response with NKA may have activated this reflex, but this is unlikely. While SP is known to activate carotid body and peripheral chemoreceptors, activation of chemoreceptors by arterial hypoxaemia will cause bronchoconstriction and this could have augmented the bronchoconstriction caused by SP. However, blood gas concentrations were not monitored during the experiments. The effect of the tachykinins on pulmonary vascular tone was also not assessed. The differential effect of NKA on Cdyn, while suggesting a preferential constriction and closure of peripheral airways, possibly due to pulmonary vascular congestion and consequent alveolar collapse, would suggest the Cdyn change with SP is a combination of pulmonary vascular changes and peripheral airway smooth muscle

effects. Indeed, SP and CGRP, which are often co-localised in sensory nerves, reduce the pressor response of the pulmonary artery to infused prostaglandinF_2 alpha (PGF_{2a}) in pigs (Adnot et al, 1991). While SP is a more potent pulmonary arterial vasodilator than CGRP, the effect is at low (<50pmol/min) infusion rates. However, at high infusion rates (1000pmol/min), in vessels that are not pre-contracted with PGF_{2a}, SP causes an increase in pulmonary arterial pressure, decrease in systemic arterial pressure and a concurrent reduction in Cdyn. While these changes are concomitant, Adnot and co-workers (1991) suggest the change in dynamic compliance causes the increase in pulmonary vascular resistance. In the rabbit isolated pulmonary artery preparation (Tanaka & Grunstein, 1985) the effect of SP is also dependent on the resting arterial tone, causing dilation in pre-contracted vessels and contraction in relaxed arterial stips. Nevertheless, Frossard & Barnes (1991) have recently demonstrated that both SP and NKA have a greater contractile effect on peripheral (non-cartilaginous bronchi) airways compared to central large (cartilaginous segmental bronchi) airways and there may be a functional difference between the central and peripheral parts of the respiratory tract.

The reduction in mean blood pressure (MBP) with intravenous SP is in accord with previous reports from other species including man (Hua et al, 1984; Fuller et al, 1987; Goel & Biggs, 1987; Joos et al, 1988; Haxhiu-Poskurica et al, 1992) indicating that SP is a potent vasodilator. However, Bayorh & Feuerstein (1985) have reported a pressor response with very low doses of SP (0.7-7 nmol/kg) in the pithed rat and potentiation of the pressor response with sympathetic stimulation. At higher doses (700nmol/kg) SP tended to inhibit the pressor response to sympathetic stimulation. In sheep SP is also a more potent vasodilator than NKA which agrees with findings in man (Evans et al, 1988), rats (Maggi et al, 1985; Couture et al, 1989) and guinea-pigs (Ezra et al, 1986). Joos et al (1988) have speculated that the reduction in Cdyn with SP may be secondary to pulmonary vasodilation, but the vasodilator effect of SP on

pulmonary arteries, as discussed earlier, only occurs in pre-contracted vessels (Tanaka & Grunstein, 1985; Adnot et al, 1991). NKA can cause mild systemic vasoconstriction in sheep and, while the effects on pulmonary arteries are not known, it would appear that the reduction in Cdyn can occur without a concurrent reduction in blood pressure. Neither peptide had a significant or consistent effect on heart rate.

In preliminary studies tachyphylaxis to the bronchomotor response to SP, but not the cardio-vascular effects, occured at high doses. By separating doses with an interval of at least 15 minutes the problem, of tachyphylaxis can be avoided, although this was not necessary at the lower end of the dose range. In guinea-pigs there is an unusual enhancement to repeated doses with rapid intravenous administration of SP (Shore & Drazen, 1989) which is possibly due to cyclooxygenase products of arachidonic acid metabolism, and Goel and Biggs (1987) did not find tachyphylaxis with SP, physalaemin or eledoisin in guinea pigs. However, Haxhiu-Poskurica etal (1992), working with the pig suggest a 10 minute interval between peptide doses and 30 minute pause between dose-response curves for different peptides to avoid problems of tachyphylaxis.

While SP had a more marked effect on bronchomotor tone when administered into the systemic venous and pulmonary arterial circulation as compared to the systemic arterial circulation, the differences were not statistically significant. However, the possible differences in response between sheep of different ages need to be assessed further and it could be that in young sheep (see Chapter 5) the increased bronchomotor response to SP is a function of accessibility to larger numbers of neurokinin receptors through the pulmonary circulation. Left ventricular administration of SP still caused a significant bronchoconstriction and it must be presumed that airway neurokinin receptors can be accessed from both the pulmonary and systemic circulation. The close anatomical relationship and extensive

anastomoses between the pulmonary and bronchial circulations make it possible for agents to come in contact with receptors from either side (Sant'Ambrogio & Sant'Ambrogio, 1982; Butler, 1991).

This circulatory accessibility characteristic of the response to SP is analogous to the different populations of C-fibre afferent receptors described in the dog and cat (Coleridge & Coleridge, 1984) that are separately accessible from both the pulmonary and bronchial circulations. In the rabbit an additional group of pulmonary-bronchial receptors, accessible from both sides of the circulation, have been described (Russell & Trenchard, 1979), but there is no information on the C-fibre receptor sub-types in the sheep respiratory system. The pulmonary C-fibre endings in the dog are stimulated 0.9-3.1s (mean 2.1s) after injection of capsaicin into the right atrium (Coleridge & Coleridge, 1977a) which is similar to the onset of changes in respiratory parameters noted in two sheep in the present study. The bronchial C-fibre endings are accessible from the pulmonary and systemic circulations, but only after a longer period after injection (Coleridge & Coleridge, 1984). However, only right atrial injection of capsaicin causes reflex broncho-constriction in the dog (Russell & Lai-Fook, 1979) and left ventricular injection has no affect on airway calibre. Nevertheless, these temporal differences in response to SP by different routes in the sheep suggest the possible involvement of pulmonary afferent receptors in the response to SP and should be investigated further.

CHAPTER 4. SUBSTANCE P AND NEUROKININ A IN ALLERGIC SHEEP

4.1. Introduction:

The bronchomotor effects of SP and other neurokinins have suggested a possible role for these neuropeptides in the aetiopathogenesis of airway hypersensitivity disorders, such as asthma in man, and there is considerable interest in the relative effects of the different neurokinins on hyper-reactive airways in man and animals (Barnes, 1986). Compared to normal human subjects atopic patients with asthma and rhinitis have higher concentrations of SP and enkephalin (ENK) in bronchoalveolar lavage samples, and furthermore there is an increase in SP and ENK after allergen challenge in atopic individuals (Nieber et al, 1992). In asthmatic human subjects there are conflicting reports on the effect of inhaled SP on bronchomotor tone (Fuller et al, 1987b; Crimini et al, 1988a) and in general NKA appears to be the more potent neurokinin (Joos et al, 1987). However, in sheep with an airway allergy to Ascaris suum antigen SP and NKA appeared to be ineffective in causing bronchoconstriction (Abraham et al, 1991) and although the response to SP can be enhanced by the neutral metallo-endopeptidase enzyme inhibitor L-thiorphan the effect of enzyme inhibition on the relative potencies of SP and NKA was not assessed. SP has been demonstrated in pulmonary oedema fluid of sheep with experimental adult respiratory distress syndrome, and generation of the peptide is increased by airway inflammation (Espiritu et al, 1992). However, SP is found inconsistently in the airways of normal adult sheep.

The conscious sheep Ascaris suum airway allergy model was developed in the late 1970s by Wanner and co-workers at the Department of Medicine, Mount Sinai Medical Centre, Miami, USA. The production of this model initially involved recognising sheep with cutaneous reactivity to ascaris suum antigen and then actively

sensitizing the airways of these animals to ascaris suum by either biweekly intramuscular injections for up to 20 weeks (Wanner & Reinhart, 1978) or twice monthly inhalations of antigen for up to 8 weeks (Wanner et al, 1979). However, this sensitisation procedure is laborious and it was discovered that a proportion of sheep already have a naturally acquired airway sensitivity to Ascaris suum and will bronchoconstrict if they inhale the antigen (WM Abrahams, per. comm.), and they also have an exaggerated response to carbachol and methacholine compared to normal sheep. This obviates the need for a sensitisation protocol.

The sheep airway allergy model of asthma has attracted a great deal of interest because detailed measurements of pulmonary mechanics can be made in the conscious animal and there are close similarities between this model and human asthma. Unlike other experimental asthma models the sheep develops lung hyperinflation in response to antigen (Wanner & Reinhart, 1978; Wanner et al, 1979) which is a common clinical feature of asthma in man. In addition, some allergic sheep develop both an early (immediate) and late (6-8 hours post challenge) pulmonary response to inhaled allergens (Abraham et al, 1983). These early and late phase responses can both be blocked by nedocromil sodium, but only the late response can be blocked by methylprednisolone. This again is very similar to the therapeutic response seen in some asthmatic human subjects. Because of these close functional similarities between sheep and man it is important to know if the sheep airway asthma model shows a similar response to the neurokinins as that reported in human subjects.

Therefore, the aim of this study was to assess the relative potencies of inhaled SP and NKA on pulmonary mechanics in a recognised model of airway allergy in sheep and to detemine if it was similar to that reported in normal sheep (Chapter 3), in other animal models of asthma and in human asthma.

4.2. Materials and Methods:

All work was carried out in the Department of Research Pulmonary Unit at the Mount Sinai Medical Centre (MSMC), Miami, Florida, USA, in collaboration with Drs. A. Ahmed and W. Abraham.

4.2.1. Animals:

Texel-cross female sheep (n=5), aged approximately 12 to 18 months, and with a mean body weight of 22.5 kg were used. All sheep were kept in a purpose built, air-conditioned, sheep housing unit attached to the laboratory and fed on ad lib hay, water and a commercial sheep cereal diet. The sheep were not fasted before experiments. The sheep had a naturally acquired airway allergy to Ascaris suum antigen (Wanner et al, 1979 ; Abraham et al, 1983, 1991) and hyper-responsiveness to inhaled carbachol. The bronchomotor response to Ascaris suum antigen was immediate, with no late phase response, and the sheep were classified as acute responders according to MSMC laboratory criteria.

4.2.2. Animal Preparation:

Sheep were restrained in the prone position in a purpose built trolley (Figure 39). The body was supported by a canvas sling and the head was taped onto a metal plate attached to the trolley, holding it firmly in place. After topical anaesthesia of both nostrils with 2% lidocaine solution, an oesophageal balloon catheter was introduced through the right nostril and positioned in the caudal oesophagus. The catheter was constructed from a 10cm rubber balloon sealed over a semi-rigid polyethylene tube (2mm internal diameter) with 15 to 20 spirally arranged holes. A semi-rigid tube (5mm internal diameter) was placed beside the oesophageal catheter to allow removal of excess secretions using a vacuum pump. The sheep were nasally intubated with a 6mm cuffed endotracheal tube. The endotracheal tube was guided into the trachea by

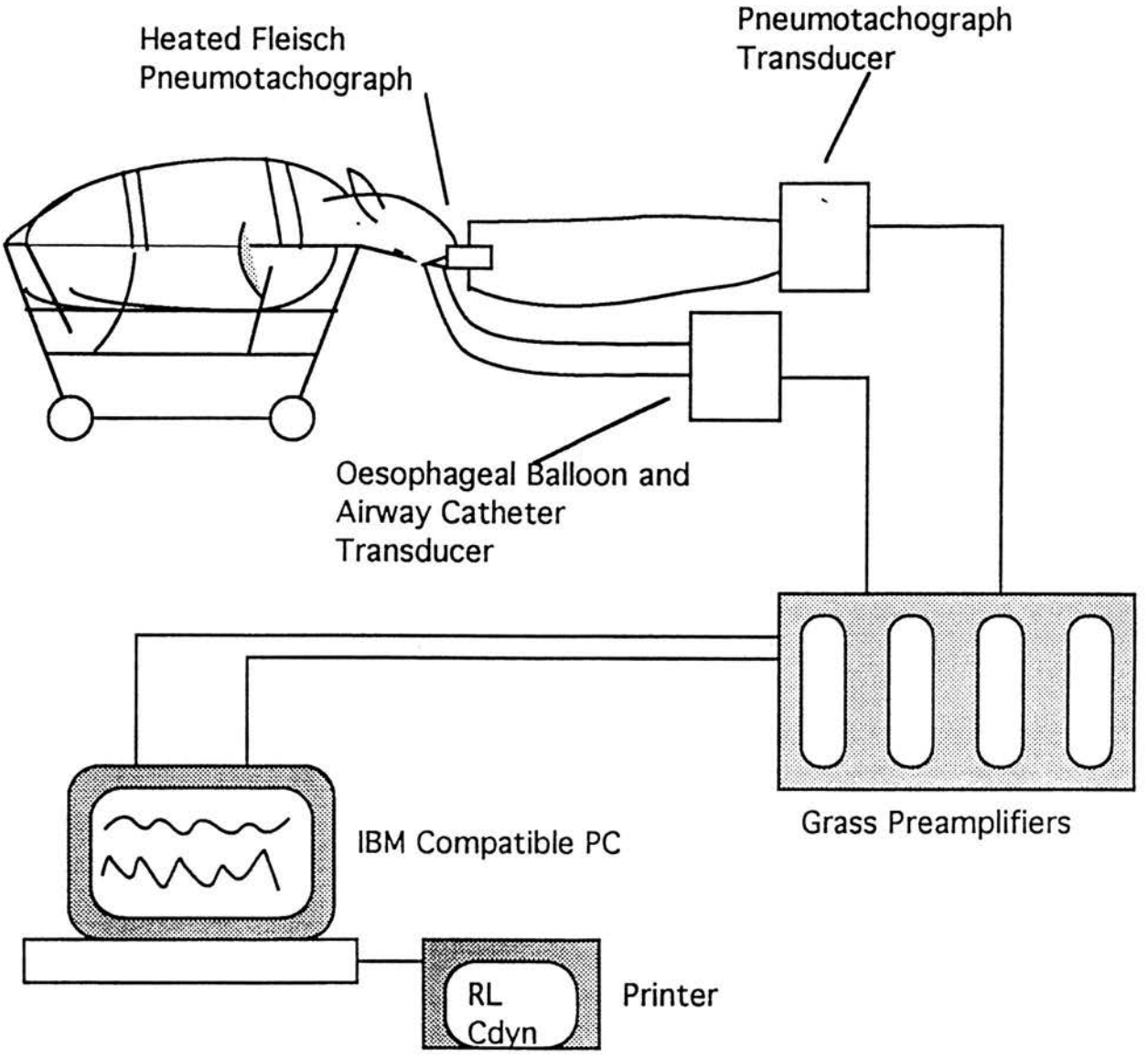


Figure 38. Diagramatic illustration of the method for measuring respiratory parameters in conscious sheep with a naturally acquired airway allergy to Ascaris suum antigen. The sheep are restrained in a shopping trolley, with the head held firmly on a metal plate with tape. The oesophageal balloon and airway catheters and the two ports of the heated pneumotachograph (attached to a nasal endotracheal tube) are connected to two matched differential presure transducers. The transducer outputs are amplified using Grass preamplifiers and the signal then passed to an analog-to-digital converter board in an IBM compatible PC. The flow signal is integrated to give tidal volume. The computer uses a pulmonary mechanics programme to calculate pulmonary resistance and dynamic compliance from the flow, tidal volume and pressure signals.

threading it over a pre-positioned length of rigid polyethylene tubing or by using a flexible fibreoptic endoscope. A lubricant gel containing lignocaine was applied to the end of the endotracheal tube to assist placement and to suppress the cough reflex. The oesophageal balloon and endotracheal tube were then taped to the sheep's head to prevent movement.

The endotracheal tube was connected to a heated pneumotachograph (Fleisch, Dyna Science Inc, Pa, USA). The pneumotachograph ports were attached to a differential pressure transducer (type P-7D, Pace Engineering, Ca, USA) from which flow and volume (integrated flow) were derived. The oesophageal balloon and a matched polyethylene catheter, placed towards the tip of the endotracheal tube, were connected to the ports of another differential pressure transducer (type P-7D, Pace Engineering, Ca, USA), from which transpulmonary pressure was measured. The signals of pressure and flow were amplified (type 7P1 pre-amplifier, Grass Instruments, Mass, USA) and recorded on-line through an analog-to-digital converter board (PDP-11, Digital Equipment, Maynard, Mass, USA) to an IBM compatible PC. Respiratory parameters were calculated and analysed using an in-house developed programme (MECHANIX, Mount Sinai Medical Centre). Pressure, flow and volume measurements were calibrated at the beginning of each day's experiments. All transducers, catheters and associated tubing were phase matched and there was no phase shift between transpulmonary pressure and flow up to a frequency of 9Hz.

4.2.3. Measurement of Respiratory Parameters:

Respiratory paramaters, including respiratory resistance, dynamic compliance, tidal volume and respiratory rate, were measured. Respiratory resistance ($cmH_2O/l/s$) was calculated on a breath-by-breath basis using a mid-tidal volume method (Wanner & Reinhart, 1978) dividing the pressure difference at the mid-tidal points for inspiration and expiration by the associated difference in flow. Dynamic compliance

(ml/cmH_2O) was calculated during the inspiratory part of the respiratory cycle by dividing inspiratory tidal volume by the pressure difference at points of zero flow.

The procedure for measuring respiratory parameters was as follows. Excess rumenal gases were first removed by compressing the abdomen. A suction tube was inserted into the endotracheal tube and connected to a vacuum source to remove excess airway secretions. The suction tube was removed and replaced by the airway catheter which was attached to one port of the transpulmonary pressure transducer. The oesophageal balloon catheter was attached to the other port, after inflating with 2ml of air. The oesophageal suction tube was attached to a continuously running vacuum pump. The pneumotachograph was attached to the endotracheal tube opening. Data were collected on a breath-by-breath basis and the mean values of 10 to 20 breaths were calculated. The MECHANIX programme allowed exclusion of breaths where there was spontaneous swallowing, or breaths where there was excessively low (less than 0.5cmH_2O/l/s) or excessively high (greater than 1 standard deviation from the mean) resistance values. The results of several runs were combined using a GRAND MEANS programme to give an overall mean value.

4.2.4. Aerosol Delivery:

Agents were delivered as aerosols via the endotracheal tube. The aerosol was produced using a disposable medical nebulizer (Raindrop, Puritan-Bennett, Kan, USA). The nebulizer was driven by compressed air at 20 psi and was connected to a dosimeter system consisting of a solenoid valve. The nebulizer was connected to a plastic T piece, one end of which was attached to the inspiratory port of a Harvard respirator (Harvard Apparatus, Inc., Mass, USA) and the other end to the sheep endotracheal tube. The pump delivered a tidal volume of 500ml at 20 breaths per minute, which was tolerated by the sheep and has been shown to have no effect on subsequent pulmonary mechanics measurements (Allegra et al, 1983). The aerosol

produced by the Raindrop nebuliser has a mass median diameter of 3.6um (geometric standard deviation, 1.9) as determined by an Andersen cascade impactor (Andersen Inc, GA, USA). As the sheep passively allowed the pump to ventilate their lungs, when disconnected they often did not revert to normal breathing for several minutes. With respect to drug administration, it was not possible to measure lung mechanics during the period immediately after cessation of drug administration.

4.2.5. Drug Administration:

The bronchomotor response to inhaled phosphate buffered saline (PBS), the metallo-endopeptidase inhibitor L-thiorphan, and two different doses of SP and NKA were assessed over a period of 5 days. Respiratory parameters were measured in the sheep after inhalation of 20 breaths of PBS and then allowed to rest for at least 30 minutes. They then inhaled 20 breaths of L-thiorphan (10 uM) and the bronchomotor response was again assessed 10 minutes later. This was immediately followed on day 1 by 20 breaths of SP or NKA (0.6 mM) and the response measured within 2 minutes of cessation of inhalation. The same doses of SP or NKA were administered in reverse order on day 2. After a days rest the procedures were repeated on day 4 and 5 using 40 breath doses of PBS, SP and NKA.

4.2.6. Data Analysis:

The data was analysed by computer and the significance of any change relevant to PBS control was determined by the paired t-test. $p<0.05$ was taken as statistically significant.

4.3. Results:

The respiratory response to inhaled substance P (SP) and neurokinin A (NKA) was assessed in five sheep at two different doses. Both peptides caused a dose-dependent increased in bronchomotor tone, but SP was more potent than NKA. The data are

shown in detail in Tables 9 and 10. Both peptides primarily affected RL rather than Cdyn. The increase in RL with SP was significantly different from baseline measurements and the response to inhaled PBS ($p<0.01$) (Figure 39). The decline in Cdyn was only significantly different from PBS at the 40 breath dose ($p<0.05$) (Figure 41). NKA significantly increased RL from baseline and PBS only at the 40 breath dose level (Figure 40) and had no significant effect on Cdyn (Figure 41). Inhalation of the metallo-endopeptidase inhibitor L-thiorphan caused a minor, but insignificant fall in resting bronchomotor tone, although a more marked increase in Cdyn was note in one sheep (#1227) on two occasions. Neither peptide affected respiratory rate.

4.4. Discussion:

SP is a more potent bronchoconstrictor than NKA in allergic sheep and this accords with the findings in normal sheep. The peptides primarily affected RL and had little effect on Cdyn suggesting their main site of action is in the central airways. This however might not represent a functional finding, but merely reflects the deposition of aerosolised peptide in the more central airways during inhalation. In normal sheep SP is equally effective in reducing Cdyn as increasing RL when given intravenously. This may be due to improved access to the peripheral airway smooth muscle through the pulmonary circulation or, as mentioned earlier, due to changes in pulmonary vascular resistance (Chapter 3).

Abrahams and others (1991) have previously reported the effects of SP and NKA, and the non-tachykinin peptide bradykinin, on RL in the sheep allergy airway model. Neither peptide affected RL values significantly, however in the presence of the metallo-endopeptidase inhibitor L-thiorphan, SP significantly increased RL. The effect of L-thiorphan pre-treatment on the response to NKA was not assessed. However, NKA, but not SP, significantly increased the airway responsiveness to

Pulmonary Resistance in Allergic Sheep

Sheep ID	Baseline	20 Breaths PBS	20 Breaths L-Thio	20 Breaths SP	% Change	Baseline	40 Breaths PBS	40 Breaths L-Thio	40 Breaths SP	%Change
Substance P										
#919	1.28	0.97	0.84	2.06	112%	0.78	0.71	0.87	1.72	142%
#1128	0.78	0.97	0.78	1.27	31%	0.78	0.82	1.16	1.42	72%
#1182	0.73	0.75	0.78	1.63	117%	1.14	0.93	0.95	2.20	136%
#1227	0.86	0.95	1.00	1.57	65%	1.22	0.84	0.77	1.79	113%
#1231	0.87	0.69	0.70	1.09	57%	0.70	1.00	0.71	1.90	90%
Mean	0.9	0.87	0.82	1.52	76% **	0.92	0.86	0.89	1.80	110%**
S.E.M.	0.1	0.06	0.05	0.16	16.5	0.10	0.05	0.08	0.13	13.3
Neurokinin A				20NKA					40NKA	
#919	0.70	0.68	0.60	0.63	- 8%	0.65	0.69	0.64	1.08	56%
#1128	1.17	1.23	0.96	0.96	- 23%	0.75	0.87	0.86	1.16	33%
#1182	0.66	0.74	0.60	0.62	- 17%	1.11	0.94	0.73	1.54	64%
#1227	1.16	1.07	1.09	1.27	18	0.98	0.96	1.08	1.22	27%
#1231	0.73	0.73	0.60	0.65	- 11%	0.60	0.69	0.67	0.86	25%
Mean	0.88	0.89	0.77	0.83	- 8%	0.82	0.83	0.79	1.17	41%*
S.E.M.	0.11	0.11	0.11	0.13	7.03	0.01	0.06	0.08	0.11	7.9

Table 9. Data for pulmonary resistance (RL) in five sheep with a naturally acquired airway allergy to ascaris suum antigen, after inhalation of 20 or 40 breaths of phosphate buffered saline (PBS), L-thiorphan (L-Thio), substance P (SP) and neurokinin A (NKA), and the percentage change in RL relevant to PBS for both peptides at both doses are shown. SP significantly increased RL (t-test; ** $p<0.01$) at both doses and NKA significantly increased RL at the 40 breath dose (* $p<0.05$).

Dynamic Compliance in Allergic Sheep

Sheep ID	Baseline	20 Breaths PBS	20 Breaths L-Thio	20 Breaths SP	% Change	Baseline	40 Breaths PBS	40 Breaths L-Thio	40 Breaths SP	%Change
Substance P										
#919	74	58	59	47	- 19%	31	41	36	29	- 30%
#1128	74	67	63	59	- 12%	48	57	42	39	- 32%
#1182	50	63	59	64	1%	49	57	58	30	- 48%
#1227	48	57	101	55	- 4%	44	43	80	41	- 5%
#1231	27	52	59	35	- 33%	33	44	66	38	- 14%
Mean	54.6	59.4	68.2	52	- 13.4%	41	48.4	56.4	35.4	- 25.8%*
S.E.M.	8.9	2.6	8.2	5	6.0	3.8	3.5	8.0	2.46	7.48
Neurokinin A				20NKA					40NKA	
# 919	36	30	32	29	- 4%	46	50	45	36	- 28%
#1128	58	76	57	61	- 20%	49	62	58	54	- 13%
#1182	42	38	54	52	36%	47	54	54	58	7%
#1227	68	54	59	53	- 4%	71	48	50	45	- 7%
#1231	46	47	54	38	- 20%	47	40	39	34	- 15%
Mean	50	49	51.2	46.6	- 2.4%	52	50.8	49.2	45.4	- 11.2%
S.E.M.	5.7	7.8	4.9	5.74	10.2	4.8	3.6	3.3	4.7	5.7

Table 10. Data for dynamic compliance (Cdyn) in five sheep with a naturally acquired airway allergy to ascaris suum antigen, after inhalation of 20 or 40 breaths of phosphate buffered saline (PBS), L-thiorphan (L-Thio), substance P (SP) and neurokinin A (NKA), and the percentage change in Cdyn relevant to PBS for both peptides at both doses are shown. SP significantly decreased Cdyn (t-test; * $p<0.05$) at the 40 breath dose, while NKA had no significant effect on Cdyn at either dose. It should be noted that in sheep #1227 both 20 and 40 breaths of L-thiorphan markedly increase Cdyn, but this was allowed to return to baseline levels before SP was administered.

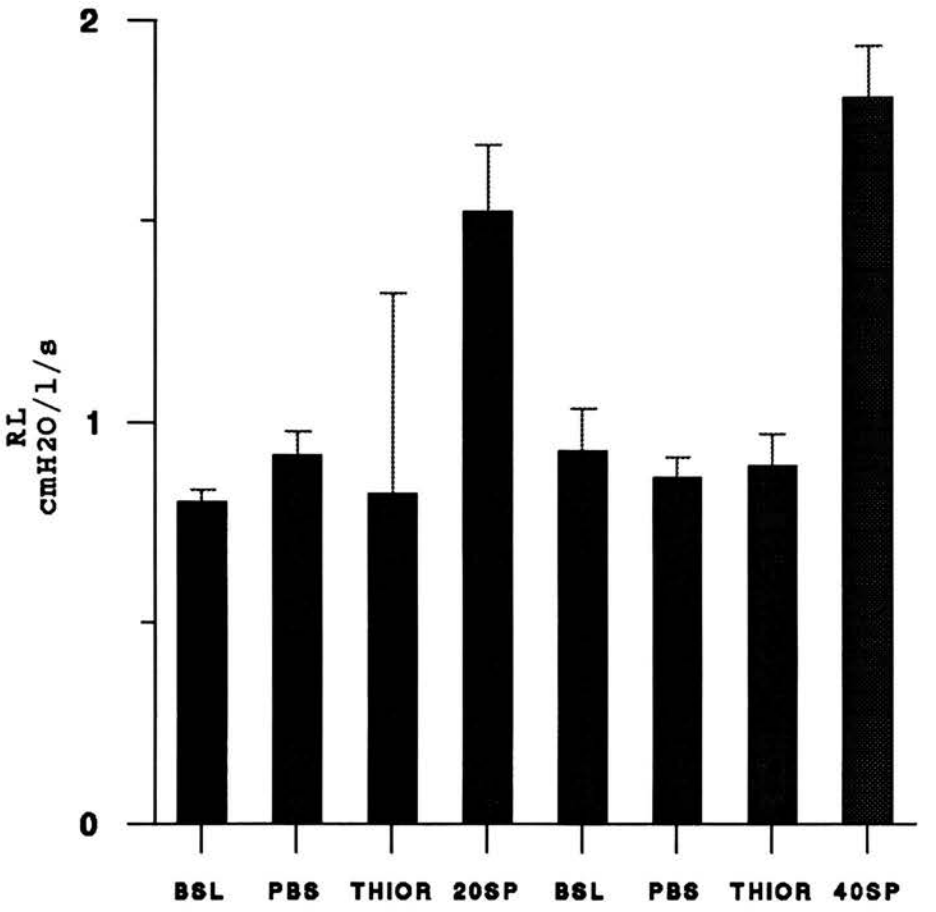


Figure 39. The effect of inhalation of 20 and 40 breaths of substance P (20SP & 40SP) on pulmonary resistance (RL) in allergic sheep (n=5). Compared to baseline (BSL) and 20 or 40 breaths of phosphate buffered saline (PBS), SP caused a significant increase in RL at both doses (t-test; $p<0.01$). THIOR-20 breaths of 10^{-5}M L-thiorphan.

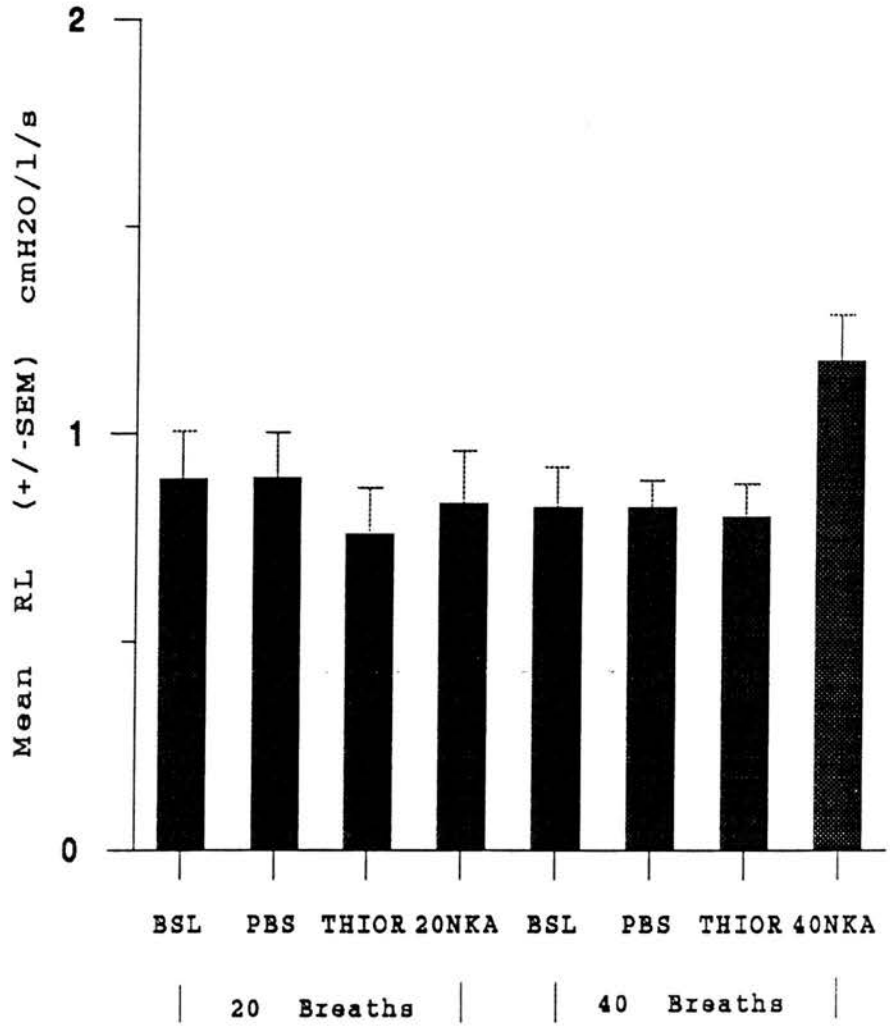


Figure 40. The effect of inhalation of 20 and 40 breaths of neurokinin A (20NKA & 40NKA) on pulmonary resistance (RL) in allergic sheep (n=5). Compared to baseline (BSL), NKA significantly increased RL only at the 40 breath dose (t-test; $p<0.05$). PBS-20 or 40 breaths of phosphate buffered saline; THIOR-20 breaths of 10-5M L-thiorphan.

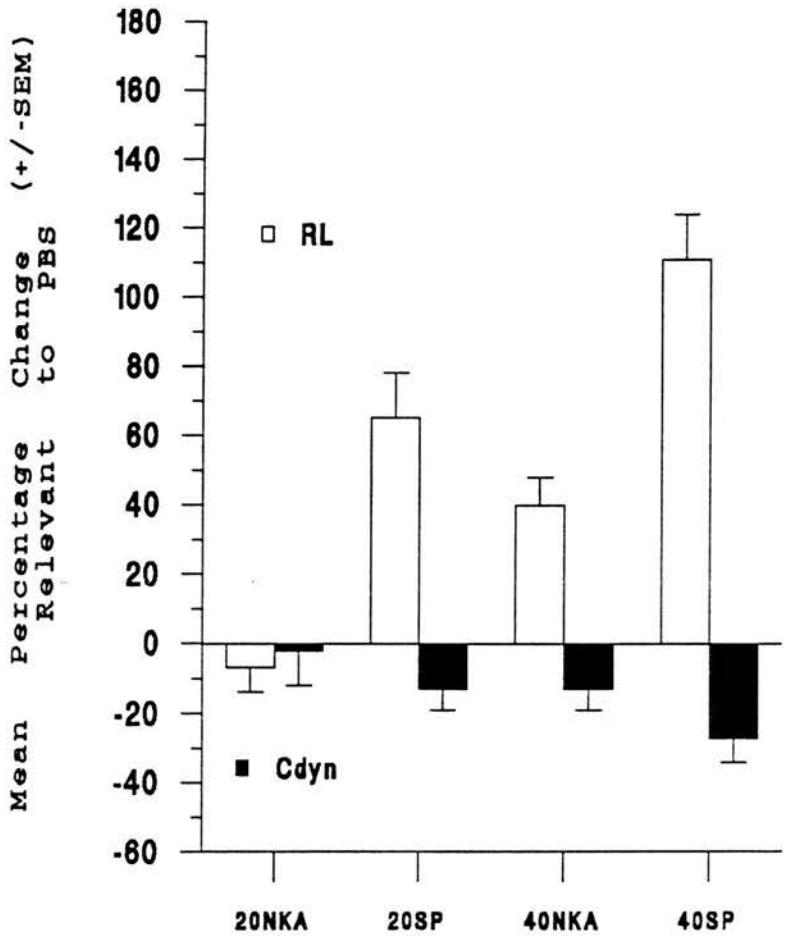


Figure 41. The effect of inhalation of 20 and 40 breaths of neurokinin A (NKA) and substance P (SP) on dynamic compliance (Cdyn) and pulmonary resistance (RL), relevant to phosphate buffered saline control, in allergic sheep (n=5). NKA had no significant effect on Cdyn at either dose, while SP significantly reduced Cdyn at the 40 breath dose (t-test; $p<0.05$). For detail on pulmonary resistance see Figures 39 and 40.

inhaled carbacol in allergic sheep and Tamura et al (1989) have demonstrated a similar increase in responsiveness to methacholine after pre-treatment with NKA in Japanese monkeys. A similar mechanism of enhanced bronchoreactivity has been demonstrated in guinea pig airways (Hsiue et al, 1992), where capsaicin pre-treatment enhances the subsequent response to both ACh and NKA. In contrast to SP, bradykinin produces a significant and long lasting (up to 15 min) bronchoconstriction in allergic sheep, which is significantly greater than that occurring in normal sheep. The functional relevance of these differing effects of peptides in sheep airways is not known. In allergic sheep the epithelial derived metallo-endopeptidase enzyme system for the degradation of SP appears to be intact, which presumably would limit the role of endogenously released SP in the bronchospasm and inflammation associated with airway allergy. Loss of epithelium in airway inflammation might enable SP to act on airway smooth muscle and the airway micro-vasculature. Of the tachykinins, SP appears to be the more important for directly affecting bronchomotor tone in allergic and normal sheep, while NKA may have a role in modifying the activity of other spasmogenic agents.

The response to tachykinins in human asthma patients and other animal models of asthma contrasts with allergic sheep and this may reflect a fundamental difference between sheep and humans. However, in human asthma the relative importance of SP and NKA in the aetiopathogenesis of the disease has not been decided. Inhaled NKA is a potent bronchoconstrictor agent in asthmatic adult humans while SP appears to be ineffective (Fuller et al, 1987; Joos et al, 1987) or causes only mild bronchoconstriction in asthmatic patients. At infusion rates of 3.3pmol/kg/min SP even causes significant bronchodilation (Fuller et al, 1987). Pre-treatment with nedocromil sodium significantly reduces the response to NKA in man (Joos et al, 1989 a,b). However, Crimini et al (1988a,b; 1990) have demonstrated a reduction in forced expiratory volume in one second (FEV1) with inhaled SP in moderate

asthmatics, which again can be blocked by nedocromil sodium and the anticholinergic agent ipratropium bromide, but not by the H1 receptor antagonist astemizole. In studies where SP was ineffective the patients were classified as mild asthmatics and consequently, although individuals in all studies were asymptomatic, fundamental differences between mild and moderate asthmatics, such as airway NEP content, might exist. It is interesting to note that in asthmatic sheep suppression of NEP activity by L-thiorphan was needed to achieve bronchoconstriction with SP (Abraham et al, 1991). Furthermore, in piglets, NEP specifically ameliorates the airway response to SP and appears to have no effect on the NKA response (Haxhiu-Poskurica et al, 1992).

Injected SP also causes bronchoconstriction in experimental guinea-pig asthma models (Schreiber et al, 1988; Nagai et al, 1990) and has been shown to augment the bronchomotor response to antigen and acetylcholine in one study (Nagai et al, 1990), but the comparable effects of NKA have not been reported. In normal guinea-pigs NKA is consistently more potent than SP. In the guinea-pig asthma model SP infusion was also found to cause a reduction in respiratory rate, which agrees with the findings in normal sheep, but there was no change in respiratory rate with SP in asthmatic sheep.

NKA has been shown to increase airway responsiveness to carbachol in allergic sheep (Abrahams et al, 1991) and to methacholine in Japanese monkeys (Tamura et al, 1989) and this may underlie its role in the pathogenesis of bronchoreactive illnesses in man and animals. It would be interesting to know if neuropeptides that have minimal or variable effects on bronchomotor tone in man, such as SP, could affect responsiveness to other bronchoreactive agents. While the role of SP in asthma in man is believed to be minor compared to NKA, there is still an overall increase in the number and length of nerve fibres immunoreactive for SP in asthmatic subjects

compared to normals (Ollerenshaw et al, 1991), while there is an absence of the bronchodilating peptide VIP (Ollerenshaw et al, 1989). Furthermore, an increased cutaneous reactivity to SP, but not NKA, in asthma patients has been demonstrated (Iwamoto et al, 1990), and while NKA is probably the more important neurokinin in man, these findings support attributing a greater importance to SP in human asthma.

CHAPTER 5. AGE-RELATED RESPIRATORY RESPONSES TO SUBSTANCE P IN NORMAL SHEEP

5.1. Introduction:

In preliminary studies it was noted that the bronchomotor response to SP was greater in young sheep in comparison with adults and that occasionally periods of apnoea after SP administration occurred in some sheep. It was decided to investigate further the extent of this difference between age groups.

While in most species, including sheep (see Chapter 3), SP is a potent bronchoconstrictor and vasodilator there is evidence that SP and several other neuropeptides may be involved in other aspects of respiration. SP, VIP and ENK have been identified in the carotid bodies and are believed to be involved in baroreceptor reflexes and in the chemoreceptor hypoxic ventilatory response (Scheibner et al, 1988; Smith et al, 1990). In normal adult man the main effect of exogenously administered SP is on ventilation and not on bronchomotor tone (Fuller et al, 1987b; Maxwell et al, 1990), although SP does affect airway smooth muscle in some asthmatic subjects (Crimini et al, 1988). The bronchomotor and ventilatory responses to SP also appear to change with age (Tanaka and Grunstein, 1990) and the increase of the normal physiological hypoxia response coincides with an age-related increase in carotid body glomus cell SP-like immunoreactivity (Scheibner et al, 1988).

5.2. Materials and Methods:

Experiments were carried out in 11 Suffolk cross and 4 Scottish Blackface female sheep. The sheep weighed 30-60 Kg and were divided into four age groups; group 1, 4-6 months of age (n=3); group 2, 6-12 months (n=4); group 3, approximately 18 months; group 4 approximately 4 years (n=4). Group 4 were Scottish Blackface as

these were the only aged sheep available from commercial sources.

All experimental methods, including instrumentation, drug delivery and data analysis, were as described earlier (Chapter 2), except blood pressure was not measured. The significance of any difference between groups was determined by comparing the mean responses at each dose level using one way analysis of variance.

The respiratory effects of rapid intravenous injection of SP (0.01-0.5umol/kg, group 1; 0.1-5umol/kg, groups 2,3 and 4) were assessed in each age group. Cdyn and RL were measured 20-30 seconds after SP injection and respiratory rate was also calculated during this 10 second period. The presence of apnoeic periods (s) was also noted and included periods of complete cessation of respiration and/or concurrent reduction in pressure and flow. Typically apnoea occured 25-30s after SP injection.

5.3. Results:

There was a dose-dependent decrease in Cdyn and increase in RL in all sheep and this was most pronounced in the youngest age group (Figures 42 and 43). This group also achieved a greater maximum bronchoconstriction compared to the other groups. The changes in RL and Cdyn in groups 1 and 2 were significantly different from that in groups 3 and 4, while the increase in RL in group 1 was significantly different from that in group 2. Group 3 had a significantly greater decline in Cdyn than group 4.

There was a dose-dependent reduction in respiratory rate in all four groups (Figure 44). Groups 1 through 4 had mean basal respiratory rates of 30, 35 (+/-2.9), 36.2 (+/-8) and 29 (+/-3.3) breaths per minute respectively and there was no statistically significant difference in basal respiratory rates between groups. The decrease in respiratory rate was similar for groups 1 and 4 and was significantly different from group 3. Three sheep in group 4 also had marked apnoeic periods (Figure 45), which

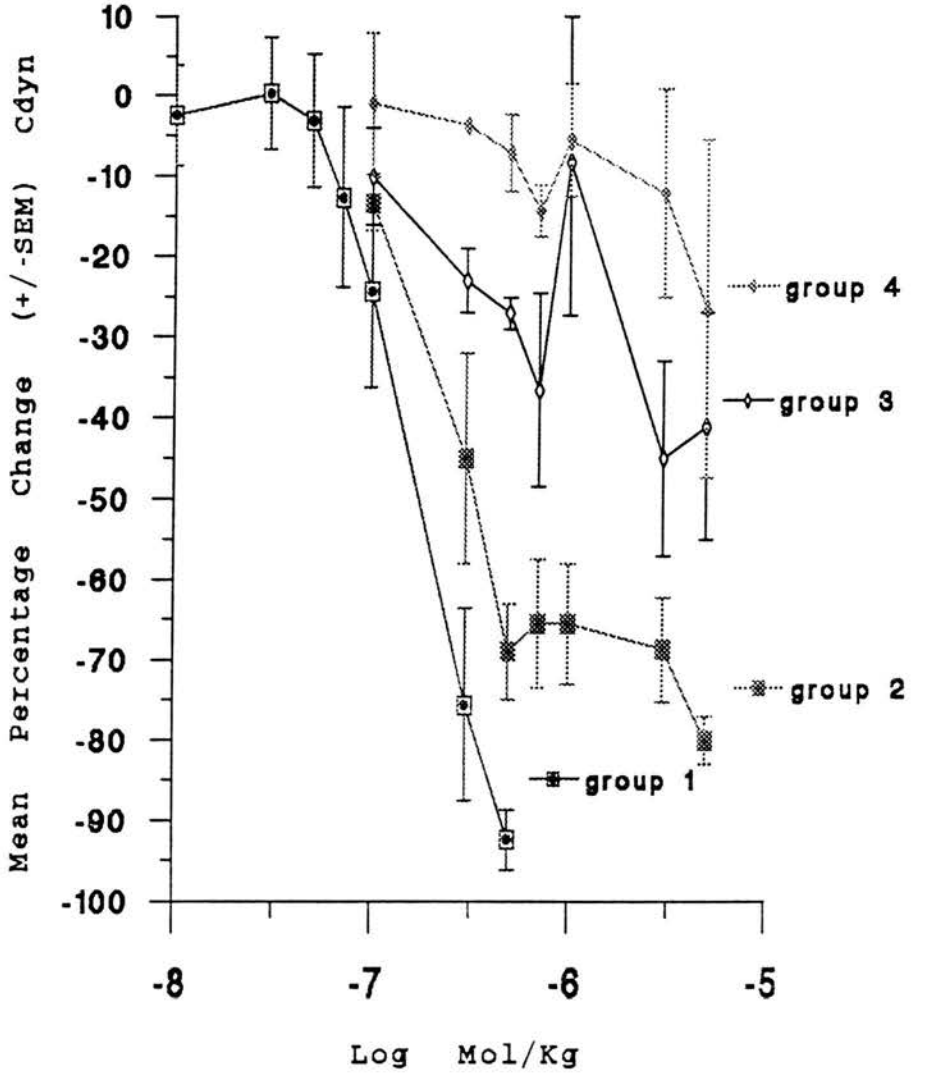


Figure 42. Percentage change in dynamic compliance (Cdyn) after intravenous administration of substance P (0.01-5 μmol/kg) in four different age groups of sheep. Group details are given in the text. The magnitude of the decline in Cdyn is age-related with the most pronounced change in the youngest sheep (group 1; 4-6mths), and only minimal change in the oldest group (group 4; >4yrs).

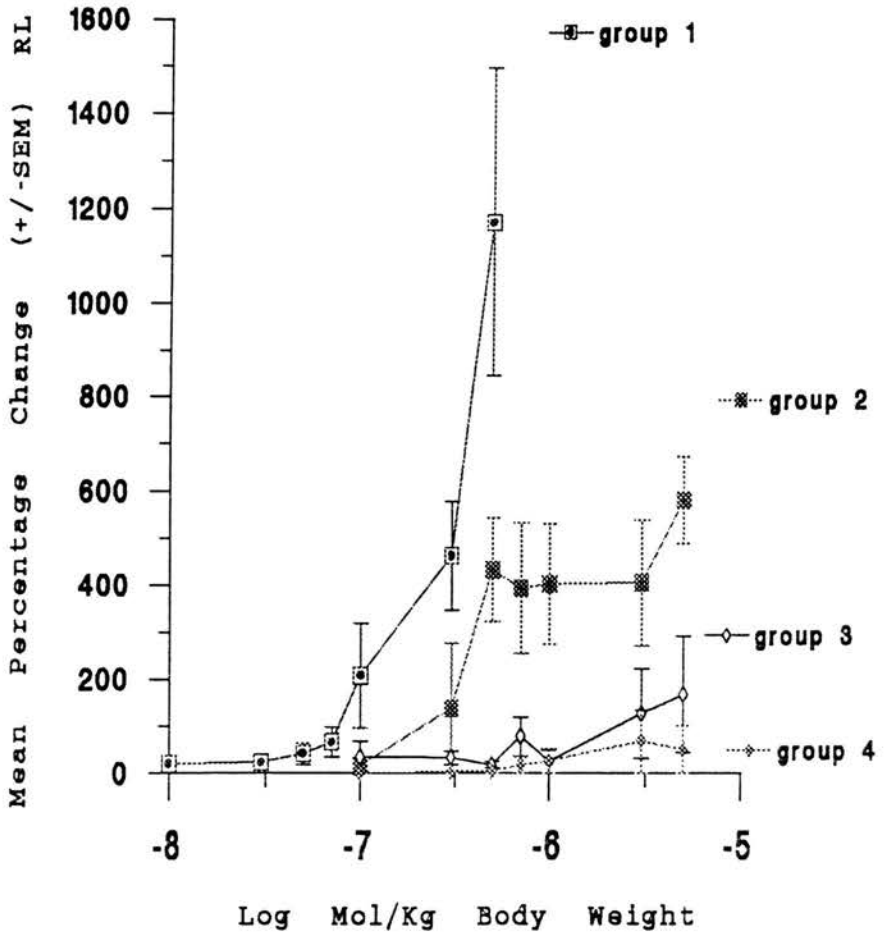


Figure 43. Percentage change in respiratory resistance (RL) after intravenous administration of substance P (0.01-5 μmol/kg)in four different age groups of normal sheep. Group details are given in detail in the text. As for the change in Cdyn (Figure 42) the magnitude of the increase in RL was age-related with the most pronounced change in the youngest sheep (group 1; 4-6mths) and only minimal and insignificant change in the oldest sheep (group 4; >4yrs).

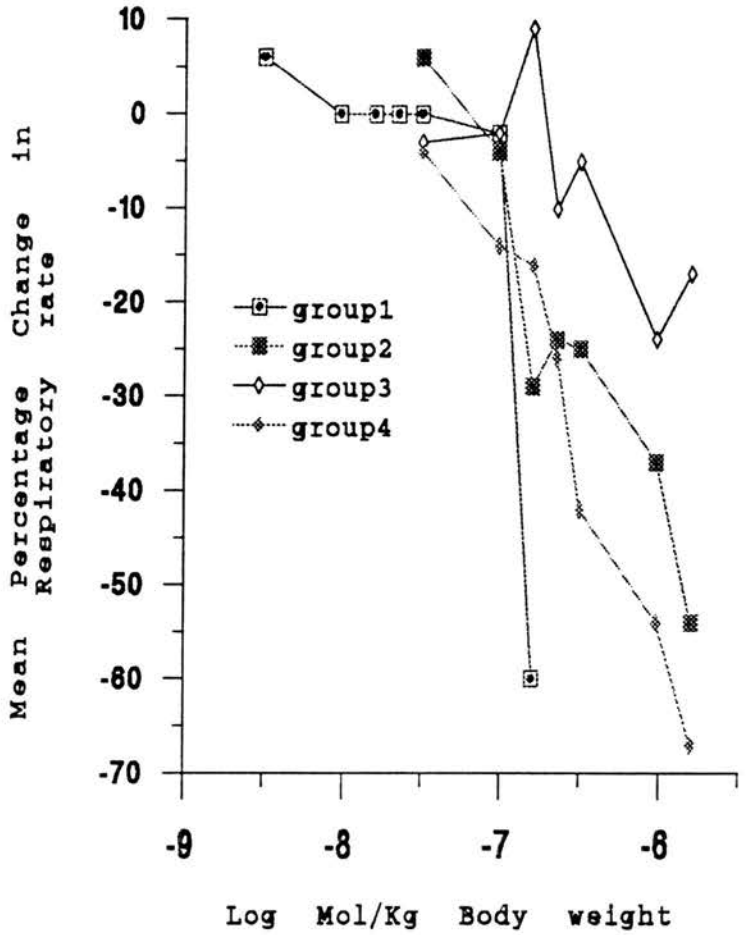


Figure 44. Percentage change in respiratory rate after intravenous administration of substance P (SP) (0.01-5 μmol/kg) in four different age groups of normal sheep. Group details are given in the text. The youngest sheep and the oldest sheep (groups 1 (4-6mths) and 4 (>4yrs) respectively) had the most pronounced reduction in respiratory rate, while the reduction in respiratory rate was less pronounced in the young adult sheep (groups 2 (6-12mths) and 3 (12-18mths)). SEM bars have been omitted for clarity.

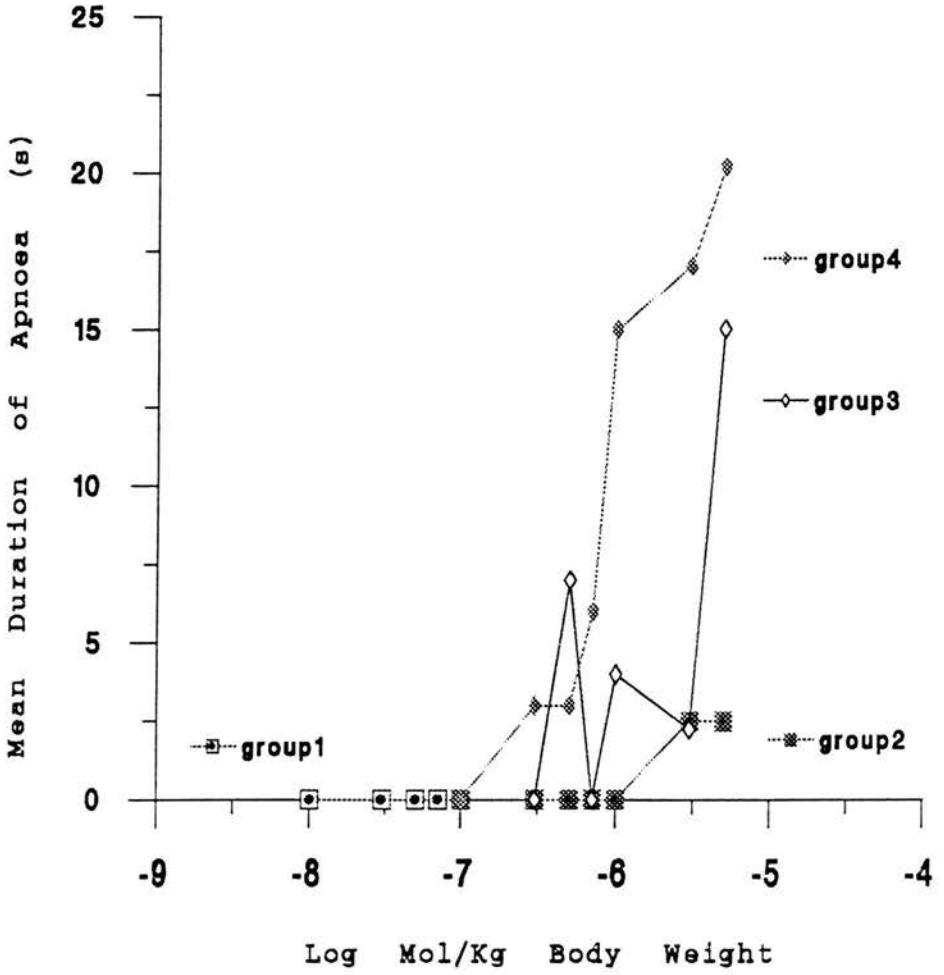


Figure 15. The development of apnoea after intravenous administration of substance P (SP) (0.01-5 μmol/kg) in four different age groups of sheep. Group details are given in the text. The two oldest groups (group3 (12-18mths) and 4 (>4yrs)) demonstrated periods of apnoea after SP administration, which were most consistent in the oldest group (group 4). At no time did sheep in the youngest group (group 1; 4-6mths) demonstrate apnoea.

were dose dependent. However, one sheep in group 4 did not develop apnoea, but had the most pronounced bronchoconstrictor response for that group. At no time did group 1 sheep demonstrate apnoea, and in groups 2 and 3 apnoeic periods were minor or inconsistent.

5.4. Discussion:

Several studies of the ontogeny of neuropeptide innervation and expression in different mammalian organ systems and different species have been previously reported. Ontogenic changes in SP concentrations have been demonstrated in baboon cerebral cortex (Beal et al, 1988), thoracolumbar spinal sympathetic preganglionic nuclei in rats (Newton et al, 1988), in several areas of the rat central nervous system (Diez-Guerra, et al, 1989; Sivam et al, 1991), chicken carotid bodies (Kameda, 1990) and guinea-pig uterus (Alm et al 1988). In rat lung SP content is constant between 3 and 20 months of age (immature to senescent), but rises rapidly in aged rats (Amenta et al, 1988). Developmental changes in NPY content and distribution have also been demonstrated in mouse hypothalamus and adrenal glands (Chua et al, 1991), rat superior cervical ganglia and perivascular nerve fibres (Dhital et al, 1988; Gurusinghe et al, 1990) and Rhesus monkey neurohypophysis (McDonald et al, 1988). The ability of SP to elicit certain types of slow potentials in the rat spinal cord is reduced and eventually lost during maturation (Gibbs & Kendig, 1992). This suggests the reduction in response to neuropeptides may be due to a loss of tissue sensitivity rather than an overall reduction in the level of tissue peptides. The results from rats further suggest that neuropeptides have an important role in developmental neurobiology.

Immunohistochemical data from man indicates there are fewer peptide-containing, including SP, nerves in the lung of young adults compared to infants, children and adolescents (Hislop et al, 1990), while there is an age related increase in NPY-like

immunoreactivity, particularily in peri-arterial nerve fibres (Allen et al, 1989). SP has also been demonstrated in human lung neuroendocrine cells (Gallego et al, 1990) with SP-like immunoreactive cells being more numerous in foetal and infant than in adult lung. Furthermore, Hislop and co-workers (1990) have noted an age-related increase in the neural expression of immunoreactivity for the bronchodilating neuropeptide VIP in human lung and have suggested that alteration in neural peptide content and/or number of neuropeptide-containing nerve fibres may underlie the age-related change in asthma symptoms seen in man. While changes in neuropeptide expression in airway nerves may have a bearing on the bronchomotor response to these peptides, or their involvement in respiratory disease, the ontogeny of neuropeptides suggests they have a role in lung development (Johnson et al, 1988; Gallego et al, 1990; Rinaldi et al, 1991).

In the present study it was demonstrated that SP is a potent bronchoconstrictor in sheep, that the response decreases with age and that in aged sheep SP causes apnoea. The effects of breed differences or the possible existence of subclinical respiratory disease in the aged sheep on the results was not assessed. In organ bath studies with isolated trachealis muscle preparation a similar lack of contractile response to SP in aged sheep was found, and the details are given in chapter 6. SP is recognised as a potent bronchoconstrictor in a variety of species, but it has little effect on bronchomotor tone in normal adult human subjects where the predominant effect is to increase ventilation and improve the hypoxic respiratory drive (Fuller and others, 1987b). This increased ventilatory response in man is due to a direct action of SP on chemoreceptors in the carotid bodies or peripheral chemoreceptors with direct input to the medullary respiratory centre (Maxwell et al, 1990). The demonstration of neuropeptides, including SP, in the carotid body and in the carotid body-like laryngeal paraganglia structures supports the theory that they are involved in the peripheral control of respiration (Dahlqvist et al, 1992). However, SP is also believed to be

involved in the pulmonary chemoreflex (Bezold-Jarisch Reflex). This is a complex reflex involving a combination of bradycardia, systemic hypotension, bronchoconstriction and apnoea followed by tachypnoea (Coleridge & Coleridge, 1984; Green et al, 1984). In experimental situations the pulmonary chemoreflex is usually activated by agents such as phenylbiguanide, capsaicin, bradykinin and several of the eicosanoids (Karczewski & Widdicombe, 1969; Kaufmann et al, 1980; Coleridge & Coleridge, 1986). Activation of pulmonary C-fibre afferents by both SP and capsaicin evokes rapid shallow breathing (Green et al,1984; Prabhakar et al, 1987) and in addition injection of capsaicin as a bolus into the right atrium, but not the systemic arterial circulation, can also result in apnoea (Green and others, 1984). Therefore, it is possible that the rapid injection of SP into the systemic venous circulation may activate this apnoeic part of the pulmonary chemoreflex. This part of the reflex may be easier to activate in adult sheep than in lambs and may constitute the primary response of the adult airway to SP. It has been shown that direct application of SP to the central nervous system stimulates respiration. However, adult human sufferers of sleep apnoea syndrome have elevated SP levels in their cerebro-spinal fluid (Gislason et al, 1992), and a role for SP in the central and peripheral control of respiration can be hypothesised.

The change with age of the sheep SP response contrasts with other species as well as with the reaction of sheep to other spasmogenic agents. Sheep have been shown to demonstrate an age-related increase in the bronchomotor response to both histamine and carbachol (Saunders et al, 1984). In rabbits the neuromodulatory action of SP on cholinergic neurotransmission and control of bronchomotor tone increases with age (Tanaka and Grunstein, 1990), but this is due to an increase in intrinsic bronchoreactivity rather than an increase in the contractile response to SP itself. In the cat carotid body there is an age-related increase in SP-like immunoreactivity coiniciding with development of the hypoxic ventilatory response (Scheibner et al,

1988), while there is an age-related decrease in SP-containing nerves in normal human airways (Hislop et al, 1990). The change in human neuronal expression of peptides and the sheep bronchomotor response to SP roughly coincides with the onset of maturity.

The apnoeic response in aged sheep, as mentioned earlier, is probably due to activation of pulmonary C-fibre afferent nerve endings, and while the functional significance of these findings is not known, these preliminary results suggest a limited role for SP in the control of bronchomotor tone in adult sheep. The possibility that SP may be stimulating lung afferent receptors also needs to be considered with respect to the bronchomotor response to SP and this subject is discussed in detail in chapter 7.

CHAPTER 6. PHARMACOLOGICAL ANTAGONISM OF THE BRONCHOMOTOR EFFECTS OF SUBSTANCE P IN NORMAL SHEEP AIRWAYS

6.1. Introduction:

The exact mode of action of SP and other neurokinins is not completely understood and there appear to be definite species differences (Barnes, 1989). In man and guinea pig the bronchomotor response to SP is believed to be largely direct (Palmer et al, 1987), involving interaction with specific neurokinin receptors on airway smooth muscle. However, there are numerous studies in other species demonstrating pharmacological antagonism (using non-neurokinin antagonists) of the bronchoconstriction caused by the neurokinins, particularily cholinergic mediated events in the rabbit, rat and pig (Tanaka & Grunstein, 1986; Joos et al, 1988; Hashiu-Poskurica et al, 1992). Furthermore, the effect of SP in asthmatic human subjects can be antagonised by nedocromil sodium, sodium cromoglycate or the muscarinic antagonist atropinic agent ipratroprium bromide (Crimini et al, 1988a,b; Crimini et al, 1990; Joos et al, 1989a,b). The close anatomical association that has been demonstrated between cholinergic and peptidergic neural elements in various tissues would suggest a functional interaction (Martone et al, 1992; Schmidt et al, 1992) and this may also be applicable to the respiratory system. More likely than not, the event that initiates bronchconstriction with SP involves a specific NK-1 receptor, although the exact location of this receptor in airways and its association with other neural and non-neural elements in the airways is not yet completely understood (Barnes, 1991).

The aim of this series of experiments was to evaluate the effects of a variety of pharmacological agents and specific neurokinin antagonists on the bronchomotor response to SP in normal sheep.

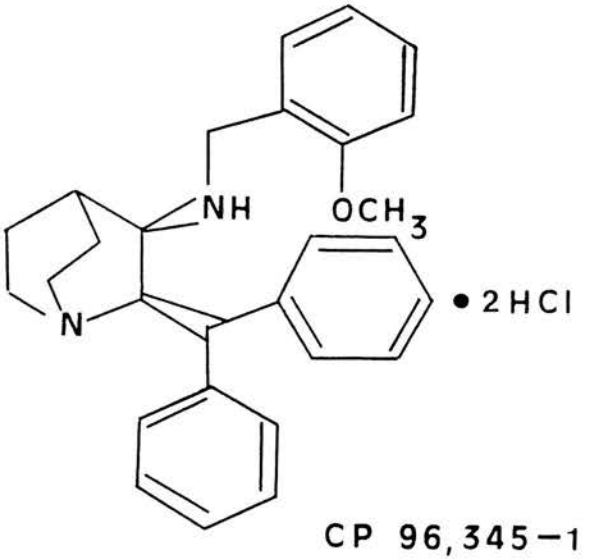
6.2. Materials and Methods:

Experiments were carried out in vivo using anaesthetised sheep and in vitro using an isolated trachealis muscle preparation. The experimental details are outlined in Chapter 2.

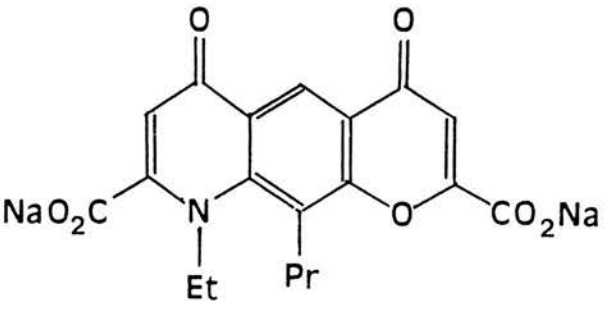
6.2.1. Pharmacological Antagonism of Substance P in Anaesthetised Sheep:

Experiments were carried out in Suffolk-cross sheep, 6-12 months old, as described earlier. A dose response curve to intravenous SP was obtained for each sheep so that a dose giving 50 to 80% (submaximal) of the maximal bronchoconstrictor response could be determined. The effects of the general muscarinic antagonist atropine, the ganglion blocker hexamethonium, the H1 receptor antagonist chlorpheniramine, the non-peptide NK-1 antagonist CP-96,345-1 ((+/-)-Cis-3(2-methoxy-benzylamino)-quinuclidine-4) (Figure 46), the peptide neurokinin antagonist spantide ([D-Arg^{1},D-Trp^{7,9},Leu^{11}]SP) and nedocromil sodium (Figure 46) on the response to a submaximal dose of SP was assessed. The decision to use a sub-maximal dose of SP was due to the tachyphylaxis noted with repeated dose-response curves of SP, and the ability to achieve reasonable repeatability of the response to sub-maximal doses at suitable dose intervals. Further details are given in chapter 3.

Atropine (1mg/kg), hexamethonium (20mg/kg), chlorpheniramine (2mg/kg), CP-96,345-1 (0.1 and 0.5mg/kg) and nedocromil sodium (0.1 and 1mg/kg) were administered as a single intravenous bolus injection, while spantide (10ug/kg/min) was administered slowly using a continuous infusion pump (Re Cy Chrom, LBK-Produkter, Sweden) connected to the cephalic intravenous catheter. All agents were administered via the right cephalic vein at least 10 minutes after the last SP dose. The subsequent SP dose was given 10 minutes after injection, or 10 minutes into the



CP 96,345−1



NEDOCROMIL SODIUM

Figure 46. The chemical structure of the NK-1 antagonist CP 96, 345-1 and the anti-asthma drug nedocromil sodium.

spantide infusion, giving an inter-SP dose interval of at least 20 minutes.

Atropine sulphate, hexamethonium bromide, chlorpheniramine maleate and spantide were purchased as indicated in Appendix II. CP-96,345-1 and nedocromil sodium were gifts from Pfizer Inc (USA) and Fisons Ltd (UK) respectively. All agents were dissolved in PBS.

6.2.2. Pharmacological Antagonism of Substance P in the Isolated Sheep Trachealis Muscle Preparation:

Experiments were carried out using trachealis muscle preparation obtained from female sheep aged 6-12 months and in a group of sheep aged over 4 years. The dose-response characteristics of this preparation to SP were investigated as was the influence of atropine, the M2 receptor antagonist pirenzepine (Research Biochemical Incorporated, UK) the peptide SP antagonist spantide and the NK-2 antagonist L-659,874 (Ac-Leu-Met-Gln-Trp-Phe-Gly-NH2) (Cambridge Research Biochemicals Ltd, UK) on the contractile response to SP.

6.3. Results:

6.3.1. In Vivo Experiments:

The effects of atropine, hexamethonuium, chlorpheniramine, CP-96-345-1, nedocromil sodium and spantide on the response to SP was assessed in normal anaesthetised sheep.

6.3.1.1. Atropine and Hexamethonium:

Atropine pre-treatment (n=6) significantly reduced the bronchomotor response to SP from a control RL/Cdyn value of +575/-70% to +12/-17% (Figure 47). In 2 sheep there was still an appreciable reduction in Cdyn (-60 & -56%) with SP after atropine

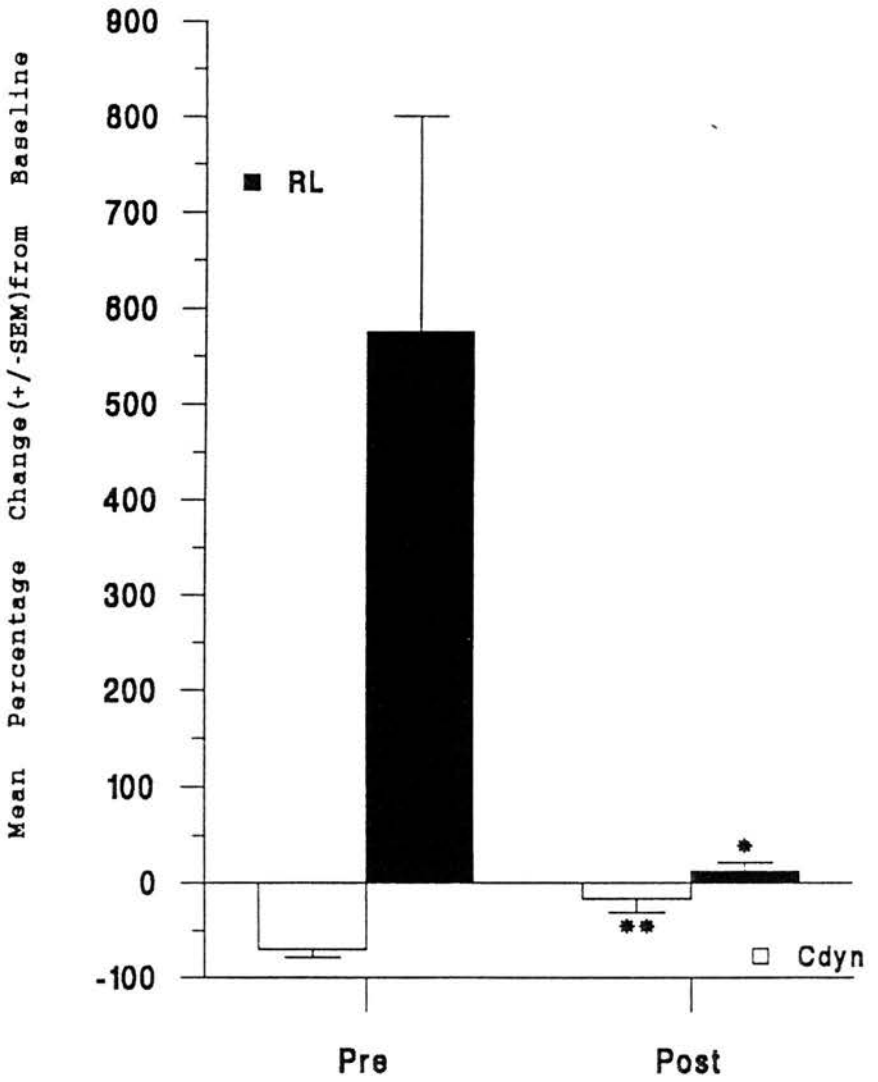


Figure 47. The effect of intravenous pretreatment with atropine (1mg/kg) on the percentage change in dynamic compliance (Cdyn) and pulmonary resistance (RL) after intravenous administration of substance P (SP) (n=6). Pre and Post represent the SP response before and after atropine. * $p<0.05$, ** $p<0.01$.

pre-treatment, but the change in RL had been abolished. Atropine itself did not affect basal bronchomotor tone.

Hexamethonium (n=3) also reduced the SP-induced change in Cdyn and RL to SP from a control value of -71+/-4 to 43+/-10% for Cdyn (P<0.05) and from 425+/-168 to 44+/-33% for RL (P<0.05) (Figure 48). Hexamethonium also markedly reduced mean blood pressure from 98+/-10 to 67+/-14mmHg (P<0.05) (Figure 49).

6.3.1.2. Histamine H1 Receptor Antagonist:

Chlorpheniramine was administered to 6 sheep. In one sheep administration of SP resulted in apnoea and bronchodilation (RL/Cdyn; -37/+15%), but after chlorpheniramine pre-treatment mild bronchoconstriction occurred (RL/Cdyn; +70/-19%). In the 5 other sheep SP caused varying levels of bronchoconstriction (RL +234 +/-135%; Cdyn -39 +/-13%) (Figure 51). Intravenous chlorpheniramine itself caused a transient rise in bronchomotor tone (RL/Cdyn; +51/-8%) and stimulated respiration (Figures 50 and 51), and then caused significant augmentation of the response to SP (RL +477 +/-205%; Cdyn -55 +/-10%) in all 5 sheep (t-test; p<0.05) (Figure 51).

6.3.1.3. Neurokinin Receptor Antagonists:

Intravenously administered CP-96,345-1 at 0.5 mg/kg (n=5) caused an initial transient rise in RL (+135%) and fall in Cdyn (-60%) in 3 of the 5 sheep, but values returned to baseline after 2 to 3 minutes (Figure 53). CP-96,345-1 reduced the change in RL/Cdyn to SP from control values of +204/-43% to +9/+3% and so effectively abolished the response to SP. The effect of 0.1 mg/kg of CP-96,345-1 was assessed in a separate group of sheep (n=4). Unlike the higher dose group there was no effect on basal RL and Cdyn (Figure 51), but it did abolish the response to SP reducing the mean RL/Cdyn values from +93/-35 to -2/+1.

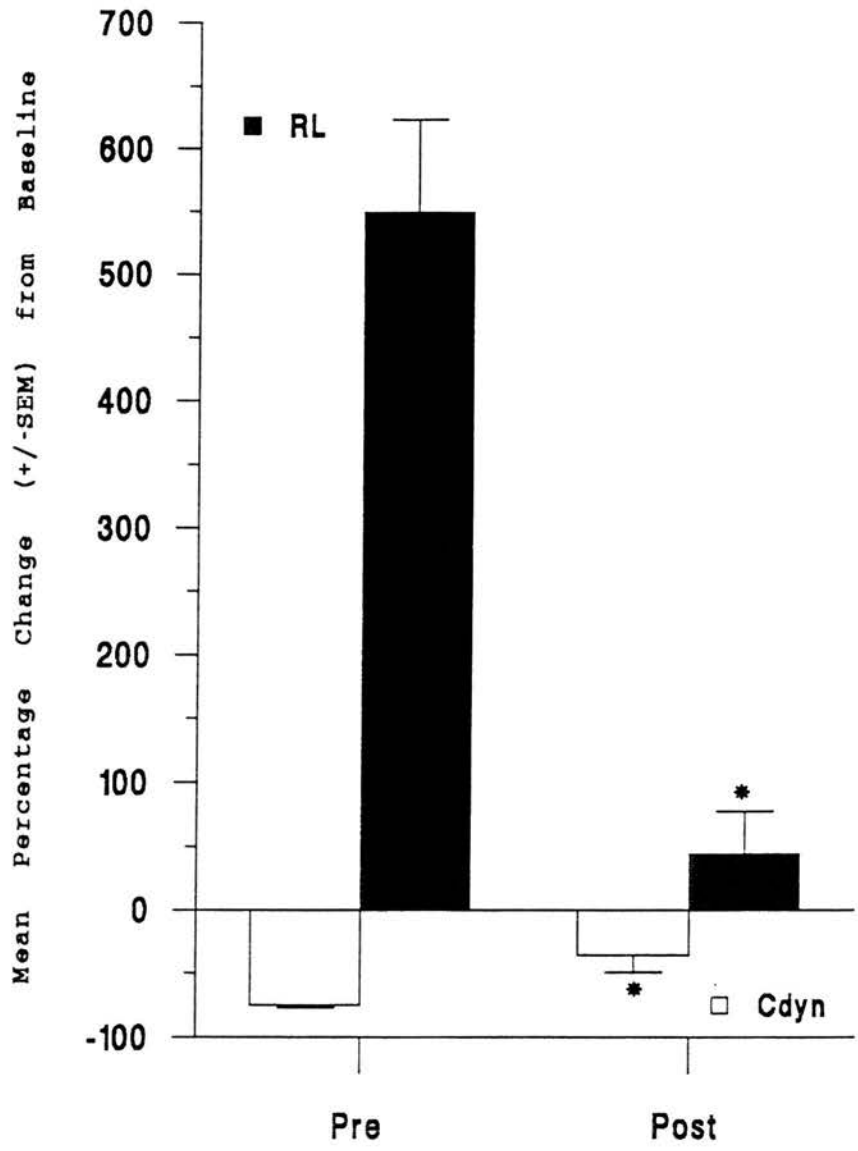


Figure 48. The effect of intravenous pretreatment with hexamethonium bromide (20mg/kg) on the percentage change in dynamic compliance (Cdyn) and pulmonary resistance (RL) after intravenous administration of substance P (SP) (n=3). Pre and Post represent the SP response before and after hexamethonium. *$p<0.05$.

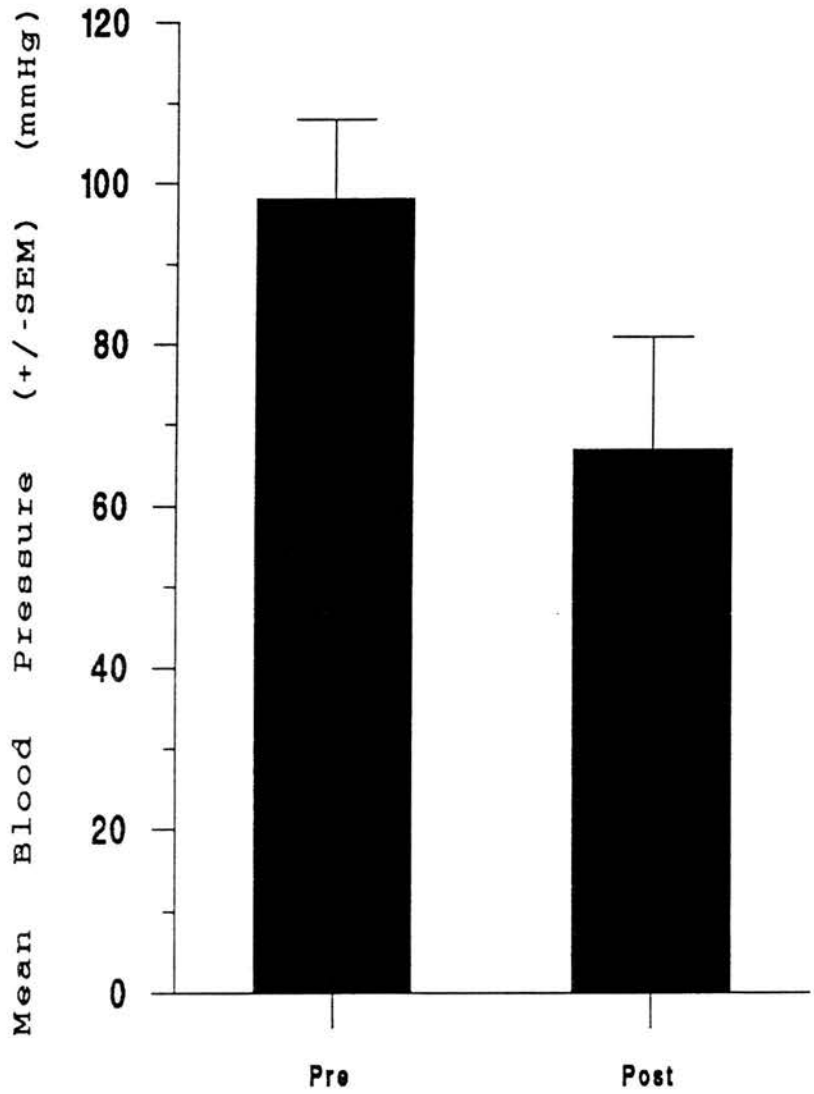


Figure 49. The effect of hexamethonium on mean blood pressure (n=3). Hexamethonium significantly ($p<0.05$) reduced the mean blood pressure from a level of 98+/-10 (Pre) to 67+/-14 mmHg (Post).

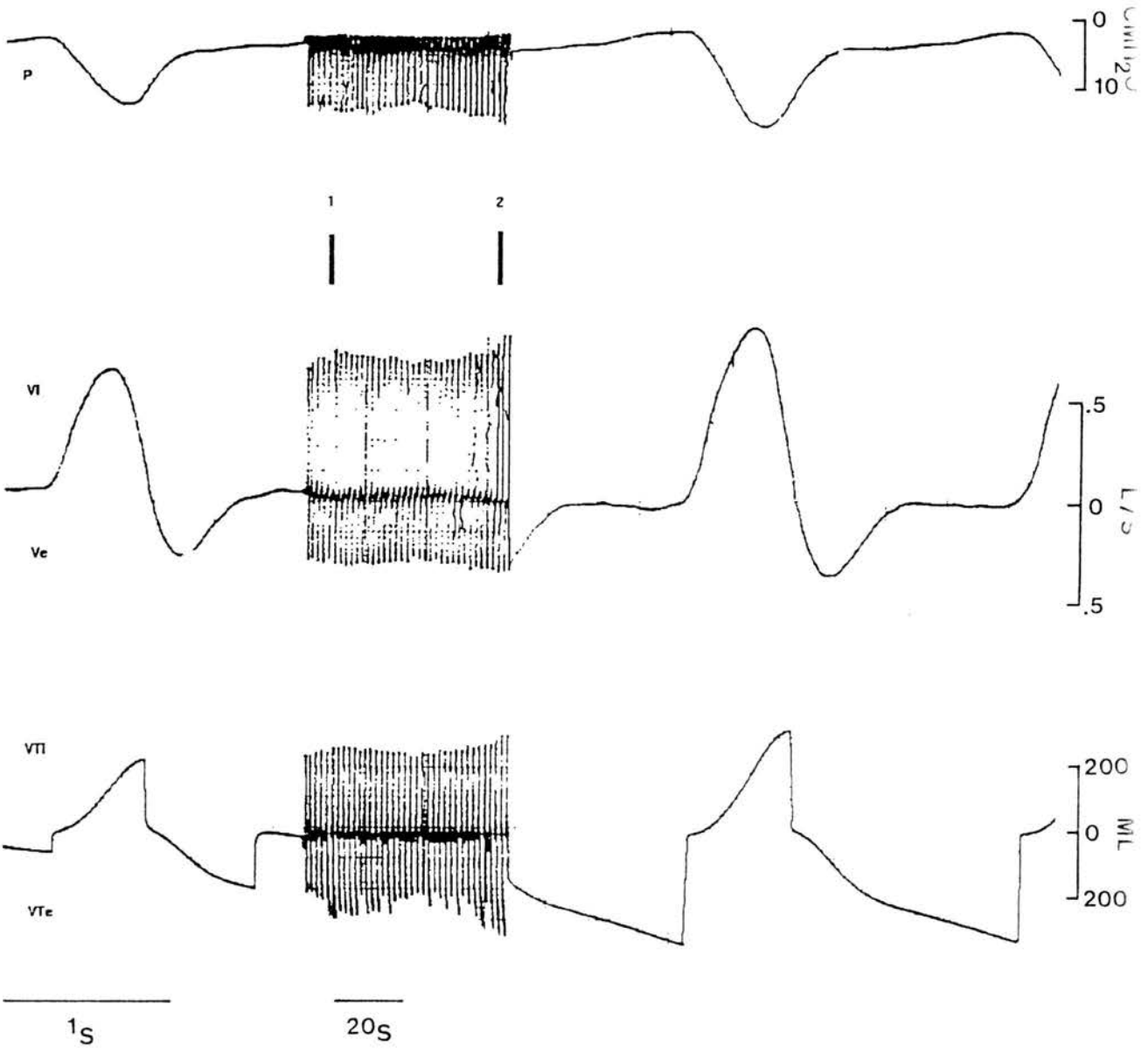


Figure 50. The effect of the H1 receptor antagonist chlorpheniramine (1mg/kg) on respiratory parameters in a normal sheep. Chlorpheniramine was injected intravenously over a 60s period (marker points 1 to 2) and stimulated respiration resulting in an increase in transpulmonary pressure (P), inspiratory and expiratory flow (Vi, Ve) and inspiratory and expiratory tidal volume (VTi, VTe). These parameters returned to baseline within a further 2 to 3 minutes in all sheep. The tracing is shown at two paper speeds.

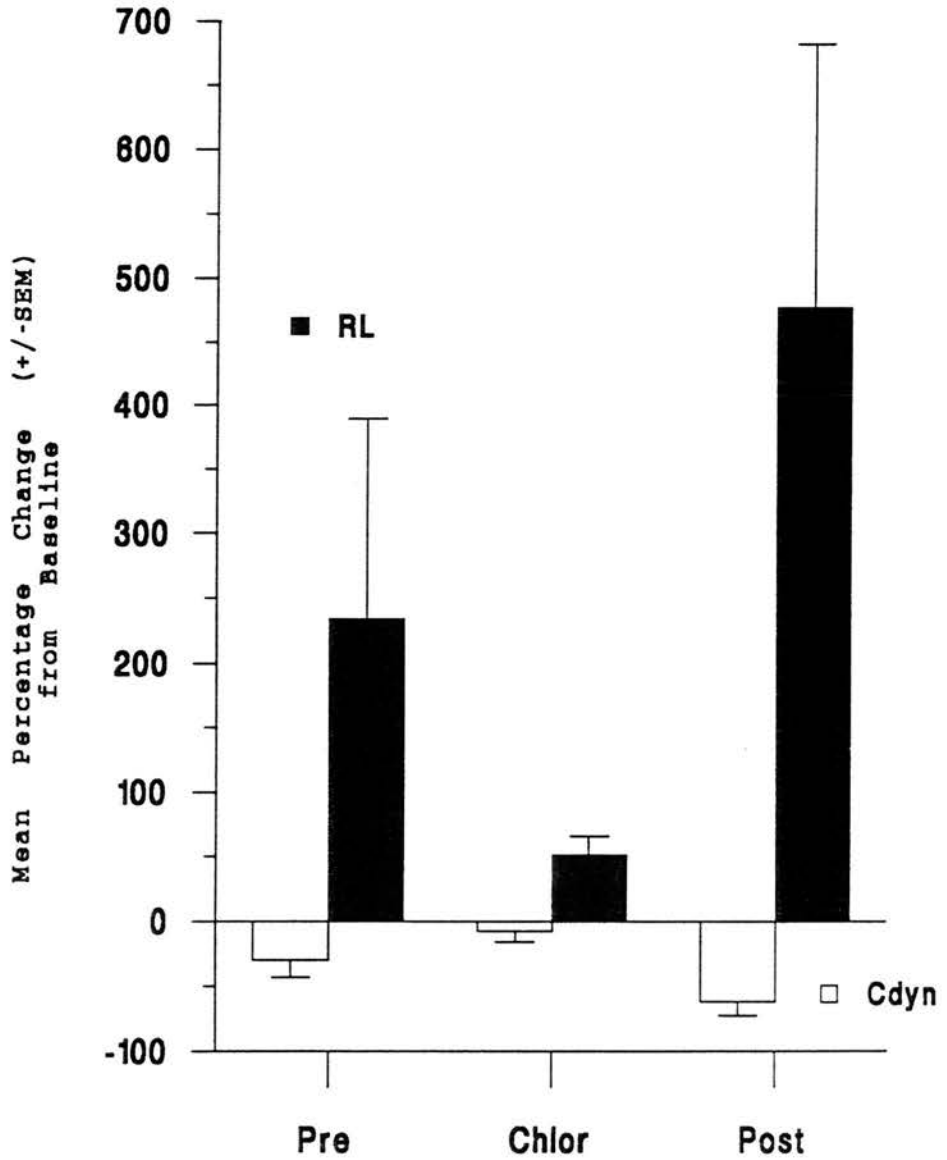


Figure 51. The effect of intravenous pretreatment with chlorpheniramine (2mg/kg) on the percentage change in dynamic compliance (Cdyn) and pulmonary resistance (RL) after intravenous administration of substance P (SP) (n=5). Pre and post represent the SP response before and after chlorpheniramine and chlor is the effect of chlorpheniramine on basal bronchomotor tone. Chlorpheniramine caused a transient bronchoconstrictor effect and then significantly augmented the bronchomotor response to SP (Paired t-test; $p<0.05$).

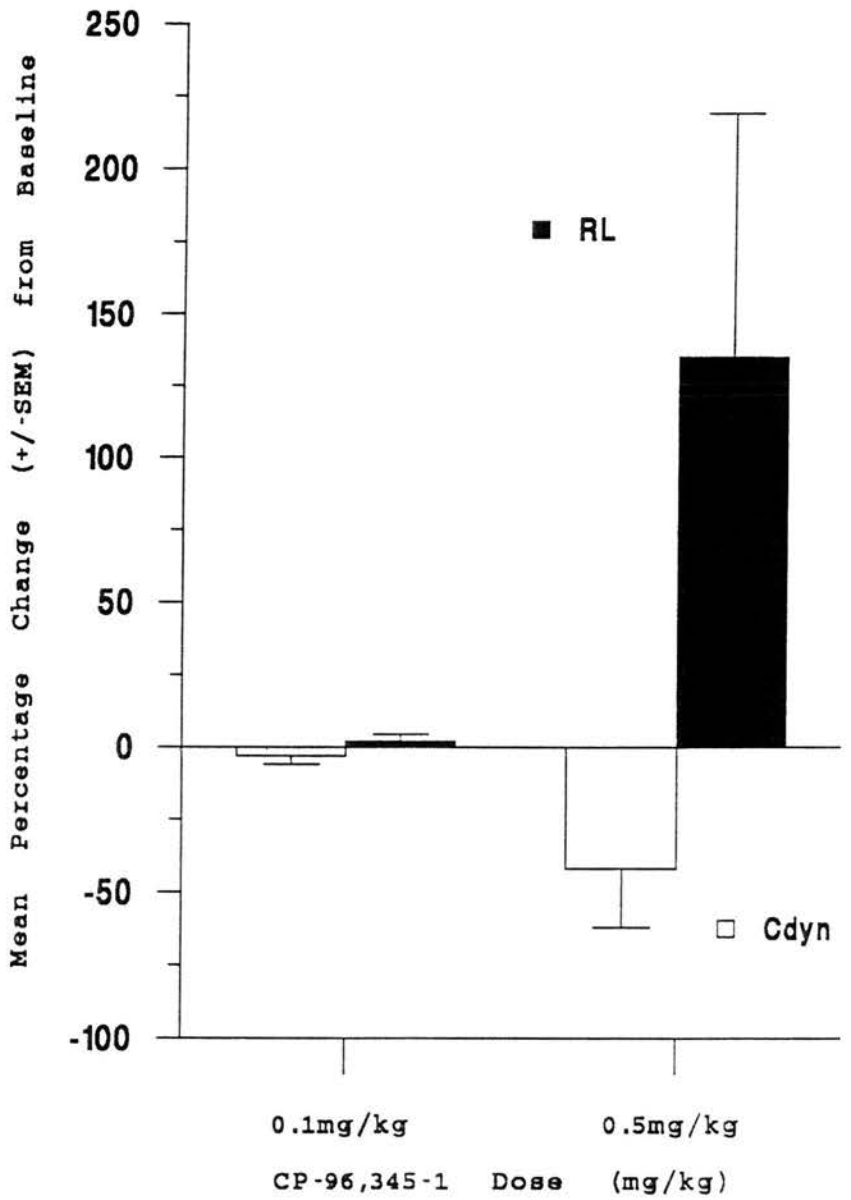


Figure 52. The effect of the intravenous administration of the NK-1 antagonist CP-96,345 at two dose levels on basal dynamic compliance (Cdyn) and pulmonary resistance (RL). The 0.1mg/kg dose had no effect on basal bronchomotor tone, but 0.5mg/kg caused a transient bronchoconstriction.

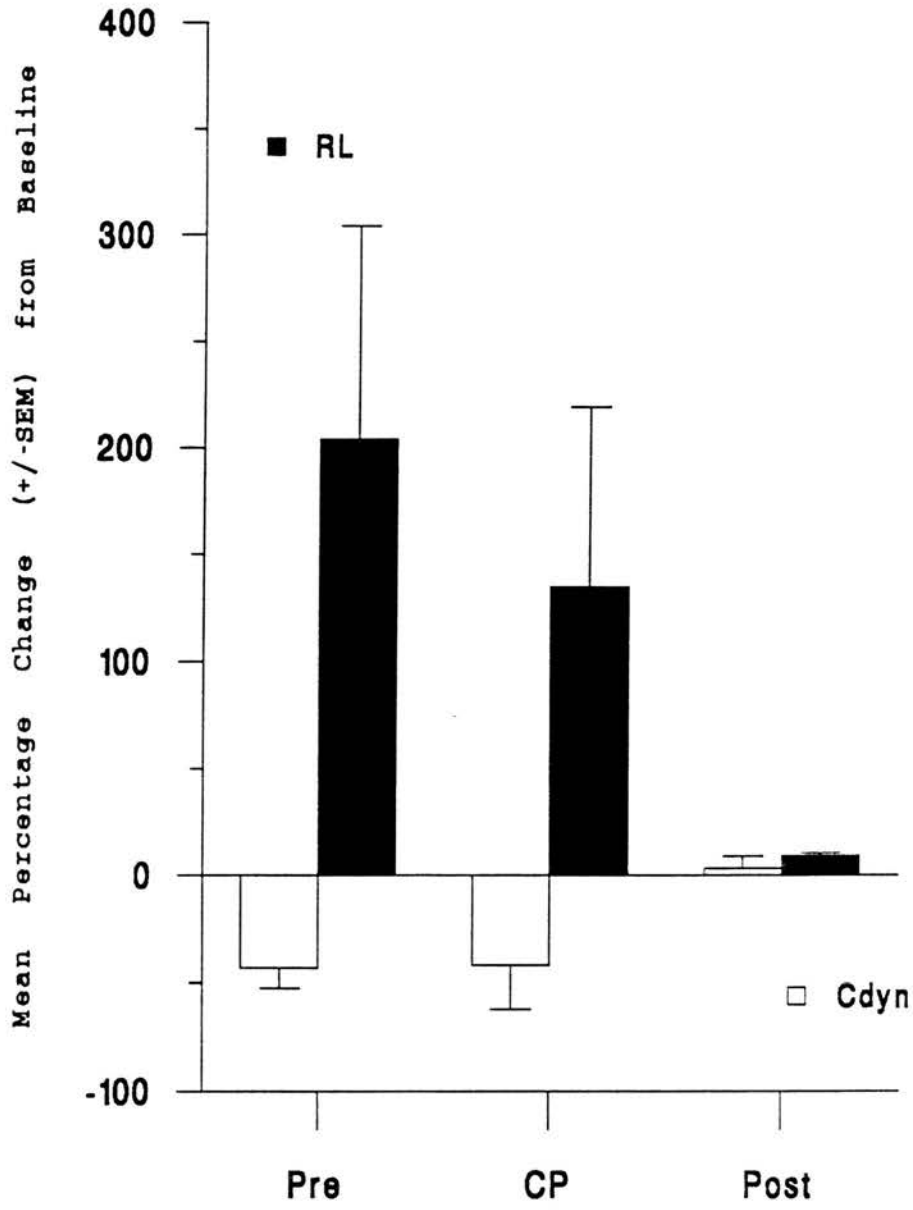


Figure 53. The effect of intravenous pretreatment with the NK-1 antagonist CP-96,345 (0.5mg/kg) on the percentage change in dynamic compliance (Cdyn) and pulmonary resistance (RL) after intravenous administration of substance P (SP) (n=5). Pre and Post represents the SP response before and after CP-96,345, and CP is the effect of CP-96,345 on basal bronchomotor tone. CP-96,345 caused a transient bronchoconstriction and then abolished the response to SP.

Infusion of spantide was carried out in 4 sheep. In one sheep SP caused apnoea of 35s which was not affected by spantide (36 s). In the other three sheep spantide reduced the effect of SP on RL (+75 +/-29 to +44 +/-11%) and Cdyn (-30 +/-7 to -26 +/-0.6%). The effect of spantide on the SP response was not significant (Figure 54).

6.3.1.4. Nedocromil Sodium:

Nedocromil sodium was administered to 4 sheep and had a variable effect on the response to SP. Nedocromil sodium itself, at 0.1 and 1 mg/kg, caused mild bronchodilation with RL/Cdyn values of -20/+11 and -37/+13.5 respectively, although one sheep (#48) had no response (Figure 55). At the lower dose two of the four sheep showed a subsequent marked increase in the response to SP over the pre-nedocromil sodium response, while in the other two sheep the response was reduced. Overall, there was no significant potentiation of the response to SP after this dose of nedocromil sodium (Paired t-test) At the 1mg/kg dose where the effect of nedocromil sodium on RL was appreciably greater, there was only a slight reduction in the response to SP in three sheep with a more marked reduction in one (Figure 55). The sheep (#48) whose basal bronchomotor tone was unaffected by nedocromil sodium had the greatest reduction in response to SP.

6.3.2. In Vitro Experiments:

The response to SP was expressed as the percentage of the response to 1umol methacholine chloride, and drug concentration refers to the final drug concentration in the organ bath. The tissue began to contract within 20 s of addition of methacholine to the organ bath, peaked after 5 min and returned to baseline tension 3 min after washout.

There was certain difficulties with the preparations response to SP. Addition of the NEP inhibitor phosphoramidon was necessary to elicit a contractile response with SP,

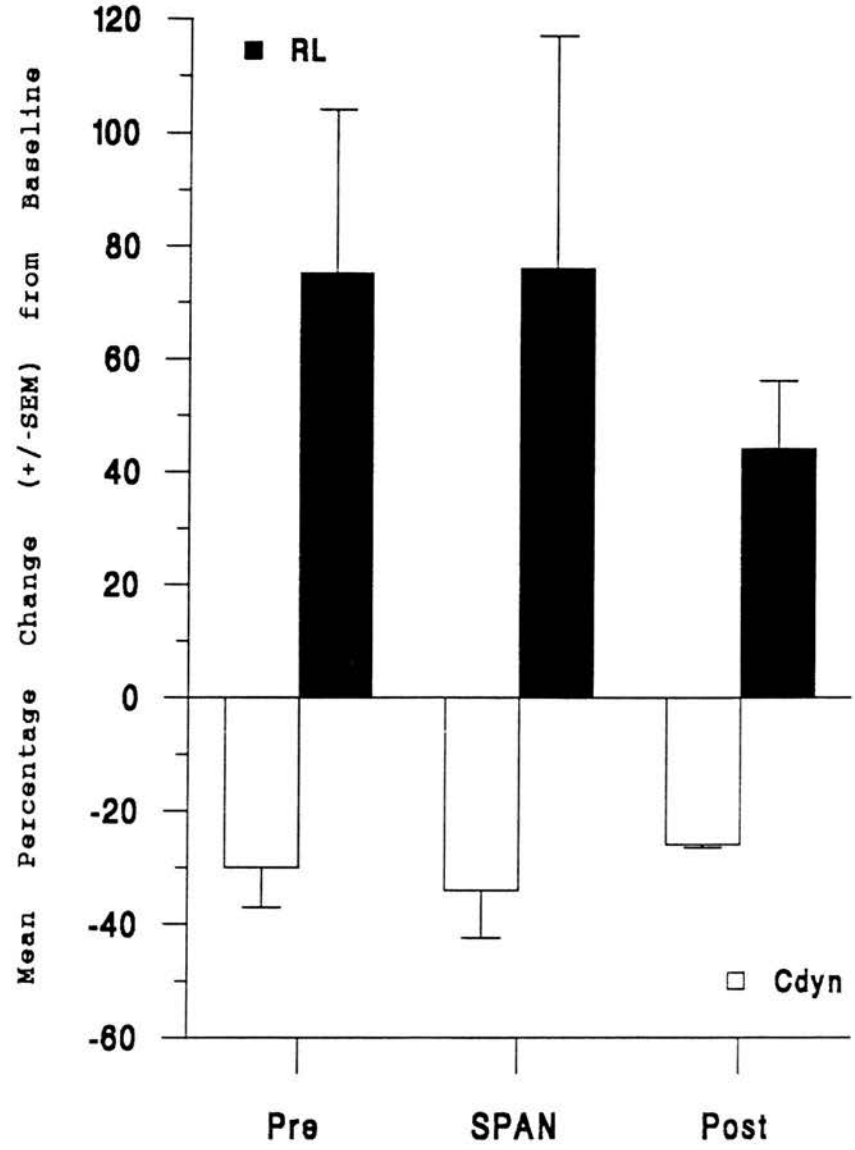


Figure 54. The effect of intravenous infusion of the neurokinin antagonist spantide (10ug/kg/min) on the percentage change in dynamic compliance (Cdyn) and pulmonary resistance (RL) after intravenous administration of substance P (SP) (n=3). Pre and Post represents the SP response before and after spantide (10 μg/kg/min for 10 min) and SPAN the response to spantide itself. Spantide infusion over 10 minutes caused a transient bronchoconstriction similar in magnitude to the response with SP and then slightly reduced the response to SP.

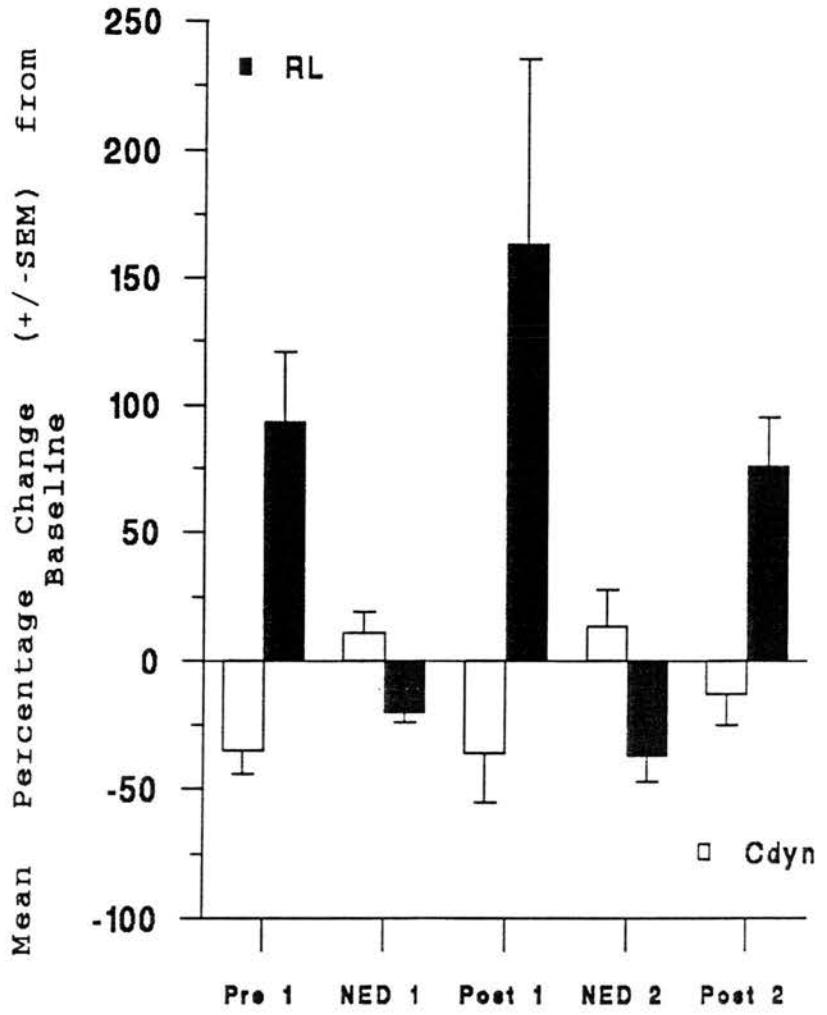


Figure 55. The effect of intravenous pretreatment with nedocromil sodium on the percentage change in dynamic compliance (Cdyn) and pulmonary resistance (RL) after intravenous administration of substance P (SP) (n=4). Pre 1 is the response to SP before nedocromil sodium, NED 1 and 2 the response to 0.1 and 1 mg/kg nedocromil sodium, and Post 1 and 2 the response to SP after NED 1 and 2 respectively. The response to nedocromil sodium itself and its effect on the response to SP were variable (see text). Nedocromil sodium augmented the increase in RL at the 0.1mg/kg dose and reduced the decline in Cdyn at the 1mg/kg dose level.

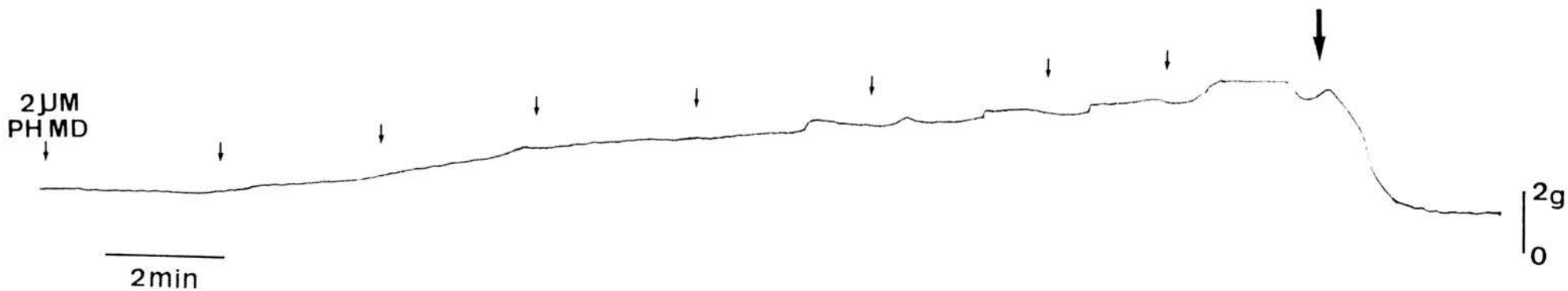


Figure 56. A typical cumulative concentration response curve obtained for substance P (SP) with the sheep trachealis muscle preparation. The tissue was pre-treated with the neutral metallo-endopeptidase inhibitor phosphoramidon (2 μM) at least 2 minutes before addition of SP. SP was added to the organ bath at two to three minute intervals to give final concentrations of 0.01, 0.03, 0.05, 0.07, 0.1, 0.3 and 0.5 μM (small arrows). Large arrow- washout with Kreb's solution.

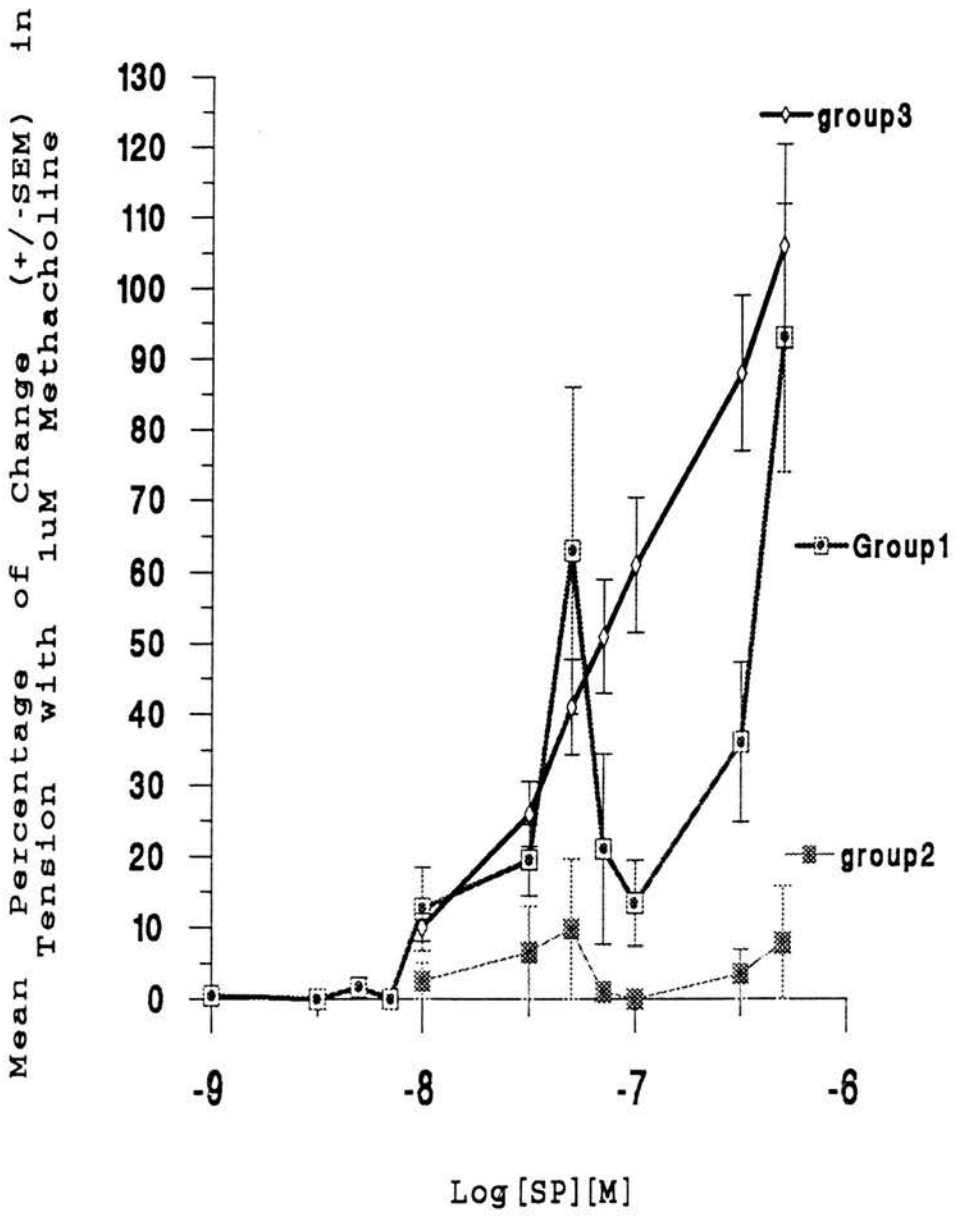


Figure 57. The contractile effect of substance P (SP) on the sheep trachealis muscle preparation, expressed as a percentage of the response to 1 μM methacholine. Concentration-response curves for young sheep (Group 1; n=21) and aged sheep (Group 2; n=12) are shown, as well as a cumulative concentration-response curve in young sheep (Group 3; n=25). In agreement with the in vivo studies it was found that SP has little effect on trachealis muscle tone in aged sheep. In the young sheep (6-12mths) the shape of the concentration-response curve depended on the method in which the SP was applied to the tissue (see text). When applying each individual concentration of peptide, in a non-cumulative manner, there was a consistent reduction of response (19 of 21 samples) at the 0.07μM and 0.1μM levels.

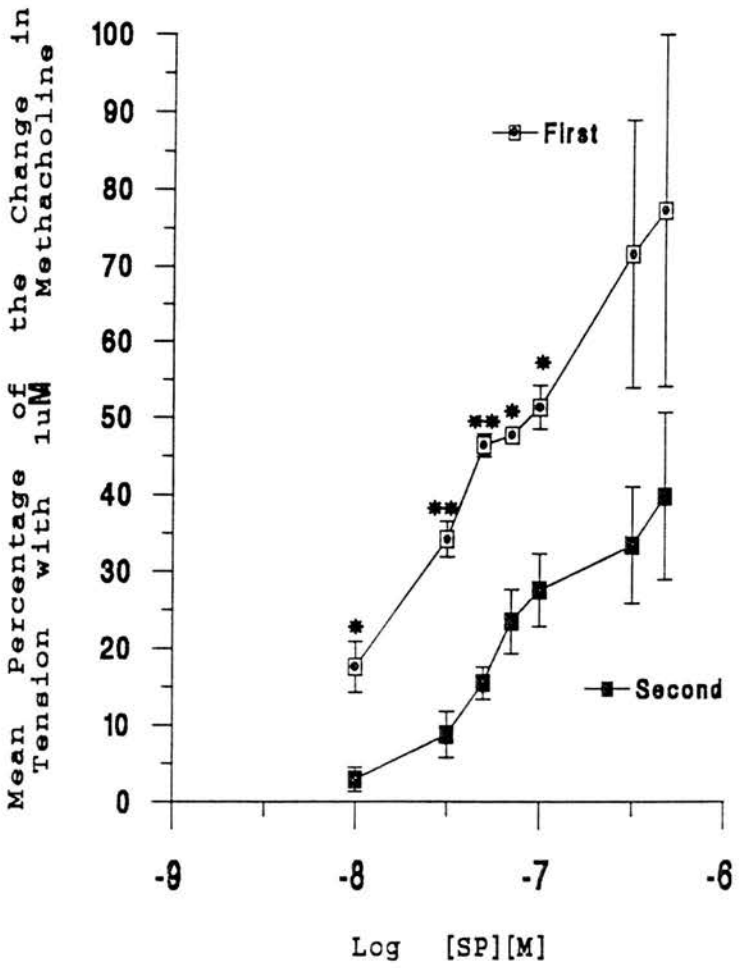


Figure 58. Demonstration of tachyphylaxis to the contractile response of the sheep trachealis muscle preparation to substance P (SP) (n=3). The contractile effect is expressed as a percentage of the response to 1μM methacholine. Two (First & Second) cumulative concentration-response curves (CRC) for SP were undertaken in each tissue sample one hour apart. There was a significant decline in the response to SP with the second CRC. On the basis of these results it was decided to investigate pharmacological antagonism of the SP response using single SP concentrations giving reproducible responses (typically 0.05μM). Paired t-test, significance of difference between the two concentrations; *$p<0.05$, **$p<0.01$.

but with a large number of samples there was no response, even in the presence of phosphoramidon. The data presented here represents the responses in 70 tissue samples, but because of the variability of the response, assessment of larger numbers of antagonists was not possible. This variability did not appear to be due to lack of efficacy of the peptide used. Where there were runs of unsucessful in vitro experiments, the peptide was subsequently assayed successfully in the anaesthetised preparation.

The response to SP began within 60 s of addition to the organ bath, peaked within 6 min and returned to baseline approximately 2 min after washout.

Concentration-response curves (CRC) for SP were obtained using a cumulative method (n=25) (Figure 56) and a single dose with inter-dose washout method (SD) (n=21). The former method was also used to assess the trachealis muscle response to SP in aged sheep (n=12). The SD method produced a standard CRC up to the 0.05uM concentration, followed by a drop-off in the response to a minimum at 0.1uM and subsequent improvement in the response at 0.5uM. The cumulative CRC gave a more standard response (Figure 57). The response at 0.07, 0.1 and 0.3uM with the cumulative CRC was significantly greater than with the single dose method (t-test; $p<0.01$ & 0.05). The response to SP in trachealis muscle from aged sheep was minimal and this is in agreement with the results of the in vivo experiments (see Chapter 5).

The reproducibility of the cumulative CRC was also assessed by undertaking two CRCs one hour apart (n=3). There was a shift in the second CRC to the right, which was significant (t-test; $p<0.01$) at the 0.01 to 0.1uM concentration levels (Figure 58). This indicated development of tachyphylaxis to SP occurs in this preparation, preventing the use of repeated CRCs to assess pharmacological antagonism.

The reduction in the response with the 0.1 and 0.5uM concentrations were further investigated in 21 tissue samples. With the SD method the first 0.05uM concentrations consistently gave a larger response than the 0.5uM (t-test; $p=0.06$) and the 0.1uM (t-test; $p<0.01$) concentrations in 19 of the 21 tissues (Figure 59a and b). The response to the second 0.05uM, administered 5 minutes later, was also larger than the 0.1 ($p<0.01$) and the 0.5uM ($p<0.1$) responses. There was also an insignificant augmentation of the second response to 0.05uM compared to the first. Because of the tachyphylaxis found with cumulative SP CRCs and the paradoxical reduced response in the mid-section of the SD CRCs, it was felt that antagonism of the contractile response to SP could only be assessed by investigating the pharmacological antagonism of the single 0.05uM SP concentration.

The effects of each antagonist on the response to SP were not assessed in tissue from the same animals. To reduce the variability of response, samples were always collected from Suffolk-cross female lambs aged 6 to 12 months. Each agent was assessed using tracheal samples collected from four different animals on the same day.

Atropine (1uM) (n=8) effectively abolished the response to SP (Figure 60), while atropine and pirenzepine at doses between 0.01pM to 1uM antagonised the response to SP in a dose dependent manner (Figure 61). The difference in response to atropine and pirenzepine was significant at the 1nM (t-test; $p<0.01$), the 10nM and 0.1uM ($p<0.05$) dose levels. The concentration of atropine and pirenzepine required to inhibit the response to SP by 50% (IC_{50}) was calculated. The IC_{50} for pirenzepine and atropine were $5x10^{-10}$ and $5.6x10^{-8}$ M respectively. The NK antagonists spantide (n=7) and the NK-2 antagonist L-659,874 (n=6) (0.1 to 4uM) were generally ineffective in antagonising the response to SP, and the reduction in response with L-

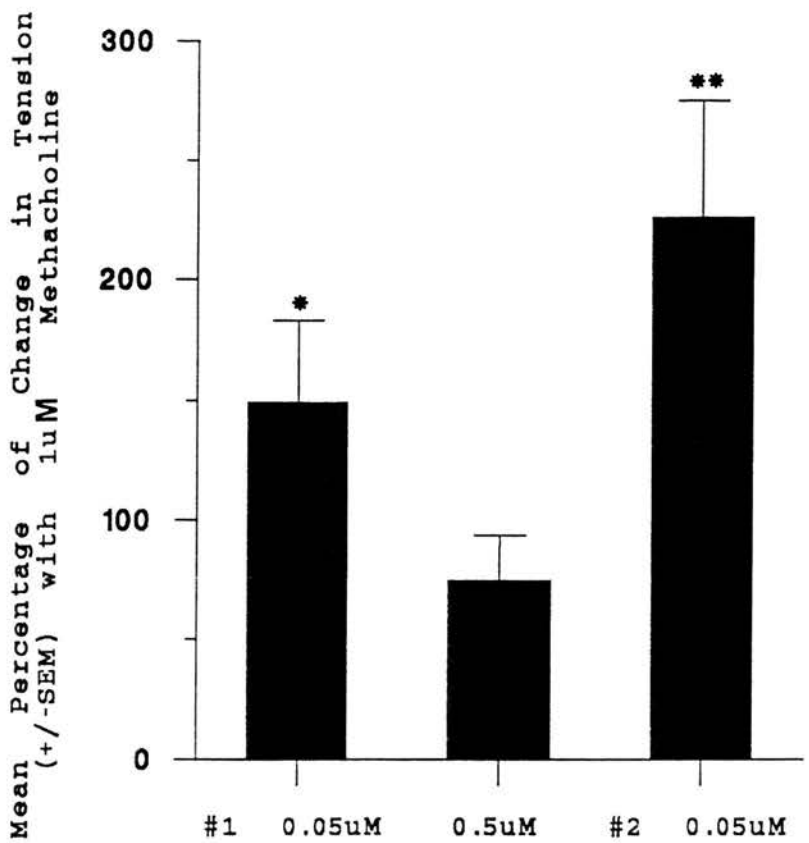


Figure 59a. The contractile effect of 0.05μM and 0.5μM substance P (SP) on the sheep trachealis muscle preparation (n=17). The first and second (#1 and #2 respectively) 0.05μM concentrations of SP were administered 5 minutes before and after the 0.5μM concentration. The 0.5μM concentration consistently resulted in a smaller response than 0.05μM and the contractile response to the second 0.05μM concentration was significantly augmented compared to the first. Paired t-test, significance of difference between 0.05 and 0.5μM concentration; *$p<0.05$, **$p<0.01$.

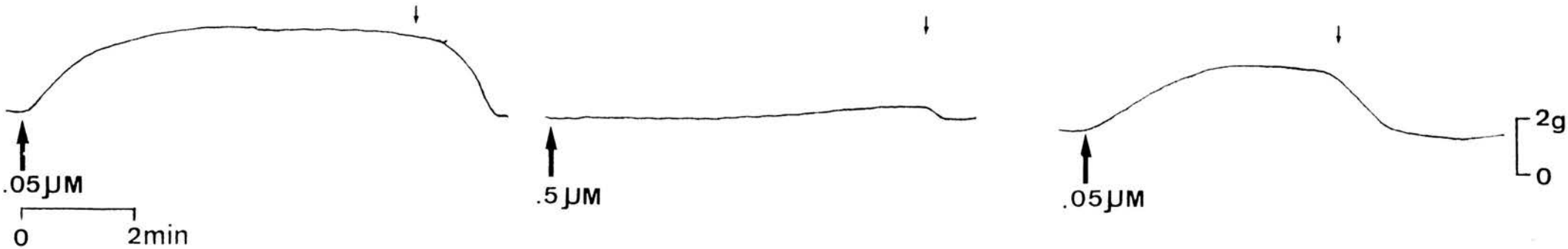


Figure 59b. Typical tracing illustrating the effect of 0.05µM and 0.5µM substance P (SP) on the sheep trachealis muscle preparation. The details are given in Figure 59a. In this sample there was a marginal response to the 0.5µM concentration. The interval between successive concentrations was approximately 5 minutes. Large arrow-application of SP; small arrow-washout with Kreb's solution.

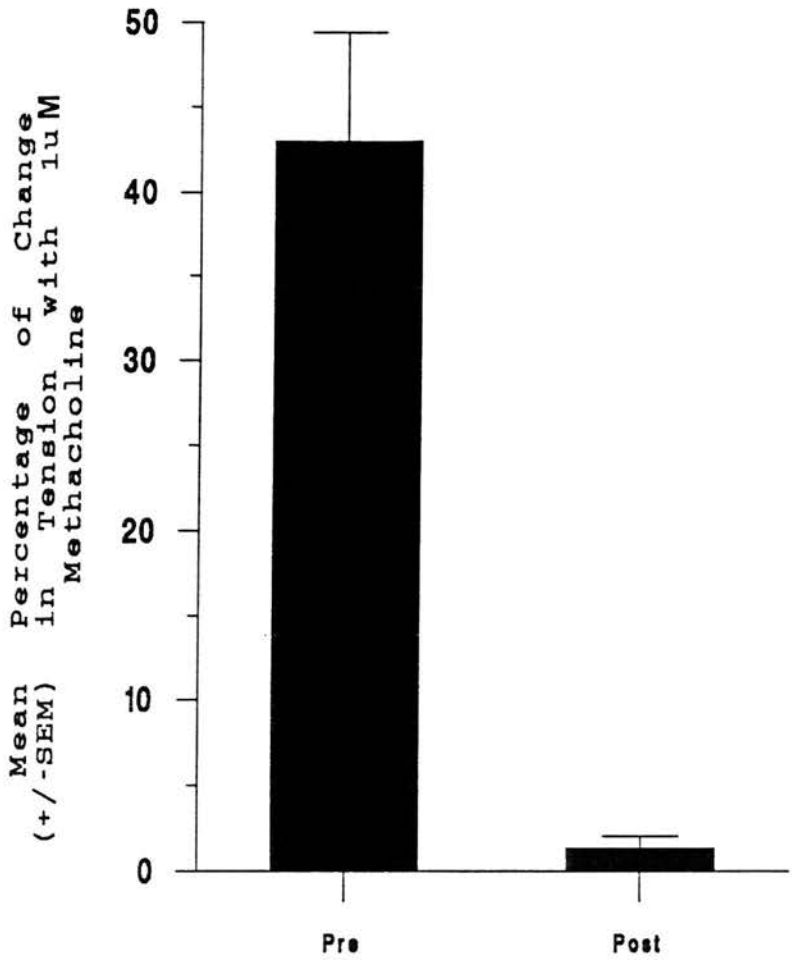


Figure 60. The effect of 1 μM atropine sulphate on the contractile effect of 0.05 μM substance P (SP) on the sheep trachealis muscle preparation (n=8). Atropine effectively abolished the response to SP (t-test; $p<0.01$). Pre and Post are the response before and after SP respectively.

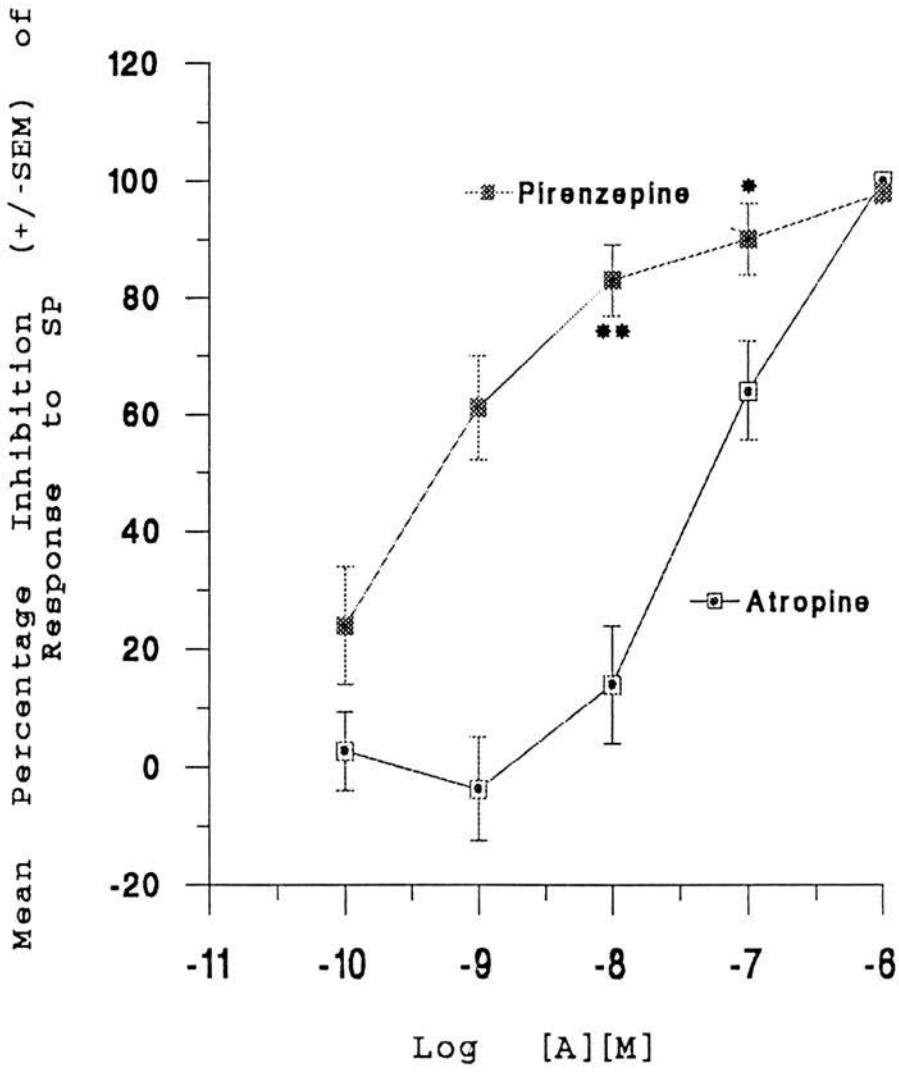


Figure 61. The effect of the muscarinic antagonists atropine (n=4) and pirenzepine (n=8) on the contractile response to 0.05 μM substance P (SP) in sheep trachealis muscle. Both atopine and pirenzepine antagonised the effect of SP in a concentration-dependent manner, with concentrations giving 50% inhibition of the agonist (IC_{50}) of 5.6×10^{-8} and 5×10^{-10} M respectively. t-test, significance of difference between pirenzepine and atropine; * $p<0.05$, ** $p<0.01$.

659,874 at the highest dose was not statistically significant (Figure 62).

6.4. Discussion:

There is widespread evidence that several of the effects of SP in the airways involve indirect mechanisms, one of which is activation of the cholinergic system. The secretory action of SP in the airways is cholinergically mediated in the cat (Kua et al, 1991), but not in the guinea-pig (Shimura et al, 1991), and in several species the bronchomotor response to different classes of neuropeptides appears to involve cholinergic neurotransmission.

Atropine significantly antagonised the effects of SP in anaesthetised normal sheep and in isolated trachealis muscle, and while the effect of atropine on SP in allergic sheep has not been reported, it effectively abolishes bradykinin-induced bronchoconstriction (Abraham et al, 1991). The effects of bradykinin can also be antagonised by nedocromil sodium, but is not affected by chlorpheniramine (Abraham et al, 1991). The ability of atropine to antagonise the actions of unrelated peptides suggests a common mode of action. The anticholinergic agent ipratropium bromide also protects against BK and SP-induced bronchoconstriction in asthmatic man (Fuller et al, 1987a; Crimini et al, 1990), while atropine pre-treatment inhibits the bronchomotor response to SP in rabbits, dogs, rats and piglets (Tanaka & Grunstein, 1984,1986; Taivan, 1987; Joos et al, 1988; Haxhiu-Poskurica et al, 1992), although Armour and co-workers (1991) failed to demonstrate antagonism of SP by atropine in the rabbit. The contractile effect of SP on bovine tracheal smooth muscle strips can also be blocked by atropine, but the effect is less marked at near-maximal concentrations, where the action of SP is mainly direct (Corson et al, 1990). Atropine is ineffective against SP induced contraction of airways and the tetrodotoxin-sensitive component of cholinergic bronchoconstriction in guinea pigs (Karlsson et al, 1984; Shore & Drazen, 1989), but it does block both BK and SP potentiation of ACh-induced

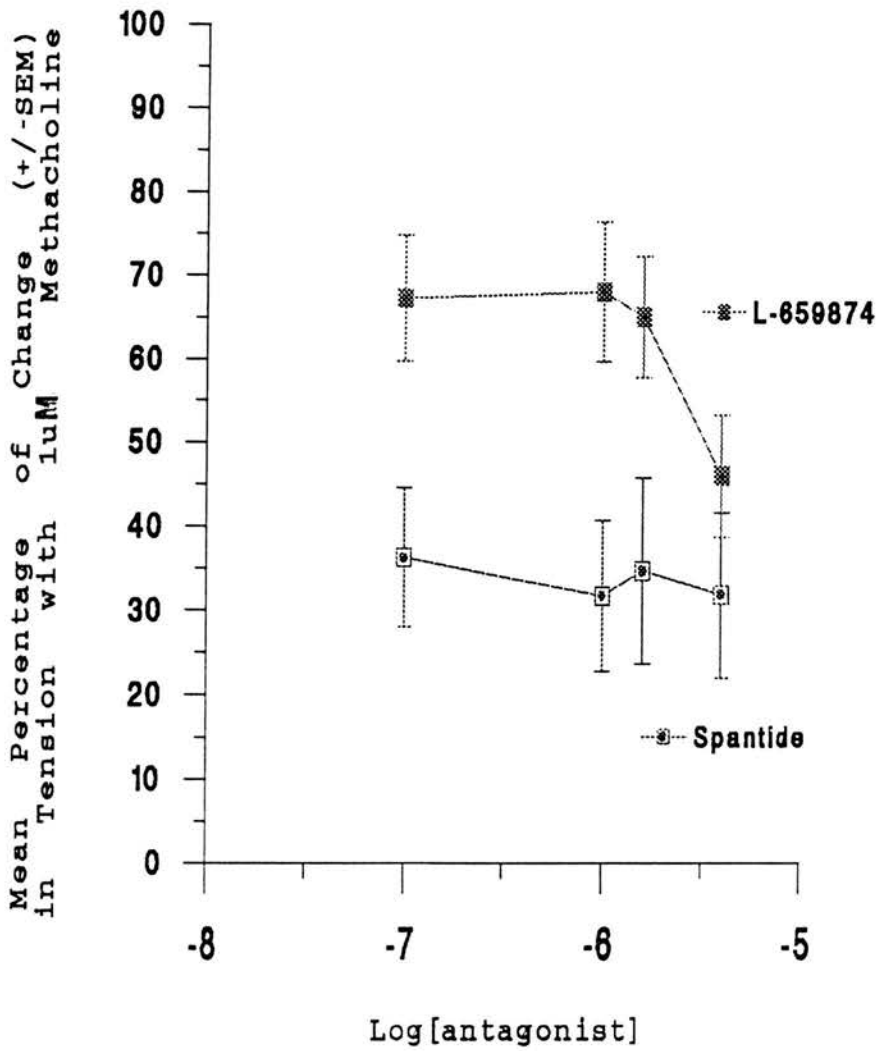


Figure 62. The effect of the general neurokinin antagonist spantide (n=7) and the NK-2 antagonist L-659874 (n=6) on the contractile effect of 0.05μM substance P (SP) on the sheep trachealis muscle preparation. Neither antagonist, up to a concentration of 4μM, significantly affected the response to SP.

bronchoconstriction (Omini et al, 1989). It is also ineffective against SP-induced contraction of isolated airways in man (Lundberg et al, 1983). Therefore, exogenously administered SP, in some species at least, acts indirectly causing bronchoconstriction by pre-junctional modulation of acetylcholine release from vagal cholinergic nerve endings. The released ACh in turn would contract smooth muscle or pre-junctionally inhibit neurotransmission in adjacent sympathetic nerves (Pendry & MacLagan, 1989). In the guinea pig ileum an analogous situation exists, where part of the smooth muscle contraction with SP, NKA and NKB is due to activation of neurokinin receptors on intrinsic cholinergic neurones, and these effects can be antagonised by the NK-1 receptor antagonist CP-96,345-1 (Legat et al, 1992).

The M1 receptor antagonist pirenzepine was more effective than atropine in antagonising the SP response in the sheep isolated trachealis muscle preparation. The M1 receptors appear to be present in airway parasympathetic ganglia and modify ganglionic neurotransmission (Minette & Barnes, 1990). Pirenzepine will antagonise vagally mediated bronchoconstriction at the ganglia level (Beck et al, 1987; Bloom et al, 1987; Yang & Biggs, 1991) and probably at the post-ganglioninic post synaptic level. The marked antagonism of the SP response with pirenzepine in sheep airways would suggest SP may be acting at the parasympathetic ganglion level rather than presynaptically on cholinergic nerve endings, and this is supported by the effect of hexamethonium on the SP response in the anaesthetised sheep. While the receptor specificity and affinity of pirenzepine, particularily at high concentrations, is questionable and it is possible that it is also blocking the pre-synaptic M2 receptor and the smooth muscle M3 receptor (Yu et al, 1992), the low IC_{50} value found for pirenzepine ($5x10^{-10}$ M) in antagonising SP would suggest involvement of the M1 receptor. Determining pA_2 values for pirenzepine, atropine and using specific M2 and M3 antagonists would have been preferable, but was not feasible. It is still conceivable that the M1 receptor mediates cholinergic bronchoconstriction in the

sheep airway and this needs to be investigated further.

Nedocromil sodium does not affect the spontaneous or BK-induced endogenous release of SP in rat trachea (Ray et al, 1991b). Nedocromil sodium does inhibit the SP potentiation of vagal-induced tracheal smooth muscle contraction (preganglionic effect) in the rabbit and has no effect on the potentiation of post-ganglionic contraction (Armour et al, 1991). It is possible that SP-induced bronchoconstriction in the sheep may be due to stimulation of myelinated and/or unmyelinated afferent fibres in the lung. However, only in one sheep in the present study could antagonism of SP by systemically administered nedrocomil sodium be shown, and this was the one animal whose basal bronchomotor tone was unaffected by nedocromil. In the three other sheep, where nedocromil caused bronchodilation, SP had a similar effect on RL and Cdyn as in the control situation and possibly would have had a greater effect if bronchomotor tone had remained close to baseline. This paradoxical effect of nedocromil and SP in the sheep needs to be investigated further in a larger sample of animals, and the effects of inhaled nedocromil sodium on systemically administered and inhaled SP should also be investigated.

The reduction of the bronchoconstrictor response to SP by the ganglion blocker hexamethonium, although not as marked as with atropine, contrasts with previous reports in other species. Hexamethonium enhances the SP-induced bronchoconstriction in the rat (Joos et al, 1988) and guinea-pig (Stewart & Fennessy, 1986; Del Monte et al, 1990) and in the latter case is due to inhibition of sympathetic innervation to the airways (Stewart & Fennessy, 1986). Hexamethonium enhances the bronchoconstriction caused by vagal stimulation at frequencies and intensity (15Hz, 0.2ms, 3s, 7-20V) associated with activation of non-cholinergic (substance P) nerves (Del Monte et al, 1990). This effect of hexamethonium appears to involve release of neuropeptides as hexamethonium inhibits neural bronchoconstriction in capsaicin-

treated animals, and also involves activation of beta-adrenoceptors in parasympathetic ganglia. The results in sheep suggest SP-induced bronchoconstriction involves ganglionic neurotransmission, and supports the hypothesis that it is of reflex origin involving activation of pulmonary vagal afferent nerves or modulates efferent cholinergic output from airway parasympathetic ganglia. The marked hypotensive effect of hexamethonium, due to sympathetic ganglion blockade, and the general interference with sympathetic ganglionic neurotransmission might also have an effect of the bronchomotor response to SP. The reduction in mean blood pressure in the present study was greater than 20 mmHg, which would affect baroreceptor reflex activity, but whether this induced reflex changes in bronchomotor tone is not known.

The possibility that histamine mediates the bronchomotor response to SP has been discussed earlier, and of particular interest is the close anatomical relationship between peptide-containing nerves and mast cells recently demonstrated in the rat mesentery (Crivellato et al, 1991). While SP can release histamine from mast cells, by activation of phospholipase A2 (Mousli et al, 1992), this has only been reported for rat peritoneal and human skin mast cells (Shanahan et al, 1985; Repke et al, 1987; Lowman et al, 1988 a,b; McLeod et al, 1990; Mousli et al, 1992) and does not appear to apply to lung cells. Conversely histamine releases SP from sensory afferent nerve endings in guinea-pigs (Saria et al, 1987, 1988a) but not in rats (White, 1988). However, histamine causes bronchoconstriction directly via H1 receptors (Saria et al, 1983), possibly indirectly by activation of vagal afferent nerve endings (Dixon et al, 1979, 1980) and bronchodilation through H2 receptors (Ahmed et al, 1980), and complex interaction does exist between histamine and peptidergic nerves in the airway. H3 receptor agonists have been shown to pre-junctionally inhibit neuropeptide release from sensory nerves (Ichinose et al, 1990) and antagonise the microvascular leakage due to activation of NANC nerves, and histamine acting on H3 receptors inhibits vagally mediated contraction. However, the present study has

shown that the H1 receptor antagonist chlopheniramine (CH) does not antagonise the effects of SP in normal sheep, and this agrees with findings in man and guinea-pigs (Lundberg et al, 1983; Shore & Drazen, 1989), but on the contrary it significantly augments the response. The response to BK in allergic sheep is also not affected by CH (Abraham et al, 1991) suggesting the bronchomotor effect of peptides does not involve histamine. However, in normal sheep there was an inexplicable increase in the response to SP after CH pre-treatment. The H2 receptor, which mediates bronchodilation, predominates in normal sheep airways (Eyre, 1973, 1975; Ahmed et al, 1980), particularily in the smaller airways (Eyre, 1969) and the H1 receptor predominates in allergic sheep (Wanner et al, 1988). Although chlorpheniramine is not a H2 or H3 antagonist, a possible, though improbable, interaction between chlorpheniramine and H2 and H3 receptors may underlie this effect.

The peptide SP antagonists were developed by inclusion of the amino acid D-Trp into the SP structure and the most potent of the original SP antagonists was spantide (also known as Spantide I), with the substitution of D-Trp at positions 7 and 9 (Folkers et al, 1984). Spantide I has been reported to be reasonably selective for the NK-1 receptor, whose endogenous ligand is SP, but also has some activity against NK-2 receptor (NKA), with pA2 values of 6.2 and 5.3 respectively (Maggi et al, 1991). Spantide has no affinity for the NK-3 receptor (NKB). The use of Spantide I has been limited by this certain lack of specificity for NK-1 and NK-2 receptors and also by its tendency to degranulate mast cells and its neurotoxicity when administered centrally. A derivative of spantide, [D-NicLys1, 3-Pal3, D-Cl_2 Phe5, Asn6, D-Trp7,9, Nle11]-SP, also known as Spantide II (Folkers et al, 1990), is less toxic, does not degranulate mast cells and is 10 times more potent than spantide I in antagonising SP at NK-1 receptors. However, it is equipotent with spantide I in antagonising NKA (Maggi et al, 1991) and has no effect on NK-3 receptors. Spantide was ineffective in antagonising the effects of SP in anaesthetised sheep and in the sheep trachealis

muscle preparation, which may reflect a lack of specificity for the sheep airway NK-1 receptor. The apparent lack of effect of spantide I in the anaesthetised sheep may be due to the low doses of antagonist used (10ug/kg/min), but the use of higher doses was restricted by cost. However, in the organ bath preparation the concentration of spantide was much greater than the muscarinic antagonists atropine and pirenzepine required to antagonise the effects of SP significantly. An alternative explanation for the lack of effect of spantide I, considering the efficacy of CP-96,345-1, may be that the material was inactive. The novel neurokinin antagonist L-659,874 is a reasonably selective NK-2 receptor antagonist, but like spantide was ineffective in antagonising the contractile effect of SP on the trachealis muscle, suggesting the NK-2 receptor does not mediate the SP response. The investigation of L-659,874 in the anaesthetised sheep was again restricted by cost.

The quinuclindine agent CP-96,345-1 is a new non-peptide SP antagonist and is highly specific for the NK-1 receptor (Snider et al, 1991). It has variable effects on events characterised by activation of NK-2 or NK-3 receptors (Snider et al, 1991; Carruette et al, 1992; Nagahisa et al, 1992; Xu et al, 1992) and on the effects of both histamine and bradykinin (Legat et al, 1992; Nagahisa et al, 1992). It is reported to be a more potent antagonist of SP than spantide I (McLean et al, 1991), but approximately 10 times less potent than spantide II with pA2 values of 6.8 and 7.6 respectively in the guinea-pig taenia coli (NK-1) preparation (Hakanson et al, 1991). CP-96,345-1 has been shown to antagonise the action of SP, NKA and NKB in inducing plasma extravasation in guinea pig skin (Nagahisa et al, 1992) and the smooth muscle contraction caused by SP and the potent NK-1 agonist septide in guinea pig trachea (Carruette et al, 1992).The effects of CP-96,345-1 are not all mediated through the NK-1 receptor and its effects on histamine and bradykinin probably involve non-specific mechanisms as they are only apparent at very high doses (Legat et al, 1992). Along with its enantiomer (CP-96,344), which is inactive at

the NK-1 receptor (Legat et al, 1992), CP-96,345-1 reduces depressor reflexes caused by stimulation of capsaicin-sensitive peripheral neurones (Griesbacher et al, 1992). Furthermore, CP-96,345-1 and CP-96,344 both cause a fall in mean blood pressure and heart rate in anaesthetised rats (Donnerer et al, 1992). The likely explanation for these cardiac effects is that they are calcium channel blockers (Schmidt et al, 1992). CP-96,345-1 at 0.1 and 0.5 mg/kg abolished the bronchomotor response to SP in sheep indicating the response is mediated via the NK-1 receptor. At the higher dose CP-96,345-1 caused a transient bronchoconstriction in 3 of 5 animals indicating possible agonist activity at higher doses, or alternatively activation of a different mechanism. In the present study the effect of CP-96,345-1 on cardiovascular parameters was not assessed. The fact that CP-96,345-1 only partially inhibits the smooth muscle contractile effects of stimulation of capsaicin-sensitive nerves (Legat et al, 1992) but abolishes SP-induced contraction, suggests it is acting only at the NK-1 receptor.

The results from these different studies suggest that while the bronchomotor effect of SP involves modulation of cholinergic neurotransmission this is still mediated through the NK-1 receptor, which may be located pre-junctionally on post-ganglionic cholinergic nerve endings or on sensory neurons in ganglia. While atropine did not abolish the response to SP in anaesthetised sheep, the effectiveness of atropine in antagonising SP suggests there is a paucity of post-synaptic NK-1 receptors, mediating smooth muscle contraction, in sheep airways and the response to SP in normal sheep must be regarded as being largely indirect. This is supported by the

organ bath studies where both the M1 receptor antagonist and the mixed muscarinic antagonist atropine effectively abolished the response.

CHAPTER 7. THE EFFECT OF VAGOTOMY AND VAGAL COOLING ON THE CARDIOPULMONARY RESPONSE TO SP

7.1. Introduction:

Neural afferent nerve impulses from the thoracic viscera are carried in the vago-sympathetic trunk. In the sheep the majority of the afferent fibres come from the thorax viscera, but an important group arise from the reticulo-rumen and other abdominal viscera (Cottrell & Greenhorn, 1987). In the case of the respiratory system three types of afferent nerve fibres and receptors have been identified. The slowly adapting receptor (SAR) is a stretch receptor that responds to changes in the mechanical structure of the airways and lung tissues during inspiration and expiration (Fahim & Jain, 1979). SARs are found from the extrathoracic to the intrapulmonary airways, but are primarily located in the trachea and the larger conducting airways. Their activity is characterised by slow adaptation of firing rate to a stimulus, such as airway distension, and this slow adaptation occurs at the beginning and end of the stimulus period. A large proportion of the receptors are active at functional residual capacity (FRC) while others are recruited during breathing. The rapidly adapting receptor (RAR), also known as the irritant receptor, show a rapid adaptation of firing response to stimuli (Sampson & Vidruk, 1975; Bergeren & Sampson, 1982). The RARs are affected by changes in lung mechanics in an irregular fashion, but are usually quiescent at FRC. The response involves an irregular burst of activity during inflation, which adapts rapidly to the discharge frequency close to the resting level or becomes quiescent. They can also be preferentially activated by intermittent lung collapse during deflation (Schultz et al, 1989). RARs are also affected by a variety of irritant agents and may be the receptors associated with coughing (Coleridge & Coleridge, 1986) and the generation of the augmented breath (Davies & Roumy, 1982).

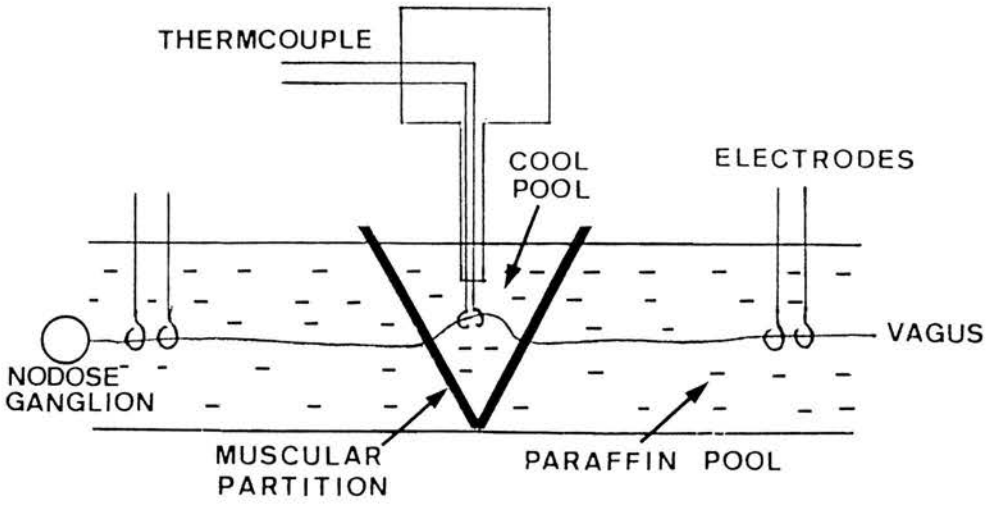
These differences in the discharge pattern of SAR and RAR associated nerve fibres are usually sufficent to allow their identification in nerve conduction studies. Both the SAR and RAR nerve fibres are myelinated and therefore have fast conduction velocities (23 and 32 m/s; dog) (Sampson & Vidruk, 1975).

The last class of respiratory afferent receptors are the C-fibre afferent receptors, also known as Paintal's J-receptors (Paintal, 1977) and comprise the majority of afferent nerve fibres originating in the thorax (Agosini et al, 1957). They are not, in general, modulated by the respiratory pattern, but some (pulmonary) do respond to hyperinflation with 2-3 times tidal volume. Attempts have been made to sub-divide C-fibres further on the basis of their accessibilty through the pulmonary and bronchial circulations, and their responses to capsaicin, phenylbiguanide and bradykinin (Coleridge & Coleridge, 1984). Pulmonary C-fibres are preferentially stimulated when agents are administered through the pulmonary circulation (right atrium), while bronchial C-fibres are activated by bronchial arterial administration (Coleridge & Coleridge, 1986). Because of the widespread anastomoses between the two circulations this sub-division is not readily accepted by some investigators (Martling, 1987). The C-fibre afferents have slow conduction velocities (0.8-2.4m/s in the dog; Coleridge & Coleridge, 1977) being unmyelinated, and are consequently different from SARs and RARs. In normal circumstances they are quiescent, with only irregular and sparse impulse traffic in nerve fibres in spontaneously breathing animals. In experimental situations activation is usually achieved by chemical stimulation with capsaicin, phenylbiguanide or bradykinin.

Despite the fact that C-fibres make up the largest number of afferent nerve fibres in mammalian lung, the function of these receptors is still unknown. They are involved in the reflex stimulation of mucus secretion in the dog trachea (Schultz et al, 1989) and in the reflex contraction that follows tracheal relaxation induced by lung inflation

(Roberts et al, 1988). The activation of C-fibres by chemicals, such as phenylbiguanide and capsaicin, elicits a chemo-reflex which involves a combination of apnoea and/or, tachypnoea, bradycardia, mucus secretion and coughing, hypotension and bronchoconstriction, although not necessarily in all species (Karczewski & Widdicombe, 1969; Coleridge & Coleridge, 1984). Furthermore, it is not known if this reflex is physiologically important and it may merely be a response to potent noxious chemicals. They are also believed to be the main site of sensory neuropeptide neurotransmitters in the airways and lung (Polak & Bloom, 1982; Cadieux et al, 1986).

Selective blockade of afferent nerve fibres in the vagus can be achieved using two techniques, vagal cooling (Figure 63) and anodal block (Sant'Ambrogio et al, 1984). Vagal cooling results in reversible blockade of nervous transmission by progressively slowing nerve impulse conduction (Figure 64). When certain temperature levels are achieved conduction ceases, or becomes minimal. Blockade is due to lengthening the period of absolute refractoriness of nerve fibres. The use of the term "selective blockade" is only applicable when we talk about myelinated versus unmyelinated nerve fibres. In the case of myelinated nerve fibres, associated with SARs and RARs, blocking temperature is largely independent of conduction velocity. Both types of myelinated fibres are blocked at similar temperatures and so their activity cannot be separated. Myelinated nerves, in general, are blocked between 20 and 8° C, leaving most unmyelinated fibres unaffected (Franz & Iggo, 1968). It is interesting to note that a proportion of unmyelinated C-fibres is blocked at temperatures usually associated with blockade of myelinated nerves (Paintal, 1967) and it is possible that a proportion of C-fibres become myelinated once the vagus has left the thorax. The majority of unmyelinated afferent fibres continue to conduct at 8°C, and the numbers progressively decrease as the temperature drops. Between 3 and 0° C impulse conduction can be abolished in most cases, although temperatures below 0°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COOL POOL
ELECTRODES
VAGUS
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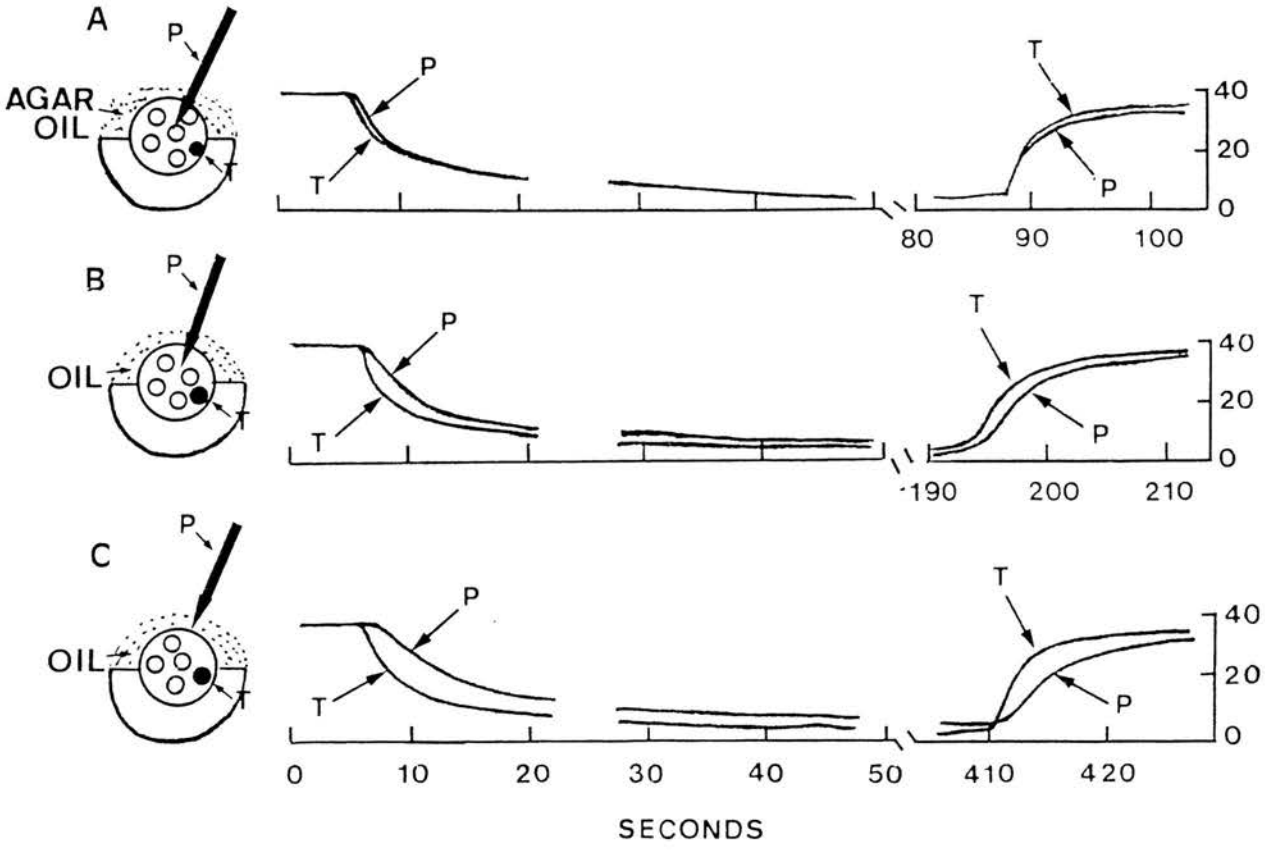
MUSCULAR PARTITION
PARAFFIN POOL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SECONDS

Figure 63 (upper and lower). Illustrations demonstrating two techniques for cooling the vagus nerve. In Paintal's preparation (upper) an immersion cooling technique is used where the nerve is surrounded by the coolant in a pool separated from the other tissues which are in paraffin pools. In Franz and Iggos' preparation (lower) the nerve is contained within a brass cooling probe and the effects of agar gel and oil on the temperature difference between the nerve bundle surface thermistor (T) and another thermistor at the core or opposite surface (P) is shown (A,B and C). With the agar gel bathed preparation (A) the maximum difference between the surface and core temperature was 1.7°C, while in B and C a difference of more than 7°C was found during cooling. This agar gel preparation, with the nerve contained in the groove of a brass cooling probe was used in the current experiments. (modified from: Paintal, 1965; Franz & Iggo, 1968).

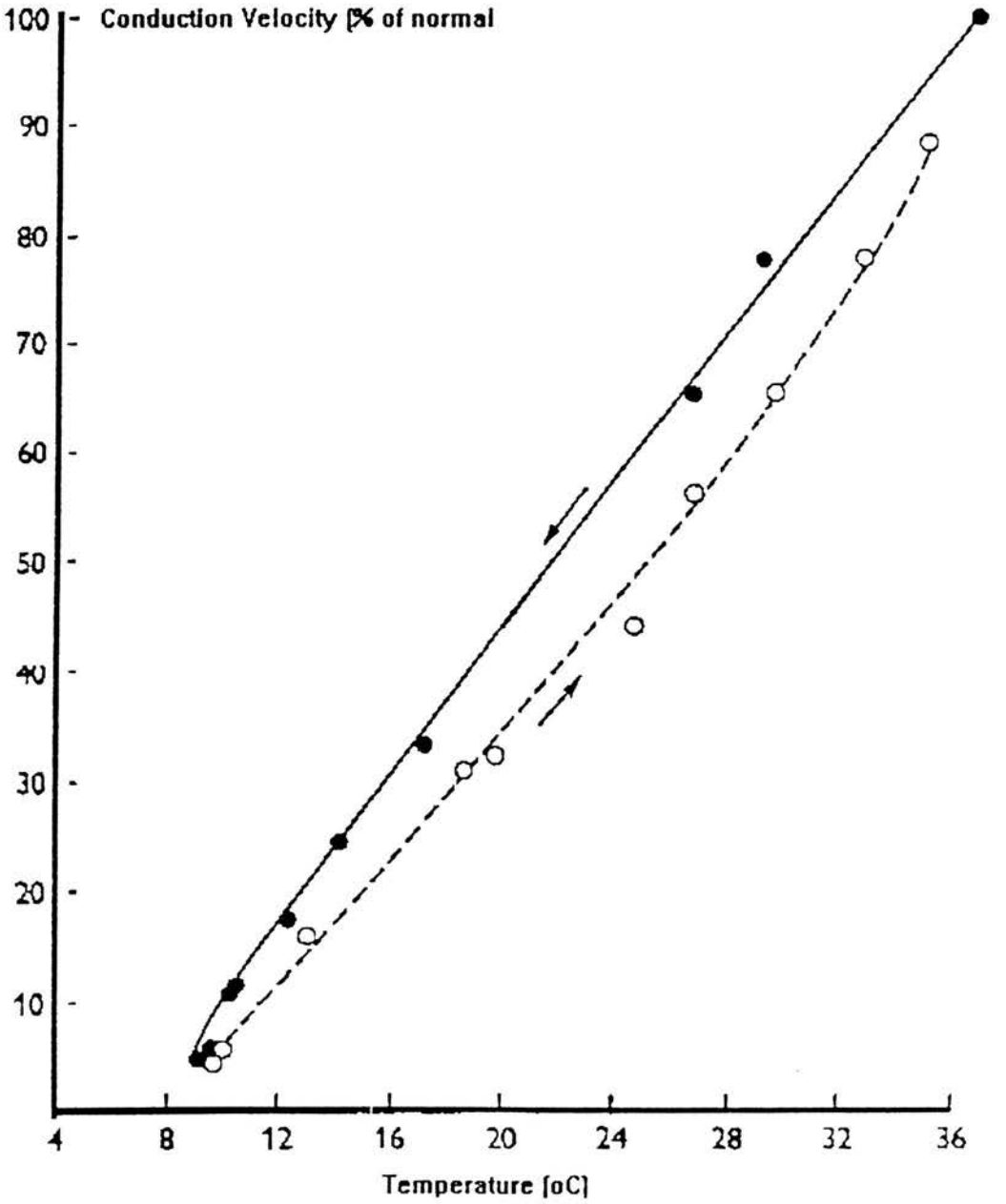

Figure 64. The effect of vagal cooling on the conduction velocity in a nerve fibre. Conduction was optimal at normal body temperature and was eventually blocked at 8.6°C. This figure shows the close correlation between temperature and nerve conduction and also the hysteresis between the effects of cooling and re-warming. (modified from Paintal, 1965).

may be required in some instances. However, freezing blockade is usually not required and will cause irreversible nerve damage. The reversibility of the procedure is dependent on the temperature used and the duration of cooling (Sant'Ambrogio et al, 1984).

Since it was demonstrated in Chapter 6 that atropine, pirenzepine and hexamethonium modified the bronchomotor response to SP, and augmented breaths were observed in some sheep after SP administration, the aim of this study was to investigate the possible role of afferent innervation in the bronchomotor response to SP.

7.2. Material and Methods:

7.2.1. Augmented Breaths:

The presence of augmented breaths (ABs), after intravenous injection of SP had been noted previously in some sheep, and consequently the incidence of ABs was assessed in all sheep studied during the course of this project. ABs are easily recognised as a double inspiration on respiratory tracings and have an associated increased flow, tidal volume and transpulmonary pressure relevant to the breaths immediately before and after the AB (Figure 65). The ABs were assessed in terms of their peak inspiratory and expiratory flow, tidal volume, transpulmonary pressure and time from injection of SP. The response was assessed after injection of 0.3 umol and 0.5 umol/kg SP and the significance of changes determined by the t-test.

7.2.2. Vagal Cooling and Vagotomy Studies:

Suffolk cross female sheep, aged 6-12 months, were anaesthetised and prepared for cardiopulmonary measurements as described previously. The cervical vagi were isolated and exposed as follows. The sheep were placed in dorsal recumbency and an

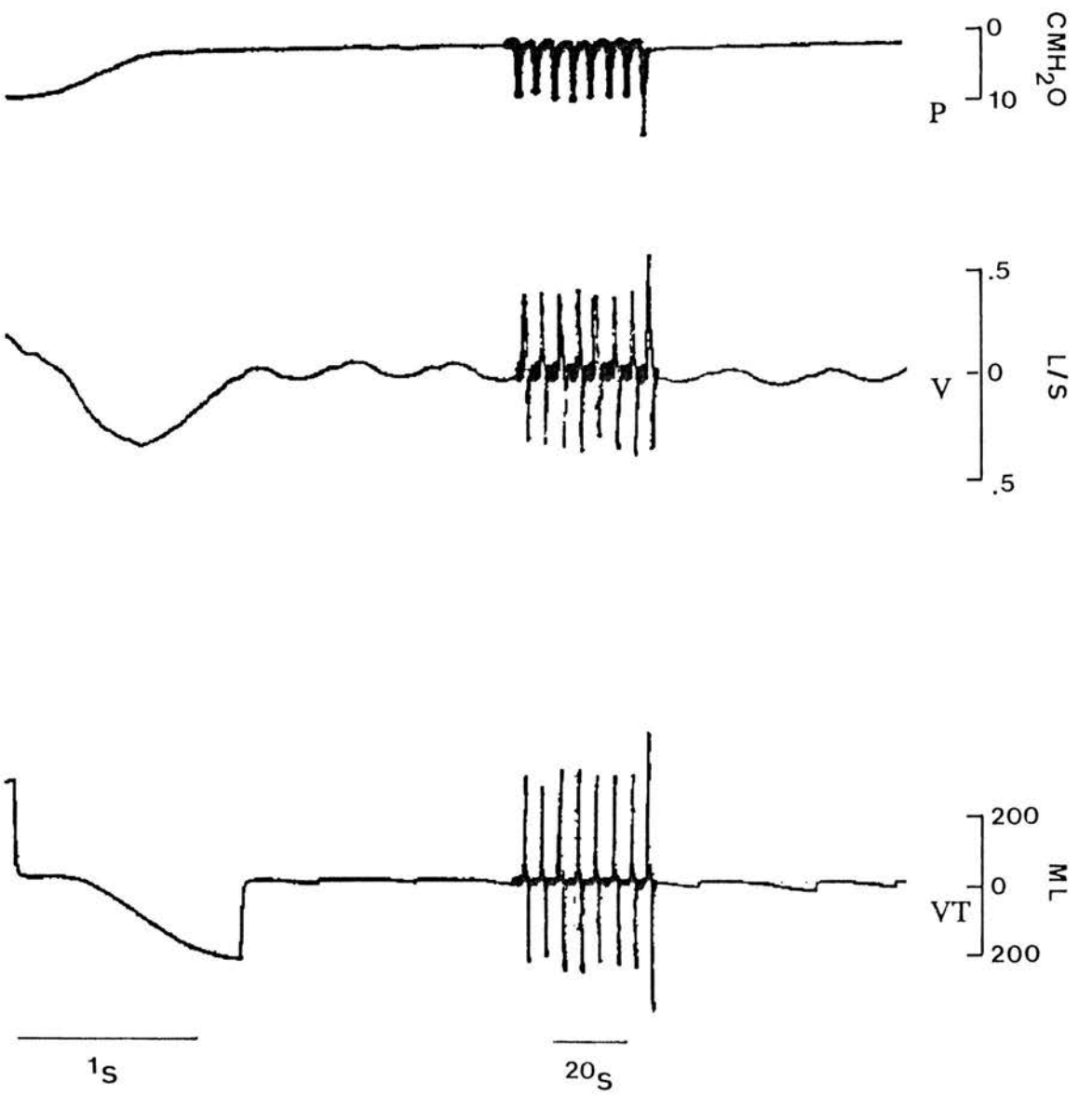

Figure 65. Augmented breath (AB) after intravenous substance P (SP) administration in a normal sheep. There is a single augmented breath, with increased transpulmonary pressure (P), flow (V) and tidal volume (VT), following a series of normal breaths. With flow the AB has increased the peak inspiratory component and has not affected expiratory flow.

8cm incision was made in the ventral cervical mid-line, equidistant from the caudal edge of the larynx and the thoracic inlet. Using blunt dissection, the right and left external jugular veins and carotid arteries were identified and separated from surrounding structures. The right and left vagi were identified adherent to the dorso-medial aspect of the carotid arteries and isolated by blunt dissection. Umbilical tape, soaked in 0.15M NaCl, was passed around each vagus for later identification. The wound incision was closed with temporary surgical clip staples and the sheep placed in the prone position.

A dose response curve for intravenous SP was carried out to establish an appropiate dose which would give a bronchoconstrictor response between 50 and 80% of maximum. The cervical incision was re-opened and the left vagus was identified. The nerve was blocked with 0.25-0.5ml of 10mg/ml lignocaine hydrochloride (Xylocaine 1%, Astra Pharmaceuticals Ltd, UK) injected directly into the nerve and the response to intravenous SP assessed. The nerve was then cut through the blocked section of nerve. The right nerve was identified and placed on a brass cooling probe (Figure 66). The nerve was sealed onto the probe with a 4% solution of agar (BDH Laboratory Supplies, UK) in 0.15M NaCl, which was at 37-40 ^{o} C. The temperature around the probe was maintained at close to 37 ^{o} C using direct heat from a lamp. The temperature of the nerve was monitored with an electrical thermistor embedded in the probe head which was attached to a volt meter (Micronta Digital Multimeter (Intertan Australia Ltd), Tandy Ltd, UK). The volt meter output was twice calibrated over the temperature range of 0^{o}to 40 ^{o}C on the day of each experiment before the probe was attached to the nerve, and at the end of each experiment. The thermistor demonstrated excellent linearity primarily in the lower part of the temperature range (0-20^{o}C) and with no variability in output throughout an entire day's experiments.

The nerve temperature was controlled by passing a coolant liquid through two tubes connected to the probe. The coolant (ethylene glycol) was pre-cooled in a reservoir tank (Colora Low Temperature Bath, Colora Messtechnik GmBH, Germany) to 1° C below the required temperature at the probe head to allow for heat loss in the tubing. The coolant was passed through the tubing with a pump which allowed the nerve to be cooled reasonably rapidly. However, the lower the temperature required the longer the delay in achieving that temperature. An additional pump tank (Colora Ultra Thermostat Bath, Colora Messtechnik GmBH, Germany) containing water at 37° C, was connected to the thermode tubing through a two-way valve to enable nerve temperature to be returned rapidly to normal (approximately 20 s).

The coolant was used to cool the right vagus to 10, 7 and 3 ° C, and the effect on the cardiopulmonary response to intravenous SP and to phenylbiguanide (PBG) (50ug/kg) was assessed. PBG was administered after SP once baseline V, VT and P levels had been achieved. Preliminary studies suggested that SP would not affect the cardiopulmonary response to PBG. Between each of the three temperatures the nerve temperature was returned to normal using the heated bath. At least 20m elapsed between each SP dose to allow for reversal of tachyphylaxis (see Chapter 3). Finally the effect of blockade of the right vagus with lignocaine and subsequent sectioning on the response to SP was assessed. In other experiments bilateral vagotomy without prior cooling of the right vagus was also carried out. Because of the problems of tachyphylaxis to SP and the problems associated with prolonged anaesthesia discussed earlier, the effect of cooling itself on the subsequent response to SP at normal temperatures was not assessed, except in sheep #42. In this animal cooling did not appreciably affect the response to SP when the nerve was re-warmed.

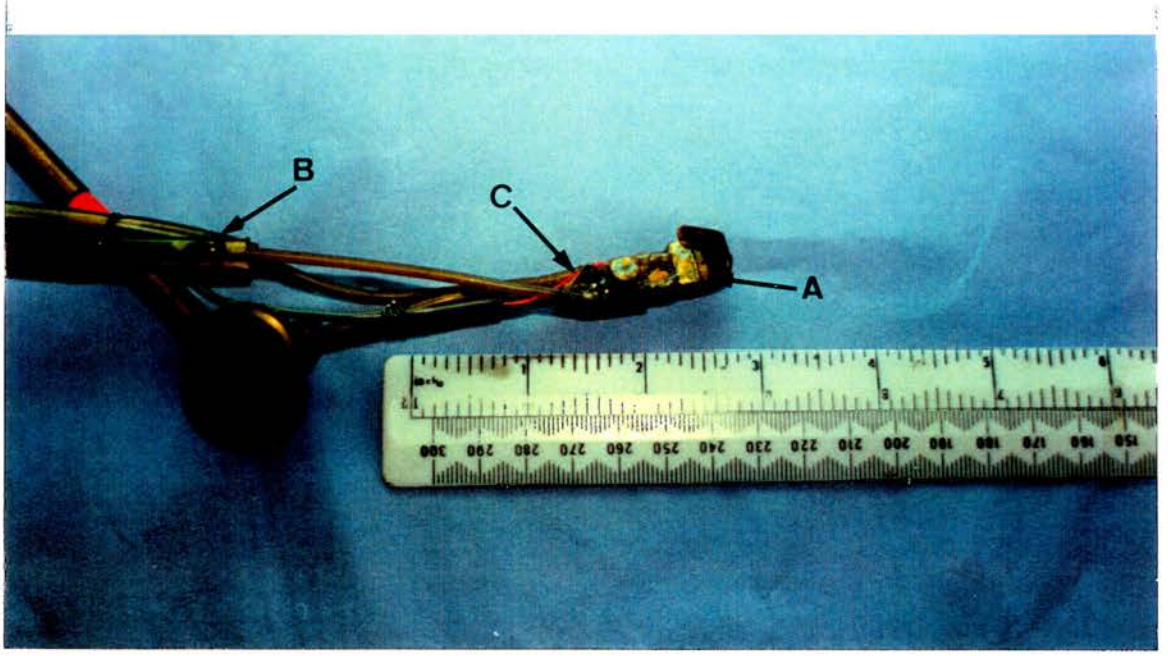

Figure 66. The cooling probe used to cool the isolated cervical vagus nerve in anaesthetised sheep. The nerve sits in the brass cooling channel (A) in a pool of agar gel. The coolant, at a pre-determined temperature, is pumped to the cooling probe head through the tubing (B) and the temperature at the cooling probe is monitored by a thermistor (C) connected to a calibrated voltmeter.

7.3. Results:

7.3.1. Analysis of Augmented Breath Activity:

The respiratory tracings of 40 sheep ranging in age from 4mths to over 4yrs were assessed for ABs. ABs were found consistently in 10 animals. Of these 10 sheep, 9 were aged 6-12mths and one was less than 6mths. ABs were not found in sheep aged 12-18mths or over 4yrs where bronchoconstriction was minimal or the response to SP involved apnoea (Chapter 5). The mean (+/-sem) time from injection of SP to the AB was 9.9 (0.69) and 11.4s (0.83) for 0.5 and 0.7 umol doses respectively, and to maximal bronchoconstriction (B_m) was 28.4 (1.37) and 28.3s (1.95). While the mean time to B_m was similar at 0.5 and 0.7 umol doses, there was no correlation between the time to appearance of the AB and time to B_m (Figure 67).

The AB consistently involved an increase in Vi, Ve, VTi, VTe and P and the percentage changes relevant to the previous breath are shown in Figures 68, 69 and 70. The increase in P was significantly different from baseline at the 0.5 umol ($p<0.05$) and the 0.7umol ($p<0.01$) dose levels (Figure 71), while the change in Vt was significant only at the 0.5umol dose ($p<0.01$).

There was no significant difference between the change in respiratory parameters with the 0.5 and 0.7umol doses of SP, but the percentage change in Vi was significantly different from Ve at the 0.5umol dose ($p<0.05$).

7.3.2. Vagal Cooling and Vagotomy:

Anaesthesia of the left vagus resulted in minor changes in the breathing pattern with increased inspiratory and expiratory flow times, characteristic of blockade of afferent input from stretch receptors. However, there was no significant change in tidal volume or transpulmonary pressure, suggesting sufficent stretch receptor afferent

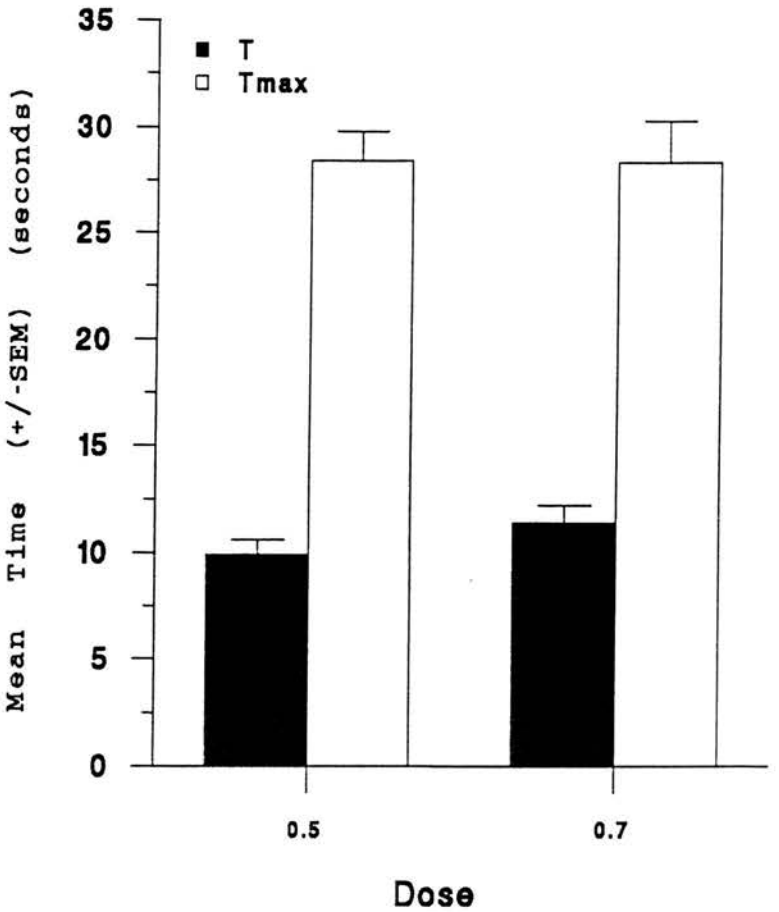

Figure 67. The time (seconds) to the appearance of augmented breaths (T), and the time to maximum bronchoconstriction (Tmax), after intravenously administered substance P (SP), at two different dose levels of 0.5 and 0.7 μmol/kg, in normal sheep (n=10). There was no significant difference in T or Tmax for both doses of SP.

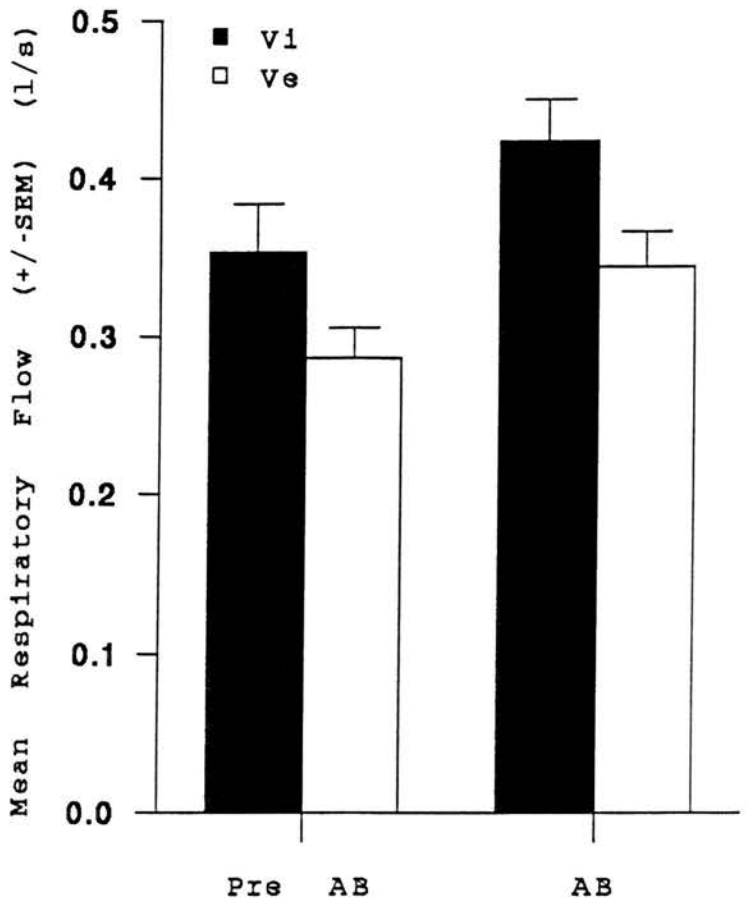

Figure 68. Expiratory (Ve) and inspiratory (Vi) flow immediately before (Pre AB) and during (AB) augmented breaths elicited by intravenous SP (0.5 μmol/kg) in normal sheep (n=10). There is an increase in Ve and Vi during the augmented breath, which is not statistically significant.

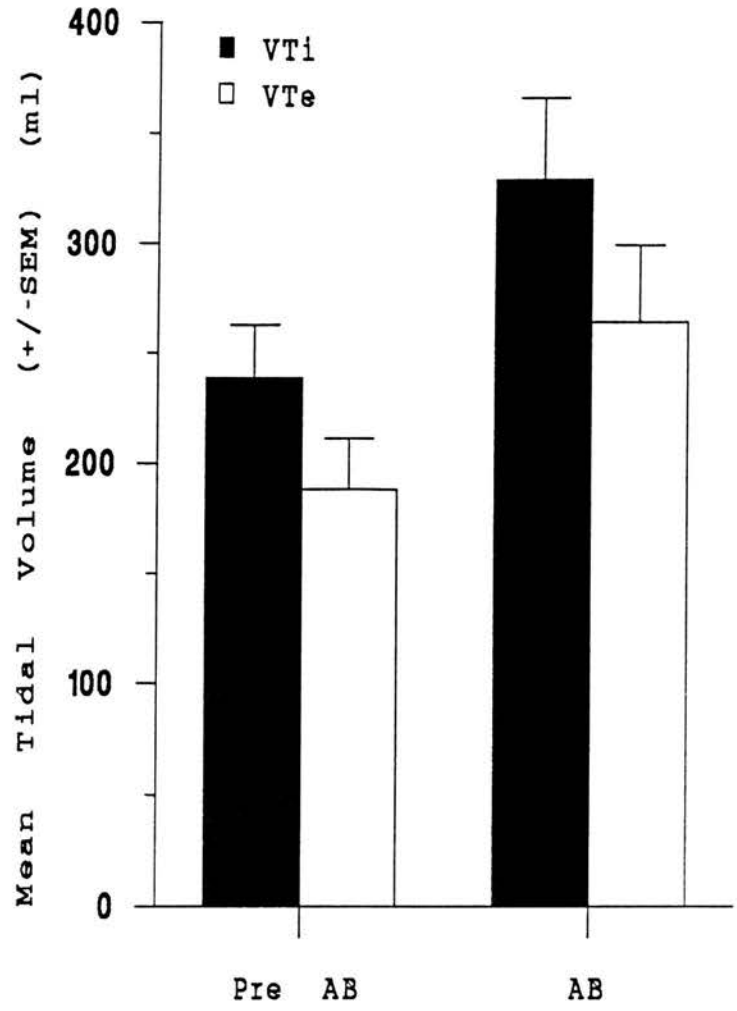

Figure 69. Expiratory (VTe) and inspiratory (VTi) tidal volume immediately before (Pre AB) and during (AB) augmented breaths elicited by intravenous substance P (SP) (0.5 μmol/kg) in normal sheep (n=10). There is an increase in VTe and VTi during the augmented breath. The change in tidal volume was statistically significant (paired t-test; $p<0.05$)

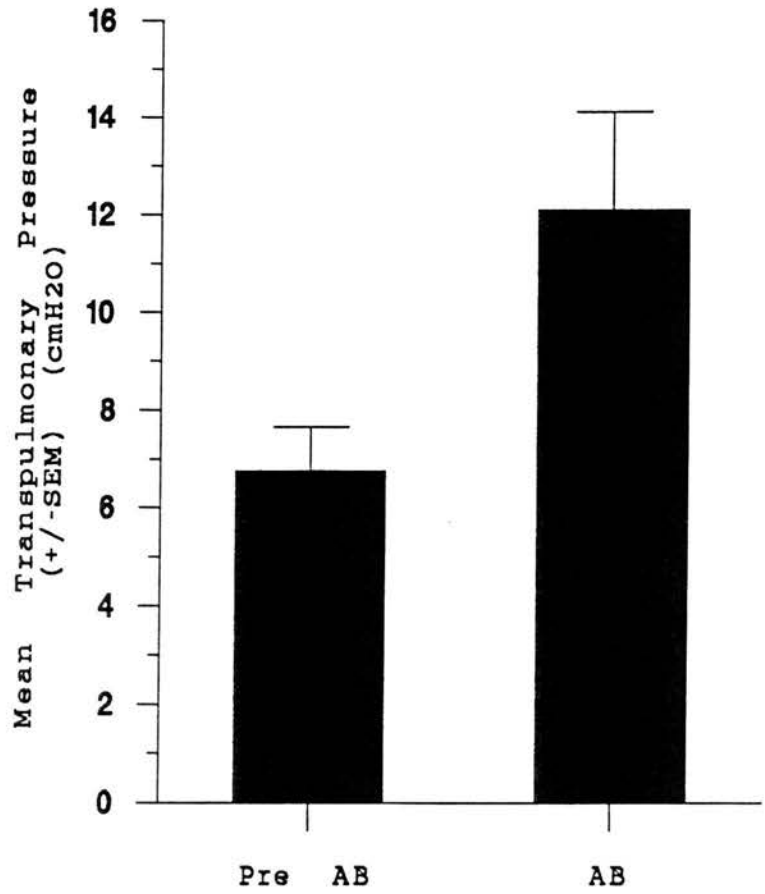

Figure 70. Transpulmonary pressure (P) immediately before (Pre AB) and during (AB) augmented breaths elicited by substance P (SP) (0.5 μmol/kg) in normal sheep (n=10). There is a significant increase in P from baseline (paired t-test; $p<0.05$).

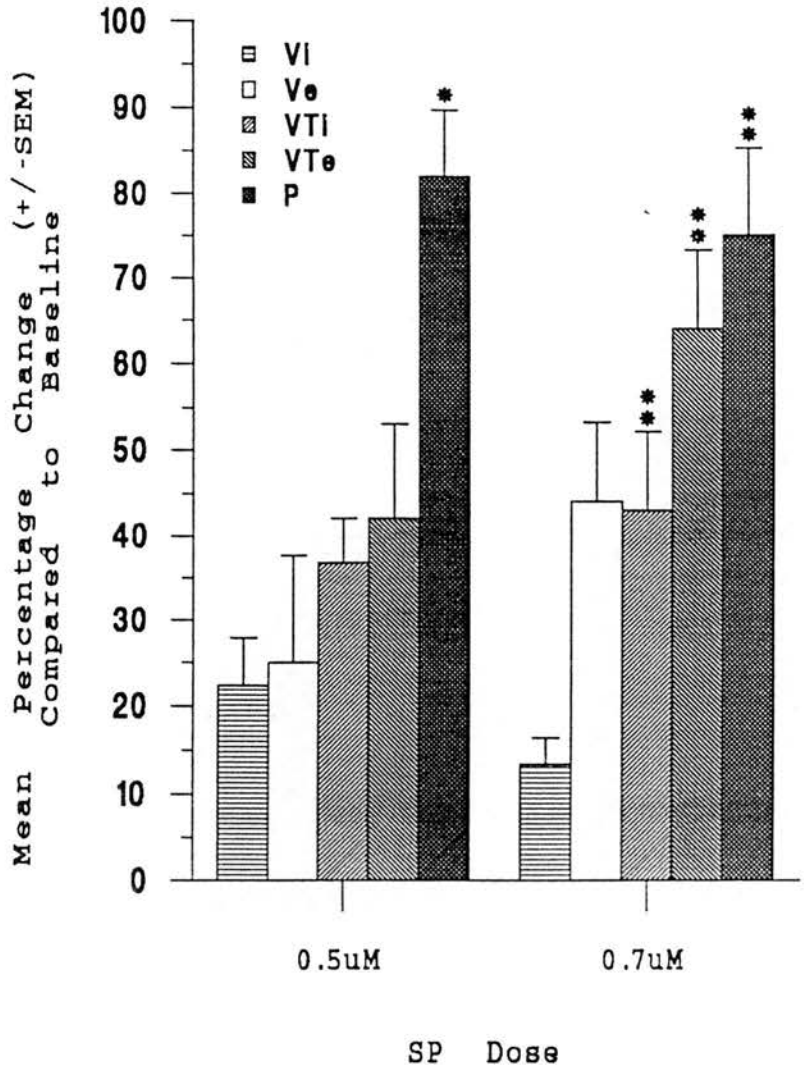

Figure 71. The differences in the magnitude of the augmented breath with intravenous SP in normal sheep (n=10) at 0.5μmol/Kg and 0.7μmol/Kg. The percentage change in expiratory flow (Ve), inspiratory and expiratory tidal volume (VTi & VTe) and transpulmonary pressure (P) was greater at the 0.7uM dose than the 0.5uM dose. t-test, significance of difference from baseline; *$p<0.05$, **$p<0.01$.

input was present in the right vagus for the breathing pattern to remain close to normal. In some individuals a normal breathing pattern resumed several minutes after left vagal blockade. Left vagal blockade had no statistically significant (paired t-test) effect on the bronchomotor response to SP or PBG.

The effect of vagal cooling on respiratory patterns and parameters are illustrated graphically in Figure 72 and representative tracings from individual animals are also shown (Figures 73, 74 & 75). There was temperature dependent increases in peak inspiratory flow, transpulmonary pressure and tidal volume and a decrease in respiratory rate. While cooling to 10 ^{o} C gave respiratory changes consistent with blockade of myelinated fibres from stretch receptors (Figure 73), further changes occured in the respiratory parameters at lower temperatures (Figure 72). Furthermore, cooling to below 10 ^{o} C in some sheep resulted in paradoxical changes in respiration characterised by an increased respiratory rate (Figures 74 & 75).

SP (0.7-1.0umol/kg; n=7) increased RL (+150% +/-29.3) and decreased Cdyn (-43% +/-4.7) at normal temperature. Vagal cooling resulted in a temperature dependent reduction in the bronchomotor response to SP with the most marked reduction at 3^{o} C (RL +6% +/-3.36; Cdyn -8.2% +/-4.24) (Figure 76). At 7 ^{o} C the change in RL was greater than at 3 ^{o} C (+20% +/-8.11), but Cdyn was similar (-8.6% +/-4.5). Stretch receptor input, as assessed by increased inspiratory and expiratory times , and increased tidal volumes and transpulmonary pressure, was abolished at temperatures below 14 ^{o} C. At 10 ^{o} C two sheep showed a reduction in the Cdyn response to SP and 3 to the RL response. After chemical (lignocaine) or surgical vagotomy (n=7) the bronchomotor response to SP was effectively abolished with RL/Cdyn of +4.3/-2.43% compared to pre-vagotomy values of +318/-56% (Figure 77). There was no statistically significant difference between the effects of cooling to 3 ^{o} C and vagotomy on the response to SP.

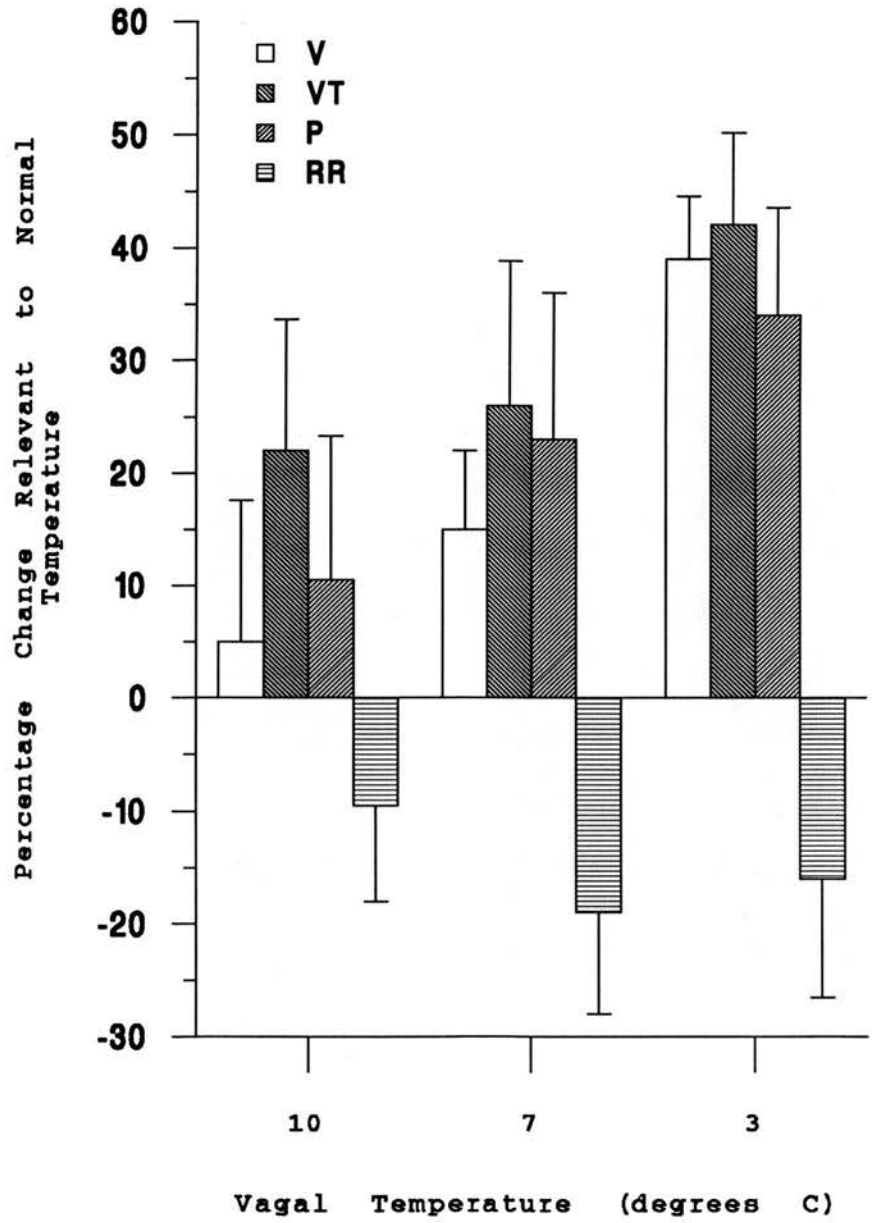

Figure 72. The effect of vagal cooling on respiratory parameters compared to normal temperature. There is a temperature dependent increase in peak inspiratory flow (V), transpulmonary pressure (P) and tidal volume (VT) and a decrease in respiratory rate (RR). This is consistent with blockade of conduction in myelinated fibres from stretch receptors, although further changes occured at temperatures consistent with blockade of C-fibre afferent conduction (<10°C).

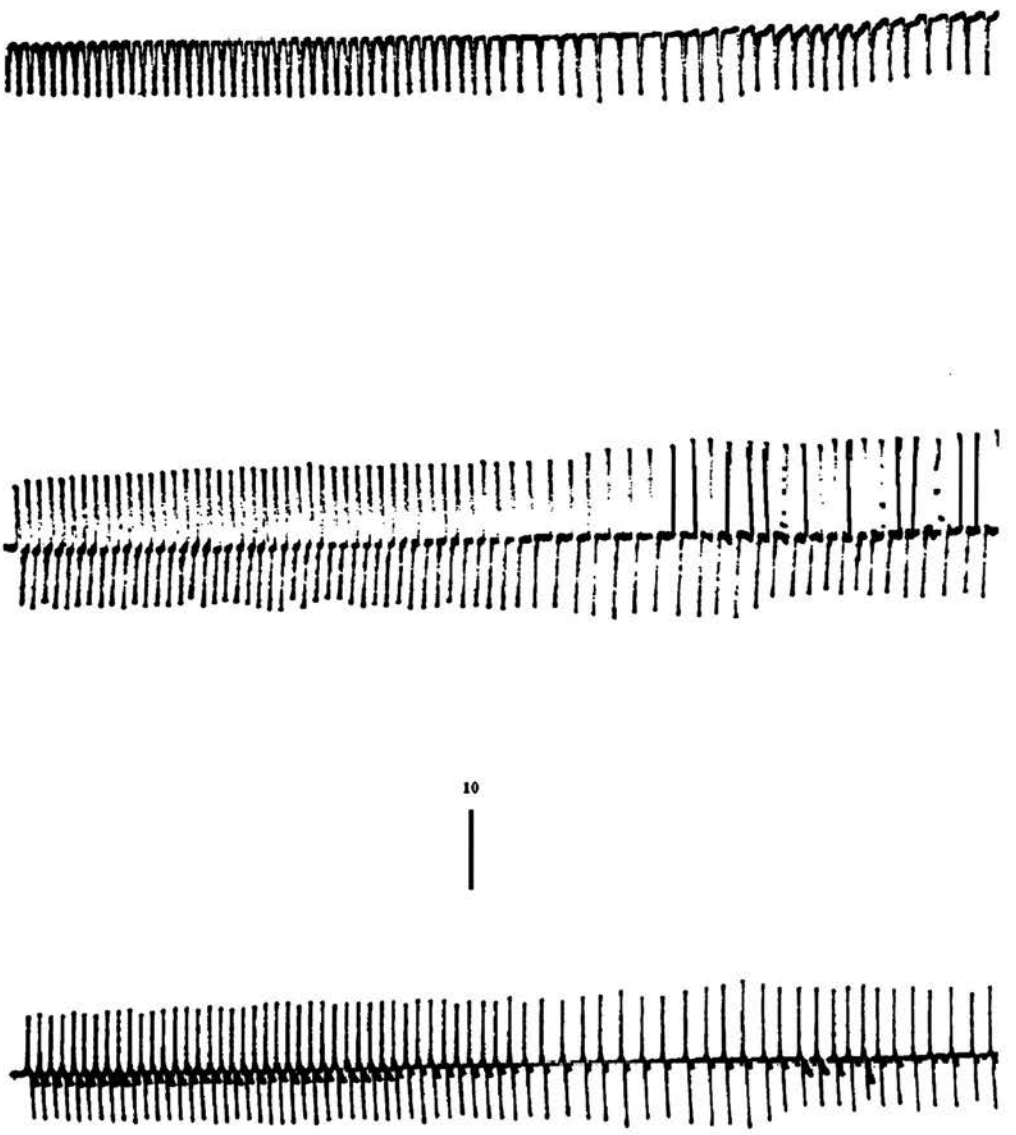

Figure 73. The effect of vagal cooling to 10°C on respiratory parameters of normal sheep. Transpulmonary pressure (upper tracing), respiratory airflow (middle tracing) and tidal volume (lower tracing). At 10°C (solid bar) there is a reduction in the respiratory rate and increase in the amplitude of the flow, pressure and volume tracings. This is presumably due to a reduction in stretch receptor input to the respiratory centres in the brainsetm and medulla. Cooling to this temperature does not always give this type of change and paradoxical alterations in respiratory parameters can occur. Examples of this effect are shown in Figures 74 and 75.

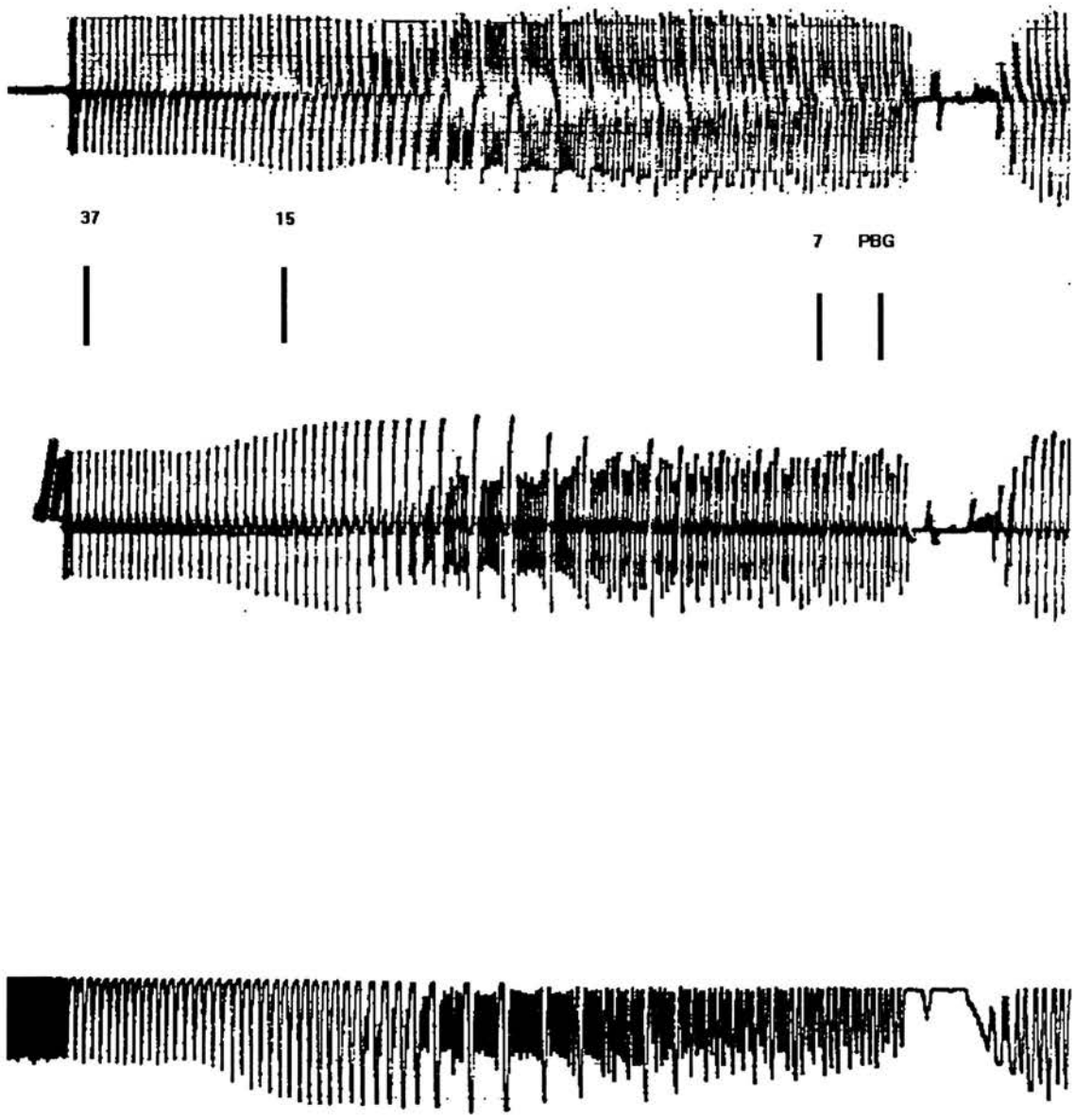

Figure 74. The effect of vagal cooling to 7°C on respiratory parameters of normal sheep. respiratory airflow (upper tracing), tidal volume (middle tracing) and transpulmonary pressure (lower tracing). It can be seen that at 15°C there is a reduction in stretch receptor input with resultant increase in flow, volume and pressure, but as vagal temperature approachs 7°C there is paradoxical increase in respiratory rate with a reduction in volume and pressure. The effect of phenylbiguanide (PBG) is also shown in this tracing. This paradoxical change in respiratory pattern did not occur in all sheep when the vagus was cooled to below 10°C.

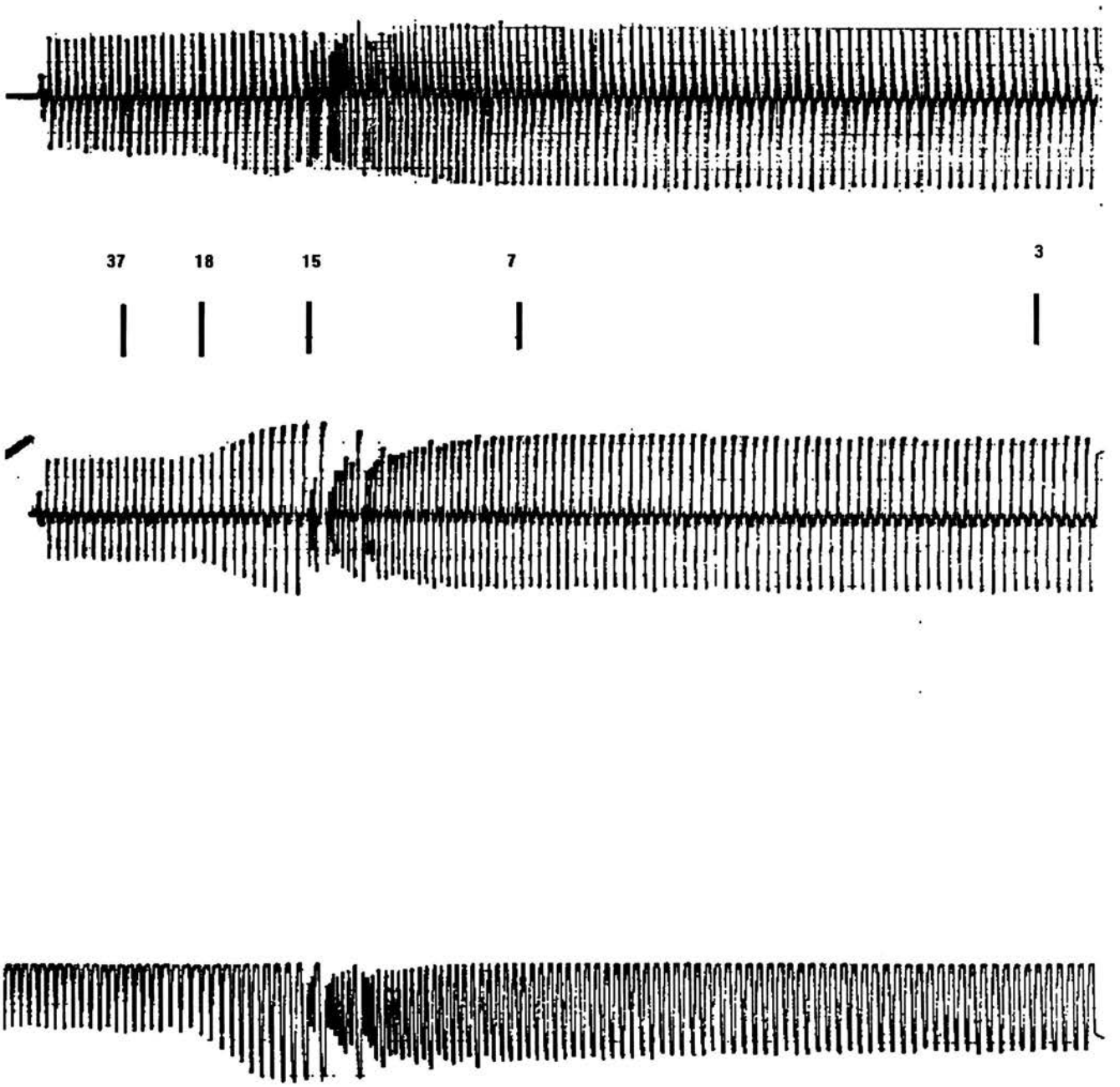

Figure 75. The effect of vagal cooling to 3°C on respiratory parameters of normal sheep. Respiratory airflow (upper tracing), tidal volume (middle tracing) and transpulmonary pressure (lower tracing). In this tracing there is a similar change in parameters at 15°C to that occuring in figure 74 and there is again a paradoxical increase in respiratory rate. However, at temperatures below 7°C breathing is more regular and the amplitudes of the pressure, flow and tidal volume tracings are greater than at normal temperature.Cooling to 7 or 3°C results in similar quantitative changes in respiratory parameters.

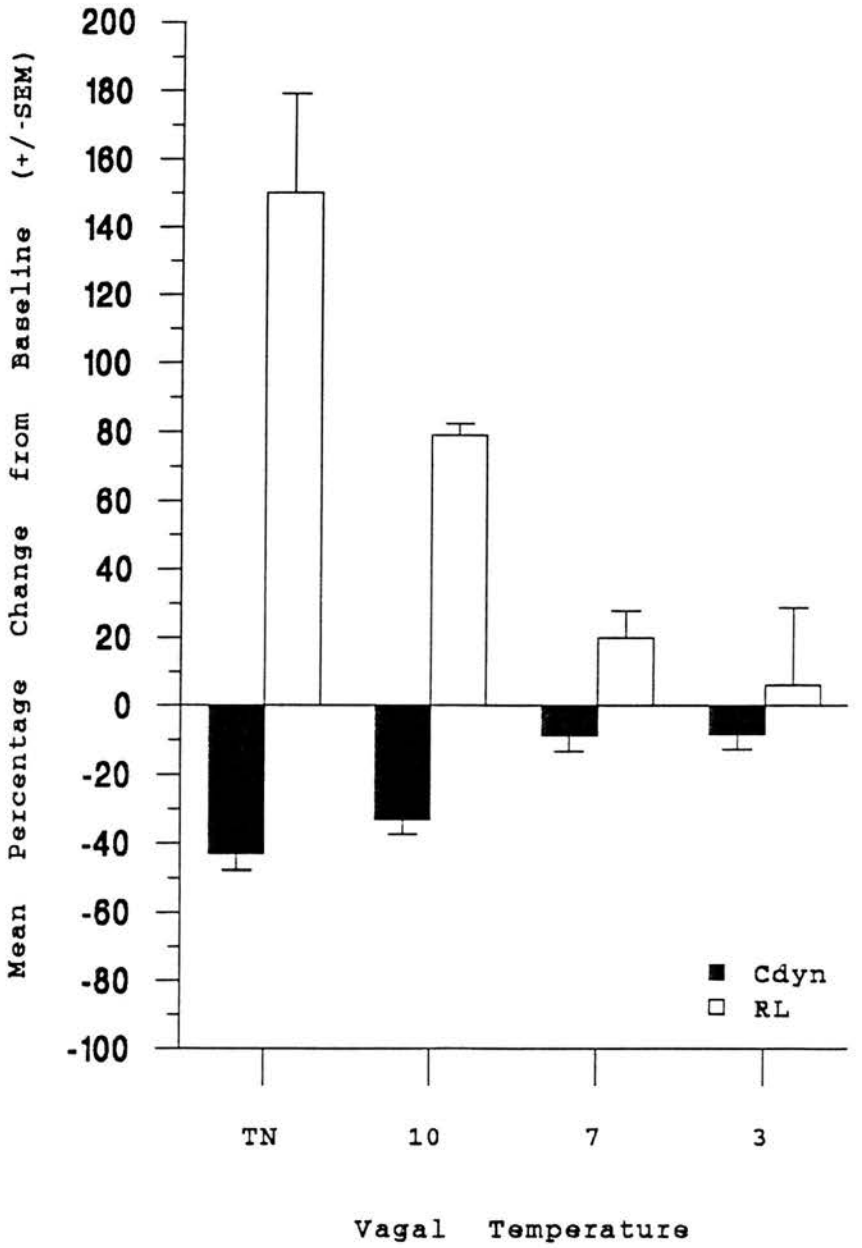

Figure 76. The effect of vagal temperature on the bronchomotor response (percentage change in dynamic compliance (Cdyn) and pulmonary resistance (RL)) to substance P (SP) in normal sheep. The reduction in the response to SP at 10°C was not significantly different from the response at normal temperature (TN). The responses at 7 and 3°C were similar and were significantly different from TN (t-test; $p<0.05$).

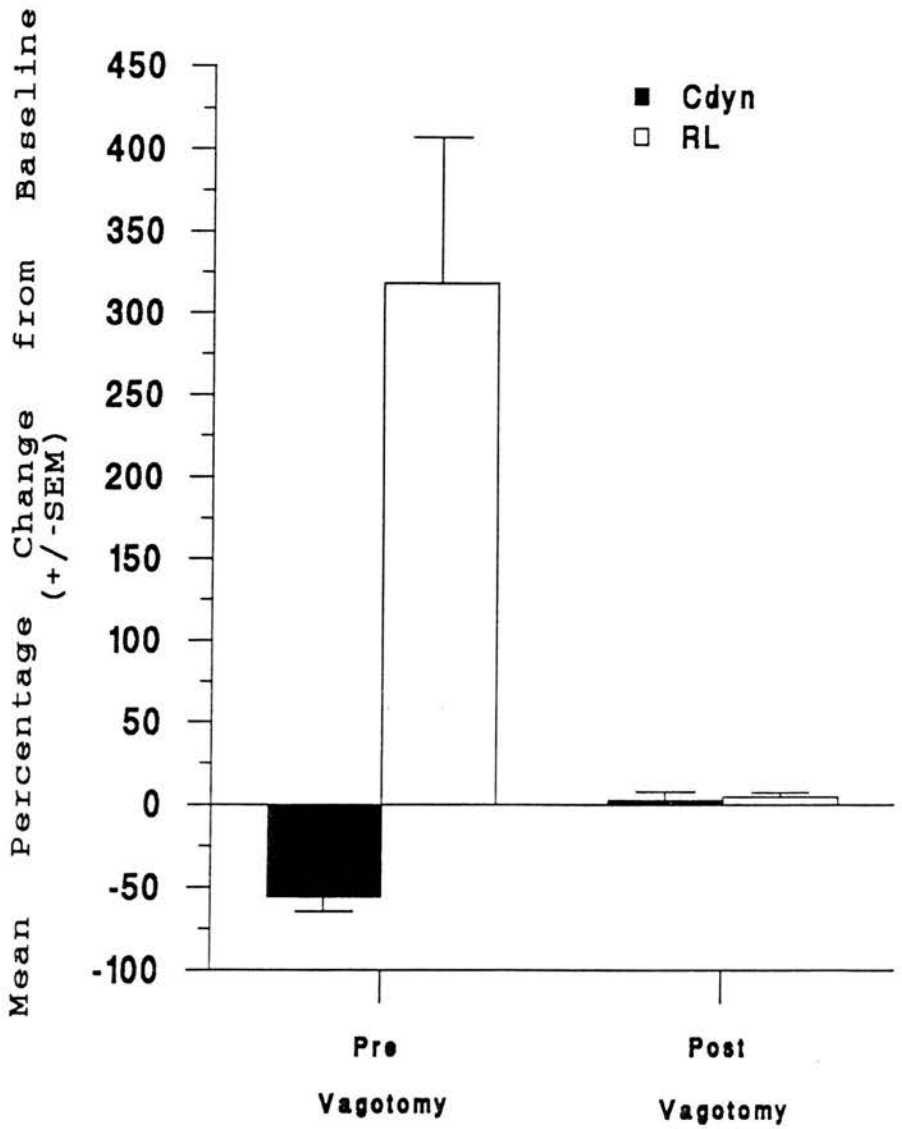

Figure 77 The effect of bilateral vagotomy on the bronchomotor response (reduction in dynamic compliance (Cdyn) and increase in pulmonary resistance (RL)) to substance P (SP) in normal sheep (n=7). Vagotomy has effectively abolished the response to SP.

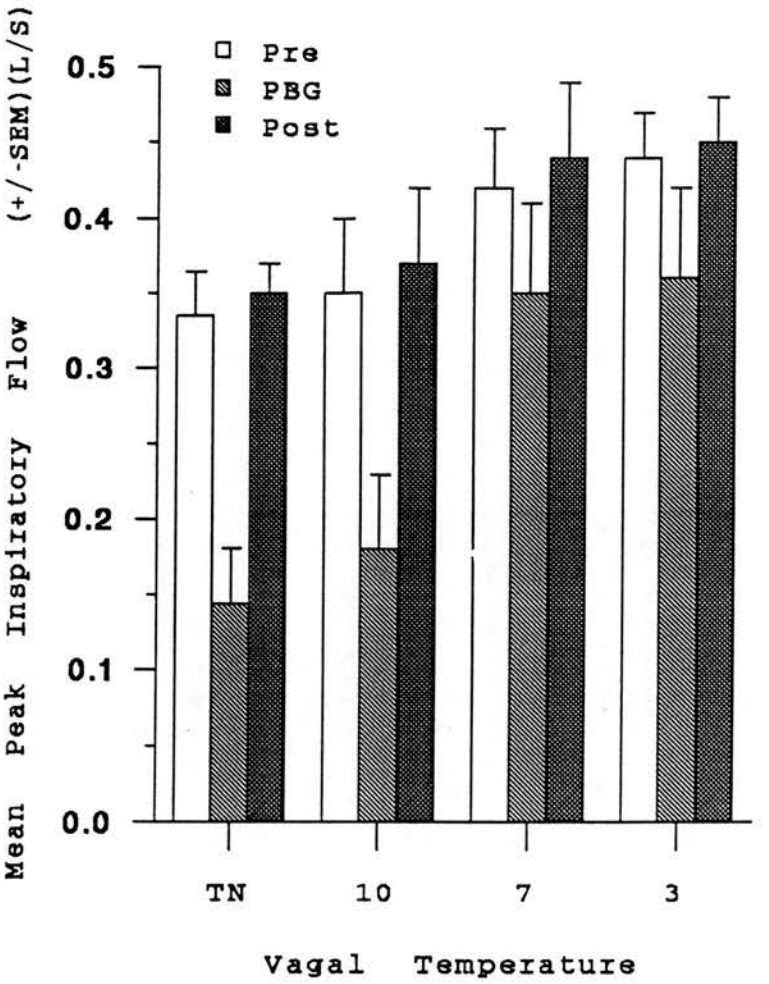

Figure 78. Effect of vagal cooling on the change in peak inspiratory flow (V) caused by intravenous injection of 50μg/Kg phenylbiguanide (PBG) in normal sheep (n=7-11). At normal temperature (TN) PBG caused a 57% reduction in V from 0.335 (Pre) to 0.144 l/s, with a transient subsequent rise in V to 0.35 l/s (Post). Vagal cooling caused a significant reduction in the change found with PBG at 7 and 3°C (t-test; $p<0.01$), but not at 10°C. There was also a temperature-dependent increase in mean basal flow consistent with blockade of stretch receptor fibre conduction.

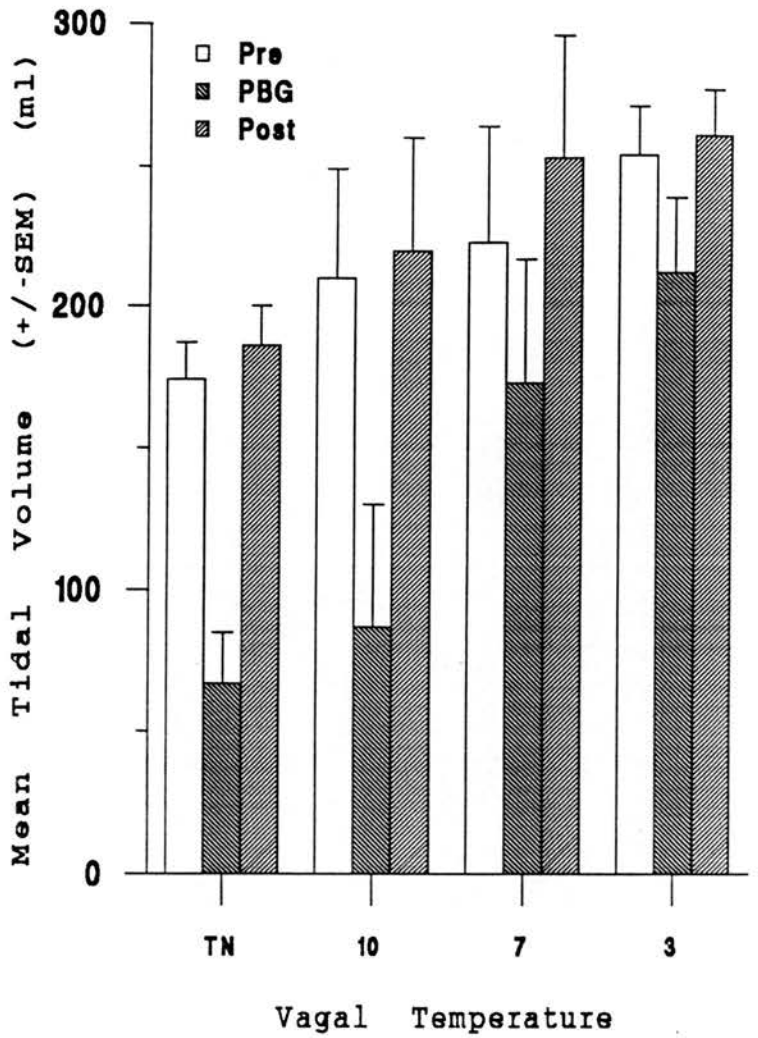

Figure 79. Effect of vagal cooling on the change in tidal volume caused by phenylbiguanide (PBG). Detail is as for figure 77. At normal temperature PBG caused a reduction of 52% in tidal volume. vagal cooling significantly reduced the change in tidal volume at 7 and 3°C (t-test; $p<0.05$ and 0.01 respectively), but not at 10°C.

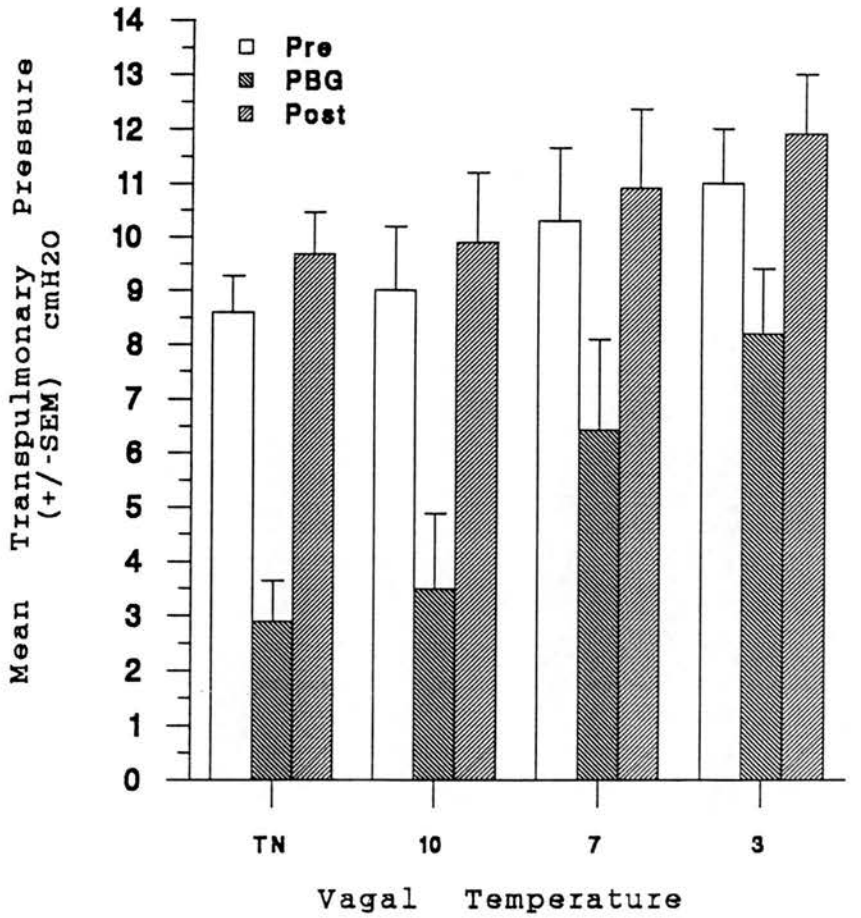

Figure 80. Effect of vagal cooling on the change in transpulmonary pressure (P) caused by phenylbiguanide (PBG). Detail is as for figure 77. At normal temperature PBG caused a 56% reduction in transpulmonary pressure. Vagal cooling significantly reduced the change in P at 7 and 3°C (t-test; $p<0.05$ and 0.01 respectively), but not at 10°C.

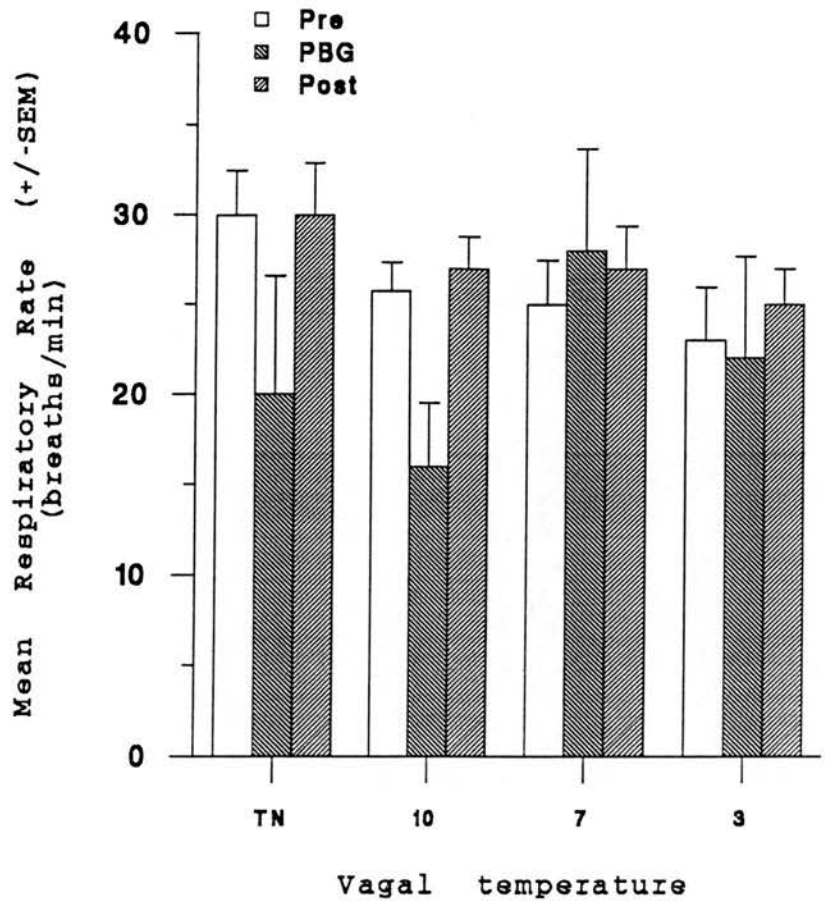

Figure 81. Effect of vagal cooling on the change in respiratory rate (RR) caused by phenylbiguanide. Detail as for Figure 77. At normal temperature PBG caused a 33% reduction in RR. While vagal cooling to 7 and 3°C reduced the change in RR by 12 and 5% respectvely this was not significantly different from the response at normal temperature or 10°C.

The effect of PBG (n=11) on ventilation, including flow (V), tidal volume (VT), transpulmonary pressure (P) and respiratory rate (RR), but not on RL and Cdyn, was assessed (Figure 78, 79, 80 and 81). PBG, at normal temperature, caused a transient reduction in all respiratory parameters and the onset and reversal of these changes were rapid. In all sheep the response occurred within 10s of injection into the cephalic vein, and began with an augmented breath followed by variable periods of apnoea (4-12s). When normal breathing resumed there was a transient rise in V, VT and P, but respiratory rate returned to the pre-PBG levels. In no animal did PBG induce tachypnoea. PBG reduced V, VT and P by 57, 52 and 56% respectively from baseline at normal temperature. At 10 ^{o} C the response to PBG was broadly similar,while at 7 and 3 ^{o} C the response was significantly reduced ($p<0.01$). The major difference betwen 7 and 3 ^{o} C was in the pressure change (-38 vs -25%). Vagotomy had no further effect on the response to PBG.

7.4. Discussion:

The appearance of augmented breaths after SP injection has been reported previously in rabbits (Prabhakar et al, 1987). An augmented breath is characterised by a deep inspiration consequent on an increase in inspiratory motor output to the diaphragm (van Lunteren et al, 1986) and may occur spontaneously during normal breathing or be stimulated by respiratory manoeuvres, various chemicals and hypoxia and hypercapnia (Glogowaska et al, 1972; Davies & Roumy, 1982; Coleridge & Coleridge, 1986; Berkenbosch et al, 1988; Marshall & Metcalfe, 1989). The precise mechanism for generation of ABs has not been determined yet, but appears to involve a variety of vagal and chemoreceptor inputs from the lung (Glogowska et al, 1972). During normal breathing the AB is activated in response to low lung compliance and its physiological function is to reverse the fall in compliance and end-expiratory lung volume associated with quiet breathing (Reynolds & Hilgeson, 1965; Szereda-

Przestaszewska et al, 1976; Coleridge & Coleridge, 1986). In addition, spontaneous ABs have been shown to increase the alveolar concentration of active forms of surfactant (Oyarzun et al, 1991). Even in asthmatic attacks in guinea pigs ABs appear in response to pulmonary compression caused by ventilation inequalities (Delmore & Koller, 1977).

This association of ABs with lung compression and deflation strongly suggest that ABs are generated by activation of pulmonary deflation receptors (rapidly adapting/irritant receptors) (Sellick & Widdicombe, 1970; Delmore & Koller, 1977) and there are direct recordings from lung irritant receptors illustrating this association. Phenylbiguanide, which is reported to stimulate C-fibre endings also elicits ABs, and as for histamine, is probably also stimulating RARs (Winning & Widdicombe, 1976; Coleridge & Coleridge, 1986). However, it is possible that stimulation of several different vagal afferent receptor types, either alone or in combination with enhanced chemoreceptor activity, may be involved in the generation of ABs (Glogowska et al, 1972; Marshall & Metcalfe, 1989). While the appearance of augmented breaths can be prevented by bilateral vagotomy (Marshall & Metcalfe, 1988; Szereda-Przestaszewska et al, 1992), re-appearance of ABs some time after vagotomy has been demonstrated in the rat and cat (Cherniak et al, 1981; Marshall & Metcalfe, 1988). Post-vagotomy ABs appear only in a small proportion of animals and are activated primarily by hypoxia and hypercapnia, possibly by affecting central control of somato-motor output to the respiratory muscles (Szereda-Przestaszewska et al, 1976).

Prabhakar et al (1987) have demonstrated that SP (100ng/Kg) can elicit ABs in anaesthetised rabbits after right atrial injection, but not after intra-aortic injection. The time to appearance of the AB was not consistent with stimulation of central structures or carotid chemoreceptors, and since the response was abolished by bilateral

vagotomy, this suggested sensory receptors accessible from the pulmonary circulation were involved. Furthermore, they demonstrated that SP stimulated activity in both rapidly adapting and C-fibre receptors suggesting both receptor types might be involved in the generation of ABs. Any role for carotid bodies in ABs might be in prolongation of the expiratory time rather than generation of the AB itself (Bowes et al, 1983).

While ABs occured only in 10 sheep and there was no definite association with maximal bronchoconstriction, the data suggests SP can stimulate pulmonary afferent receptors, which may in turn be involved in control of bronchomotor tone. The effects of vagotomy and vagal cooling on the bronchomotor response to SP tends to support this finding. However, the effect of SP, and other tachykinins, on bronchomotor tone in the guinea pig and rat cannot be abolished by vagotomy (Goel & Biggs, 1987; Joos et al, 1988). It is interesting to note that no ABs occurred in sheep over 12 months of age (n=9), and whether or not this is associated with the ontogenic change in the bronchomotor response (Chapter 5) is not known.

The degree of selectivity of vagal cooling in blocking myelinated and unmyelinated nerve fibres is questionable, as there is undoubtedly a wide temperature sensitivity range for both type of fibres (Franz & Iggo, 1968). However, at temperatures below approximately 10°C the majority of the myelinated fibres cease to conduct while conduction in the majority of unmyelinated C-fibers, and the unmyelinated efferent fibres, remains intact (Paintal, 1967; Sant'Ambrogio et al, 1984). The data from sheep strongly support the hypothesis that the response to SP involves a vagal reflex and on the basis of the cooling data the primary afferent input appears to be from the C-fibre afferents. However, as can be seen from Figure 72, cooling to below 10 ^{o} C still induced changes in respiratory parameters consistent with blockade of additional myelinated fibres from stretch receptors, and it could be presumed that sufficent

numbers of these fibres were conducting at 7 ^{o} C to be involved in the reflex response to SP.

Phenylbiguanide was used in this study, as a known stimulator of C-fibre afferent receptor activity, to assist in validating the response to vagal cooling. PBG elicits the Bezold-Jarisch reflex through activation of 5-HT_3 receptors and its cardiopulmonary effects of bradycardia and tachypnoea or apnoea is predominantly vagally-mediated (Evans et al, 1990). However, a proportion of the response to PBG is not vagally mediated and possibly involves activation of central 5-HT_{1A} receptors (Bogle & Ramage, 1989). While PBG has been reported to decrease conductance (reciprocal of resistance), Cdyn and tidal volume and increase the respiratory rate in rabbits (100ug PBG; 1.5-3.0 Kg body weight) (Karczewski & Widdicombe, 1969) its effect on bronchomotor tone in sheep was not assessed. However, a more recent study failed to reduce conductance (reciprocal of resistance) with PBG (128ug/kg) although it did cause a consistent dose-dependent reduction in Cdyn (Armstrong et al, 1990). Cooling of the vagi to 8-10^{o}C significantly reduced the bronchomotor response to PBG and the changes in breathing pattern, while vagotomy effectively abolished the response (Karczewski & Widdicombe, 1969). A similar effect was achieved with vagal cooling and vagotomy in sheep, suggesting the technique was reasonably effective in differentially blocking unmyelinated nerve fibre activity, but it is not possible to say if C-fibre ending stimulation, per se, results in bronchoconstriction.

SP and other sensory neuropeptides such as CGRP have been localised in unmyelinated C-afferent nerve fibres and are generally regarded as the putative neurotransmitters in these nerves. While endogenous SP is believed to be involved in, for example, afferent nociceptive neurotransmission, the effect of exogenous SP on C-fibre activity has only recently been investigated. In several types of rat dorsal horn preparations SP, NKA and CGRP enhance C-evoked firing in dorsal horn nociceptive

neurons, but have no effect on A-evoked (myelinated fibres) or on spontaneous activity (Nussbaumer et al, 1989; Kellstein et al, 1990). These C-evoked responses can be blocked by the SP antagonists [D-Pro2 ,D-Trp7,9] -SP and spantide, and the opiates, galanin, somatostatin and gamma-aminobutyric acid (GABA).

More likely than not in the spinal cord, the effect of SP is due to postsynaptic facilitation of the effects of agents such as L-glutamate rather than presynaptic modulation of neurotransmitter release from the C-fibres. As yet there is little direct evidence that exogenously applied SP itself increases intrinsic C-afferent fibre activity (see review by Maggi & Meli, 1988), but in specialised rabbit retinal ganglion cells (sensory neurons) SP increases both evoked and spontaneous discharges (Zalutsky & Miller, 1990) possibly through putative autoreceptors (Spigelman & Puil, 1991). It would also appear that C-fibre stimulation facilitates nociception centrally and that this involves release of SP (Wiesenfeld-Hallin et al, 1990). Maggi & Meli (1988) have speculated that a possible target for sensory neuropeptides are the sensory nerves themselves and there is indirect evidence that tachykinins, including SP, activate sensory afferents from abdominal visceral organs (Lew & Longhurst, 1985; Maggi et al, 1986, 1987). If this is the case, the putative receptor, at least in the urinary bladder, would appear to be NK-2 (Maggi et al, 1987). Activation of visceral sensory afferents by SP and NKA could additionally release neurokinin peptides centrally according to the mechanism described by Wiesenfeld-Hallin et al (1990) and affect vagal efferent outflow to the airways. Haxhiu et al (1989) have demonstrated that SP can act on structures in the ventral medulla to increase cholinergic output (antagonised by atropine) to the trachealis muscle causing bronchoconstriction. An indirect mode of action for the tachykinins is further supported by the findings that the cardiovascular depressor effect of tachykinins involves a vagal reflex (Bezold-Jarisch reflex), activated indirectly through NK-3 receptors in capsaicin-insensitive sensory fibres (Couture et al, 1989). Moreover, it has been shown that SP can

modulate the activity of vagal and sympathetic innervation of the heart and may be important in controlling reflex cardiac effects associated with activation of cardiac afferent nerve endings (Smith et al, 1992).

Whether similar mechanisms apply to pulmonary vagal C-fibre afferents, parasympathetic ganglia and the central ramifications of pulmonary vagal afferents is not known. However, there is ample evidence that in the airway sensory afferents function in much the same way as spinal afferents. Neurokinin release from non-adrenergic non-cholinergic excitatory nerve fibres (NANCis) can be antagonised by GABA, NPY and selective u-opoid receptor agonists such as [D-Ala2, NMePhe4, Gly5]ENK (Belvisi et al, 1990; Stretton et al, 1990a; Ray et al, 1991a,b). Furthermore, in guinea-pig airways exogenously administered SP and histamine require intact capsaicin-sensitive sensory afferents for at least part of their action (Biggs & Ladenius, 1990). Since histamine causes reflex bronchoconstriction possibly by activating rapidly adapting receptors (Karczewski & Widdicombe, 1969; Armstrong et al, 1990) it is possible that SP operates through this mechanism. However, at temperatures that block the majority of RAR fibre conduction (10 ^{o} C) the bronchomotor response to SP in sheep is largely intact, and complete abolition of the response is only achieved at 3 ^{o} C or after vagotomy. Sodium cromoglycate effectively blocks activation of C-fibre afferent endings by capsaicin in dog lung (Dixon et al, 1980) and the bronchoconstrictor effect of inhaled SP and NKA patients with mild to moderate asthma can be abolished by pre-treatment with the closely-related agent nedocromil sodium (Crimini et al, 1988a,b; Joos et al, 1989 a,b). The effect of nedocromil sodium on the bronchomotor response to SP in anaesthetised sheep was, however, equivocal (Chapter 6).

Determining whether or not SP affects afferent nerve fibre terminals, particularily C-fibre endings, in the sheep lung to cause reflex bronchoconstriction will require

recording activity in single afferent nerve fibres, as chemical manipulation of nerve fibre activity and vagal cooling alone are not sufficent. However, there are difficulties in interpreting the results of such experiments. The ability of a variety of agents, such as histamine, to increase vagal afferent input may be due to mechanical distortion of the area around receptors caused by direct contraction of smooth muscle (Sano et al, 1992). Demonstrating that the bronchomotor response with any agent is abolished by vagotomy is stronger proof that the mechanism has a reflex origin. The data from this study in sheep suggests there is a potential role for this reflex mechanism in the bronchomotor response to exogenously administered SP in the sheep.

CHAPTER 8. GENERAL DISCUSSION, FURTHER WORK AND CONCLUSIONS

8.1. General Discussion:

This study was developed to investigate the bronchomotor response of the sheep to exogenously administered neuorkinin peptides. Since the late 1970s there has been extensive interest in the potential role of these peptides in the pathological responses to airway diseases, including bronchoconstriction, airway oedema, mucus secretion and recruitment and activation of inflammatory cells. In general, the most important neurokinin in the airways of species examined to date seems to be NKA, suggesting the NK-2 receptor predominates in mammalian airways. With the increased use of the sheep as an experimental animal for investigating respiratory function, and more importantly, with the development of various sheep models of human respiratory conditions (Abraham et al, 1983; Wanner et al, 1979,1988) it was necessary to determine if the sheep demonstrated a similar sensitivity to the neurokinins as had been reported for other species.

The data from this study demonstrates that the order of potency for the bronchomotor response to the neurokinins in sheep is fundamentally different from that reported for other species, and the sheep response is similar to that recently reported for pigs (Haxhiu-Poskurica et al, 1992). While the lack of response with NKB roughly agrees with reports for other species (Advenier et al, 1987; Naline et al, 1989), SP is a significantly more potent contractor of airway smooth muscle than NKA. The fact that a similar order of potency is found in asthmatic sheep is of further importance considering its extensive use as a model of asthma in man.

With the increasing availability of specific neurokinin receptor antagonists the pharmacology of the neurokinins in the airways has come under closer scrutiny. In the

majority of studies the effects of neurokinins have been shown to be direct, involving interaction with neurokinin receptors on target organs (Petitet et al, 1991). In the respiratory tract the current consensus is that the neurokinins contract airway smooth muscle through activation of the NK-1 and NK-2 receptors on the muscle cells (Barnes 1991). However, there is evidence that an indirect component may be involved in some species and, in particular, several of the airway effects of the neurokinins may involve modulation of acetylcholine release from cholinergic nerve endings (Tanaka & Grunstein, 1984, 1986; Joos et al, 1988; Haxhiu-Poskurica, 1992). The results of the present study in sheep suggest the bronchomotor response to SP is largely indirect, and again this is roughly in agreement with reports in the pig and rabbit. Moreover, in sheep the response appears to involve a vagal reflex, and apart from demonstration of vagally-mediated SP-induced augmented breaths in rabbits, neurokinin-induced vagal reflex bronchoconstriction has not been demonstrated previously in other species.

8.2. Experimental Problems:

There were several problems found with the experimental techniques throughout this study.

With the anaesthetised preparation the problems with tachyphylaxis to SP and prolonged anaesthesia have been outlined. These limited the scope of experimental design that could be undertaken, in particular, the production of repeatable dose-response curves. In addition the problems with prolonged anaesthesia and tachyphylaxis prevented the repeated checking of the recovery of the bronchomotor response after re-warming the vagus nerve to normal body temperature. The continuous demonstration of the reversibility of the vagal cooling technique would have satisfactorily validated the results. However, by limiting the number of SP doses

administered the potential problems with tachyphylaxis and extended anaesthesia could be reduced.

The main problems that occurred with this project and the most disappointing part of the study involved the organ bath studies. The difficulty in obtaining consistent responses of trachealis muscle preparations to substance P limited the extent to which its pharmacology in the sheep airway could be assessed. The tissue samples that failed to contract with SP all contracted with methacholine, and as mentioned in Chapter 6, the substance P used was active in the live animal preparations. Samples were obtained from a local abattoir and precautions were taken to sample only sheep under 12 months of age (except when looking at the response of samples from old sheep), but it is possible that samples were inadvertently obtained from "older lambs".

A proportion of the immunostaining results were disappointing, as a sub-epithelial and intra-epithelial SP and CGRP-immunoreactive sensory nerve network was not demonstrated, and the density of nerves immunoreactive for these peptides was low. However, the demonstration of a rich supply of NPY-immunoreactive nerve fibres in smooth muscle was encouraging.

With the sheep asthma model there were no opportunities to assess the effects of various pharmacological antagonists or the response to intravenously administered SP. The technique itself was limited in its sensitivity as quite often the changes in RL and Cdyn could only be measured several minutes after drug administration, due to breath-holding and oesophageal regurgitation. It is possible that the peak bronchoconstriction was not measured in some animals because of this delay.

8.3. Further Work Required:

The mechanism of action of SP in causing bronchoconstriction in sheep requires additional investigation. The vagal reflex bronchoconstriction found with exogenously administered SP is a novel mechanism of action for a neurokinin peptide. Recording the activity of single afferent vagal nerve fibres in the presence of SP would assist in clarifying this issue. Although demonstrating activation of vagal afferent nerve fibre receptors by SP would not in itself prove a vagal reflex mode of action for SP as contraction of smooth muscle itself would increase afferent nerve fibre activity. However, preliminary studies are currently being planned to investigate the effects of neuropeptides on vagal afferents, and these studies will initially assess the effects of neurokinins on the activity of rapidly adapting receptors in rat lung.

The in vitro assessment of the contractile response to SP on the trachealis muscle could be investigated further. However, the variability in the response to SP found, and the tachyphylaxis that occurs, makes investigation in this preparation difficult. Determining whether or not the effect of SP involves neural or muscular receptors could be assessed using electrical field stimulation techniques and monitoring the effects of SP in the presence of neurotoxins, such as tetrodotoxin. As a clearly distinct neuronal receptor, mediating smooth muscle contraction, has been identified in guinea pig ileum (Laufer et al, 1985), this may explain the effect of muscarinic antagonists on the bronchomotor response to SP in sheep.

The ontogenic changes in the bronchomotor response to SP is a further novel finding and again work is in progress to investigate the ontogenic changes in peptide expression in the airways of sheep. The demonstration of developmental changes in

the response to spasmogenic neuropeptides, particularly in close association with the onset of sexual maturity, may have important implications in our understanding of the developmental changes seen with bronchoreactive diseases, such as asthma, in man (Hislop et al, 1990). More recently Berto and co-workers (1993), in a longitudinal study looking at young adult patients who had had childhood asthma and whose symptoms had ameliorated with age, found an improvement in their FEV values to a level similar to that found in the general population. It would be of additional interest to characterise the expression of neuropeptides in neural and non-neural elements of allergic asthmatic sheep.

8.4. Conclusions:

From the results of this study it can be concluded that the order of potency for the bronchomotor effects of neurokinins in sheep is different from that in most other species, and this order of potency also applies to asthmatic sheep. There is also an age-related alteration of the response to SP which roughly coincides with the onset of sexual maturity. Considering the increased use of sheep as experimental animals in respiratory physiology and pharmacology and the development of several types of sheep models of human respiratory diseases, these differences between the species need to be considered.

The mechanism of action of SP appears to be largely indirect, involving a reflex vagal bronchoconstriction mediated through the NK-1 receptor. There may be an additional indirect mechanism involving modulation of acetylcholine release from cholinergic nerve endings or modifying neurotransmission in airway parasympathetic ganglia. The mechanism of action of SP-induced bronchoconstriction in the sheep shows some similarity to that reported for the rabbit and pig, but is different from most other species, including man, where the mechanism is believed to be direct.

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APPENDICES

APPENDIX 1. PEPTIDE IMMUNOHISTOCHEMISTRY IN THE SHEEP AIRWAY AND LUNG

1. Introduction:

The investigation of the pharmacology and physiology of neuropeptides in most organ systems has tended to be undertaken prior to the demonstration of those particular peptides in the system being examined. Before beginning the study investigating neurokinins and their effect on sheep bronchomotor tone, there had been no investigation of neuropeptides and their localisation within the sheep respiratory system. To date only a single minor study has looked at the localisation of SP in sheep airways (Mariassy et al, 1990). Therefore, the aim of the present study was to investigate the localisation of various neuropeptides in the sheep airway and lung and to obtain qualitative information on the relative importance of these peptides in the innervation of airway smooth muscle. As there have been numerous reports on the histochemical characteristics of neuropeptides and their ubiquitous distribution in mammalian airways, it was presumed they would also be present in the sheep respiratory system.

Since the development of immunohistochemical techniques (Coons & Kaplan; 1950) numerous neuropeptides have been demonstrated in mammalian airways and lung tissue (Lundberg et al, 1984; Hislop et al, 1990), and in associated non-respiratory structures such as the carotid body (Lundberg et al, 1979). These peptides have been localised in both neuronal and non-neuronal structures in the respiratory system and in the extra-pulmonary neuronal networks innervating this system.

In the respiratory tract SP and VIP are found in autonomic nerves of the airway walls (Polak & Bloom, 1982). SP in particular is found in association with airway smooth

muscle and the airway epithelium, while VIP is usually found in nerve fibres close to blood vessels and mucus glands. SP has also been demonstrated in solitary neuroendocrine epithelial cells and neuroepithelial bodies in human lungs, with the greatest number of immunoreactive cells in foetuses and infants (Gallego et al, 1990). Within identified neural structures SP is found predominately in the vagal sensory jugular and nodose ganglia and in the vagus nerve itself (Hayashi et al, 1983). It tends to be co-localised with calcitonin gene-related peptide (CGRP) in sensory nerve fibres (Lee et al, 1985; Ju et al, 1987), particularily those located in the sub-epithelial layer in the airway mucosa (Luts & Sundler, 1989; Dey et al, 1990), and together they are the predominant sensory neuropeptides in the respiratory system. While CGRP immunoreactive nerve fibres are found in airway smooth muscle, they also have a close association with small blood vessels (Carstairs, 1987).

SP and CGRP are putative neurotransmitters for the afferent innervation of the respiratory system (Cadieux et al, 1986). The vagus is the main source of SP and CGRP detected in the lung, but a proportion of the total peptide found originates from dorsal root (sensory) ganglia sources (Saria et al, 1985; Springall et al, 1987). The main pulmonary sites for these sensory neuropeptides are in the central larger airways and associated blood vessels and to a lesser extent in the smaller airways and alveoli (Mak & Barnes, 1988).

Outside the respiratory system neuropeptides have been demonstrated in other structures that are involved in the control or modulation of respiration. In the carotid body large numbers of enkephalin (ENK) immunoreactive cell bodies and nerve fibres are found, while vasoactive intestinal polypeptide (VIP) and to a lesser extent substance P (SP) are found only in nerve fibres (Lundberg et al, 1979; Wharton et al, 1980; Smith et al, 1990).

SP and VIP have also been localised in sensory afferent neurons within sensory ganglia, dorsal root ganglia and the dorsal spinal cord (Harman & Keen, 1981; Buck et al, 1982). SP immunoreactive vagal fibres have been traced to the nucleus tractus solitarius, the dorsal motor nucleus of the vagus and the nucleus ambiguus in the medulla, showing central SP-peptidergic structures are involved in control of visceral function (Funakoshi et al, 1989).

An association between SP-containing and catecholamine-containing neurons has also been demonstrated in the CNS (Pickel, 1979), but whether or not a similar situation exists in the respiratory system is not known. However, it has proved difficult conclusively to demonstrate functional sympathetic innervation of the mammalian respiratory. Lastly, neuropeptide Y (NPY) has been co-localised with nor-adrenaline and has also been found in post-ganglionic parasympathetic nerves innervating the larynx (Johansson, 1986; Domeij et al, 1991).

2. Materials and Methods:

2.1. Tissue Fixation:

Tissue was obtained from sheep aged 6 to 12 months at a local abattoir. Material was removed within 10 minute of slaughter. Sections of trachea, two to three cartilage segments wide, a 10 mm wide section of the right apical lobe bronchus and three to four 10-20 mm wide sections of the right apical lung lobe were collected and immediately placed in a solution of saturated (15%) picric acid, formaldehyde (40%) and 0.2M phosphate buffered saline (PBS) (Zamboni's fixative) which had been kept at 4°C. Tissue was kept in fixative for 24 hours, then washed three times, 5 minutes each time, in

0.1M phosphate buffered saline and stored in 0.1M PBS and 0.05% azide at 4℃ until processed for immunostaining.

2.2. Tissue Sectioning:

The tissue was sectioned into suitable sized blocks and frozen onto corks on cryostat chucks in isopentane cooled with liquid nitrogen. The tissue was immersed in isopentane for a maximum of 2 minutes and placed in the cryostat cabinet to allow tissue temperature to equilibrate with the cabinet temperature (-26℃). 10um thick sections were cut and placed on gelatin-alum coated glass slides. A sufficent number of slides for the peptide immunostaining (3-5), negative controls, haematoxylin and eosin staining were cut for each tissue. The slides were then placed in a mositure chamber box to prevent the tissue from drying out. Tissue morphology was assessed, at the time of cryostating, by rapid staining of representative sections with toluene blue. Only sections of good morphological quality were used for immunostaining.

2.3. Immunostaining:

Several sources of primary antibodies were used. Primary antibody was either recieved as gifts or purchased from commercial sources and the details are given in Table 1. Antibodies against antigens for substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and met-enkephalin (met-ENK) were assessed. All primary antibodies had been raised in rabbits. The primary antibody was diluted, according to the manufacturers/suppliers instructions, with 0.1M PBS (neat antibody) and divided into 5ul aliquots and stored in 1ml Eppindorf tubes at -40℃. Prior to immunostaining neat primary antibody was diluted to a 1:10 dilution with 0.1M PBS and kept at 4℃. The antibody was then diluted with 0.1M PBS, containing 0.5% Triton

Antigen	Source	Optimal Dilution	Host Species
Substance P	Prof. J.M. Polak, London, UK	1:400	Rabbit
Substance P	Dr G.T. Pearson, Edinburgh, UK	1:400	Rabbit
Substance P RPN.1572	Amersham, UK	1:400	Rabbit
Calcitonin Gene-Related Peptide	Dr G.T. Pearson	1:400	Rabbit
Neuropeptide Y	Dr G.T. Pearson	1:400	Rabbit
met-Enkephalin RPN.1562	Amersham, UK	1:400	Rabbit
Vasoactive Intestinal Polypeptide RPN.1582	Amersham, UK	1:400	Rabbit

Appendix I; Table 1. List of the primary antibodies used to investigate the localisation of neuropeptides in sheep airways. The antigen to which the antibody was raised, the source (supplier), the optimal dilution and the animals in which the antibodies were raised are shown. All the antibodies gave optimal immunostaining, with minimal background immunofluorescence, at the 1:400 dilution. Of the three SP antibodies used the one donated by Dr GT Pearson gave the best results, and detail in the text refers to the results with this antibody.

100-X and 10% normal sheep serum, to the working dilution 30 minutes prior to application to the tissue.

The slides were pre-incubated in neat normal sheep serum for 30 minutes at room temperature to help reduce background staining. After washing once with 0.1M PBS, three to five tissue sections from the trachea, lobar bronchus and peripheral lung was covered with each of the primary antibodies. The optimal dilution for each antibody was determined in preliminary experiments (see Table 1). Volumes of between 40 and 60ul were required to cover the sections. Sections were returned to the moisture chamber boxes and left to incubate for 20 to 24 hours at room temperature. Except for SP, pre-adsorption controls were not carried out for all antibodies in sheep tissue. SP antibodies were incubated at room temperature for 30 minutes with 10, 50 and 100umol SP before application to the tissue, and then processed in the normal manner. However, pre-absorption controls for the primary antibodies had been carried out for SP, CGRP and VIP in dog skin (Hartland & Corcoran, pers. comm.) and for SP, CGRP, NPY, VIP and ENK in horse jejunum (Pearson & Woodman, pers. comm.), and demonstrated the antibodies to be highly specific for their respective antigens. Negative controls included tissues incubated with antibody diluent, 0.1M PBS and secondary antibody only and the diluents with secondary antibody.

After incubation the slides were washed three times, for five minutes each time, in 0.1M PBS. The slides were air- dried and then covered with a secondary anti-rabbit fluorescein isothiocyanate (FITC)-labelled antibody raised in goats (Cappell, NC, USA) diluted to 1:10 with 0.5% Triton 100-X PBS. The secondary antibody was left in contact with the tissue for 90 minutes and washed off, again three times for five minutes each time in 0.1M PBS. The slides were air-dried and mounted in buffered glycerine and covered with

glass slides. The slides were examined immediately and photographed if necessary. Otherwise the slides were stored in cardboard trays at -40°C, to preserve FITC flourescence, until they could be examined.

2.4. Assessment of Immunostaining:

Slides were examined for peptide-like immunoreactivity using an Olympus microscope (BH2, Olympus Optical Company, UK) fitted with a reflected light fluorescent attachment (BH2-2RFL) and an IF 490 excitation filter (wavelength 400-490nm) suitable for FITC studies. Slides were qualitatively assessed for the degree of background staining and the level of auto-fluorescence. Immunoreactive nerve fibres were photographed with an automatic exposure photomicrographic system (PM-10AK, Olympus).

Preliminary experiments had shown that fixing in ice-cold Zambonis fixative, pre-treatment of slides with normal sheep serum and addition of normal sheep serum to the primary antibody and the surfactant detergent Triton-100X to PBS used to dilute both the primary and secondary antibodies all improved the quality of tissue fixation and reduced the amount of background staining.

3. Results:

As it cannot be assumed that the primary antibodies are recognising specific peptide antigens the term peptide-like immunoreactivity (-Li) is used as convention.

Using the techniques outlined above, the level of background fluorecence was acceptable and did not interfere with identification of peptide containing structures. However, tracheal tissue contains large amounts of collagen which autofluoresces under ultra-violet light and can cause some problems with interpretation (Figures 1, 2 & 4 as examples).

Nerve fibres imunoreactive for NPY, CGRP, SP and VIP, but not ENK were found in sheep airways. The major site of immunoreactivity was in the trachea, with, apart from some VIP-Li in lobar bronchus, no detectable immunoreactive nerves in the smaller airways or peripheral lung.

In the trachea NPY-Li fibres were the most numerous with a dense inter-connecting network of fibres in the trachealis smooth muscle (Figure 1). There was also some NPY fibres around seromucous glands in the submucosa (Figures 2 & 3), but no evidence of NPY in the airway epithelium or the lamina propria. CGRP-Li was primarily found in single nerve fibres and nerve bundles in close associated with blood vessels (Figure 4) and as fine single nerve fibres in adjacent connective tissue (Figure 5). SP-Li fibres were found in similar location to CGRP fibres but distinct nerve bundles were not identified (Figure 8 & 9). SP-Li was also found close to glands in the submucosa (Figure 10). Smooth muscle fibres were sparse for both these sensory neuropeptides, although occassional distinct single immunoreactive fibres were noted between smooth muscle bundles (Figures 6, 7) or at the junction between smooth muscle and connective tissue (Figures 8, 9 & 11). No SP or CGRP-Li was detected in the epithelial layer. VIP-Li was found only in the smooth muscle layer of one lobar bronchus section and in epithelial neuroendocrine cells in one tracheal section. Met-ENK-Li was not detected in any of the sections.

4. Discussion:

Of the neuropeptides studied, the predominant neuropeptide in sheep airway smooth muscle appears to be NPY. NPY is often co-localised with noradrenaline in airway smooth muscle (Johansson, 1986; Luts and Sundler, 1989; Sonea et al, 1991a) and

modulates neurotransmission in NANCex neurons, being important in suppressing nerve-mediated contraction (Belvisi et al, 1989a; Grundmar et al, 1990; Stretton et al, 1990b). The demonstration of an intense NPY-Li neural network suggests the sheep airway smooth muscle may have a functional sympathetic innervation and this needs further investigation.

Although there were more CGRP then SP-Li nerve fibres, the sparse nature of both in the airway smooth muscle would suggest a minor role for these peptides in broncho-constriction. However, airway smooth muscle cells are connected together by gap junctions and smooth muscle activation does not require direct innervation of individual muscle cells (Daniel, 1988). A sparse neural network may still be sufficent to cause marked contraction of smooth muscle bundles. In most species studied CGRP appears to be the more commonly respresented sensory neuropeptide (Sonea et al, 1991b) and this appears to also apply to the sheep. In general SP in airways has been found co-localised with CGRP, but there is an additional population of CGRP neurons that do not contain SP (Lee et al, 1985; Ju et al, 1987; Luts & Sundler, 1989). The failure to demonstrate sub-epithelial SP and CGRP containing nerve fibres in the sheep contrasts with other species. In the horse CGRP-Li fibres, and to a lesser extent SP-Li fibres, form a dense arborising network close to the basement membrane and fibres penetrate between epithelial cells to lie close to the airway lumen (Sonea et al, 1991b) and a similar situation is described in other species such as the ferret (Luts & Sundler, 1989) and cat (Dey et al, 1991). Morphological changes due to poor fixation of the surface structures may explain this difference found with sheep. Haematoxylin and eosin staining of respresentative sections, however, demonstrated normal morphological features and it is ulikely this would explain the diference. It is possible, but very unlikely, that sensory afferent nerve fibres in the sheep airway do not terminate close to the airway lumenal

21. Normal Sheep Serum; Scottish Antibody Production Unit, UK

23. Pentobarbitone Sodium (5-ethyl-5-[1-methylbutyl]-2,4,6-trioxohexahydropyrimidine sodium); $C_{11}H_{17}N_{23}ONa$; MW 248.3; RMB Animal Health Ltd, UK

22. 1-Phenylbiuanide; $(C_6H_5(=NH)NHC(=NH)NH_2$; MW 177.21; Aldrich Chemical Company Ltd, UK

24. Phosphoramidon (N-(a-rhamnopyransosyloxy-hydroxy-phosphinyl)-Leu-Trp); $C_{23}H_{34}N_{10}OP$; MW 543.5; Sigma Chemical Company Ltd, UK

25. Picric Acid (2,4,6-trinitrophenol); $(NO_2)3C_6H_2.OH$; MW 229.11; BDH Laboratory Suplies Ltd, UK

26. Pirenzepine Dihydrochloride (5,11-dihydro-11-[(4-methyl-1-piperazinyl)acetyl]-6H-pyridol[2,3-b][1,4]benzodiazepin-6-one dihydrochloride; $C_{19}H_{21}N_5O_2.2HCl$; MW 442.31; Research Biochemicals Inc, USA

27. Potassium Chloride; KCl; MW 74.55; BDH Laboratory Supplies Ltd, UK

28. Potassium Dihyrogen Orthophosphate; KH_2PO_4; MW 136.09; BDH Laboratory Supplies Ltd, UK

29. Sodium Azide; NaN_3; MW 65.01; BDH Laboratory Supplies Ltd, UK

30. Sodium Chloride; NaCl; MW 58.44; BDH Laboratory Suplies Ltd, UK

31. Sodium Dihydrogen Orthophosphate Dihydrate; $NaH_2PO_4.2H_2O$; MW 156.01; BDH Laboratory Supplies, UK

32. Sodium Hydrogen Carbonate; $NaHCO_3$; MW 84.01; BDH Laboratory Supplies Ltd, UK

33. Spantide; [D-Arg^1,D-$Trp^{7,9}$, Leu^{11}]-Substance P; MW 1497.9; Cambridge Research Biochemicals Ltd, UK

34. Substance P; Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2; MW 1347.8; Cambridge Research Biochemicals, UK

35. DL-Thiorphan (DL-3-mercapto-2-benzyl-propanoylglycine) $C_{12}H_{15}NO_3S$; MW 253.3; Sigma Chemical Company Ltd, UK

36. Triton X-100; Bdh Laboratory Supplies Ltd

2. PHOSPHATE BUFFERED SALINES (PBS)

2.1. Agent Dilution PBS

Composition is given in mmol/litre

NaCl, 136; KCl, 2.68; $Na_2HPO_4.2H_2O$, 6.46; KH_2PO_4, 1.46

2.2. PBS for Zambonis Fixative (See Appendix I)

Composition is given in mmol/litre

Na_2HPO_4, 61; $NaH_2PO_4.2H_2O$, 32.1

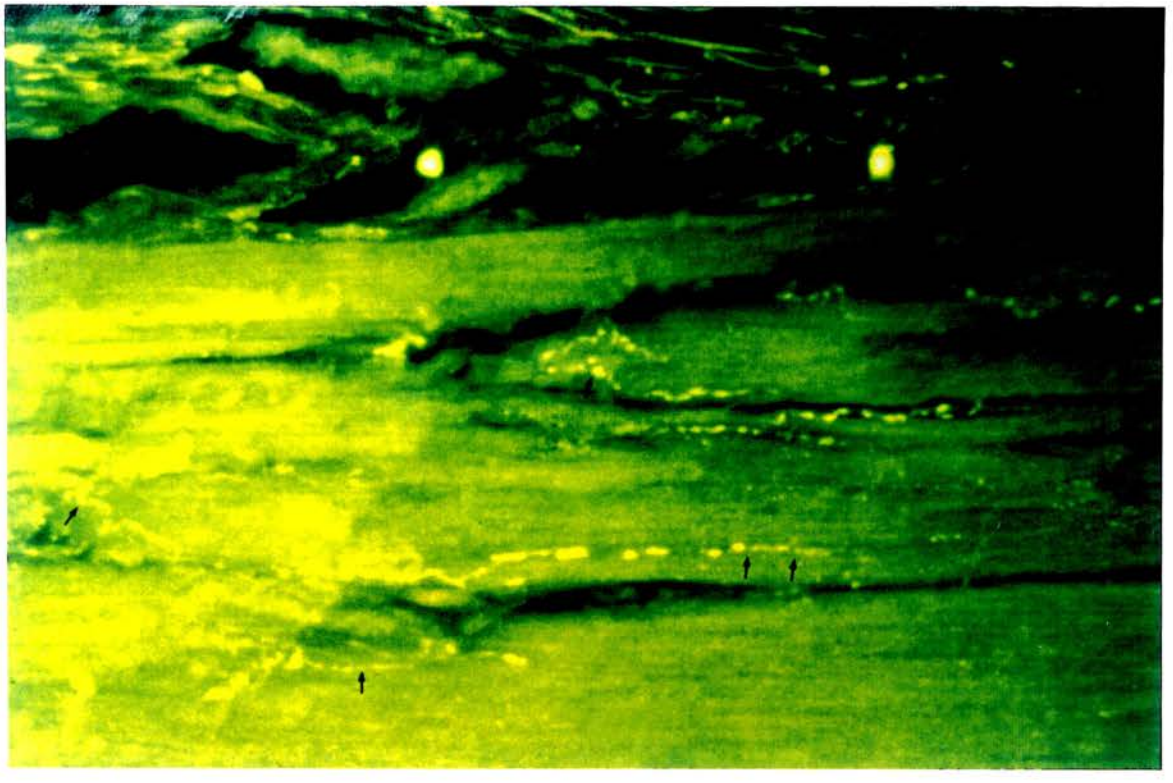

Figure 1. A section of the trachea incubated with NPY antiserum showing numberous nerve fibres (arrows) with prominent varicosities throughout the smooth muscle layer. There is little immunoreactivity in the underlying connective tissue layer (x200).

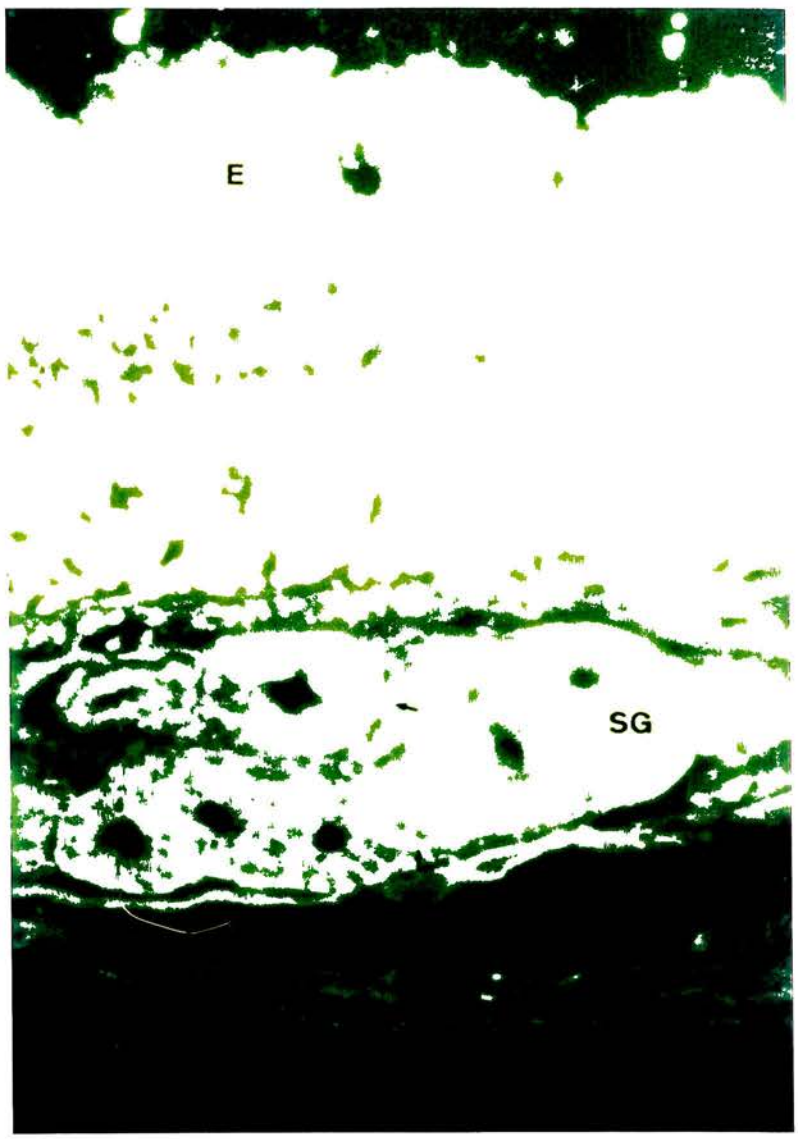

Figure 2. Section of the trachea including epithelium (E), sub-mucosa mucous glands (SG) and smooth muscle (SM), incubated with NPY antiserum. There are sparse NPY-like immunoreactive nerve fibres close to the mucous glands (small arrows) and in the smooth muscle layer (large arrow). No immunoreactive nerves are visible in the epithelial layer and lamina propria (x200).

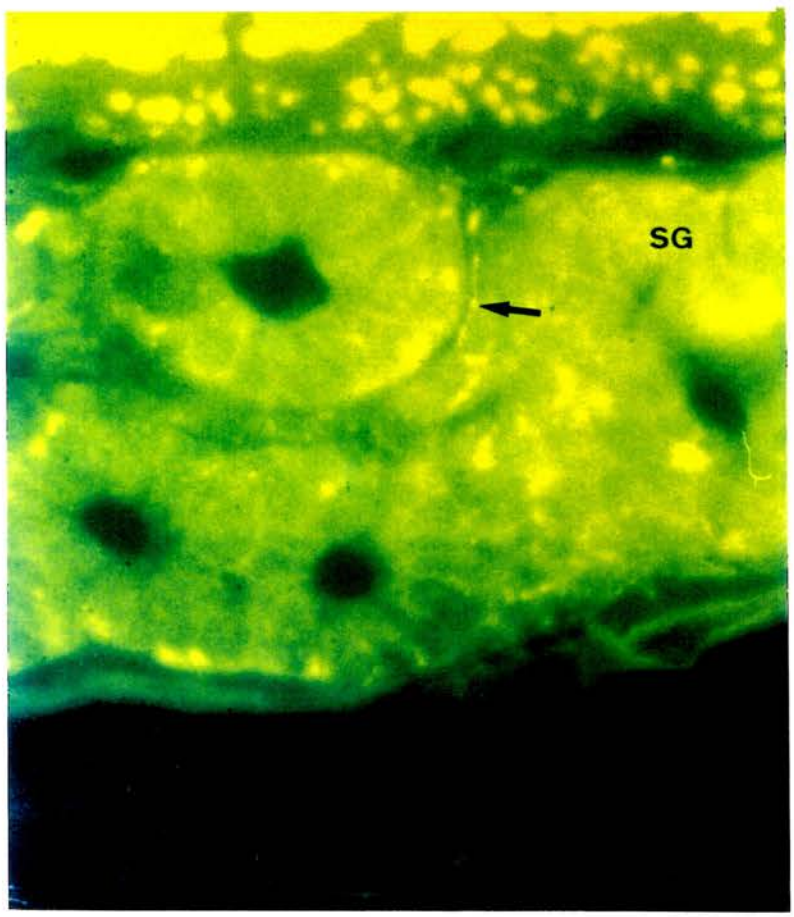

Figure 3. Enlargement of the submucosal mucus gland (SG) area in figure 2, showing NPY-like immunoreactive nerve fibre (arrow) (x400).

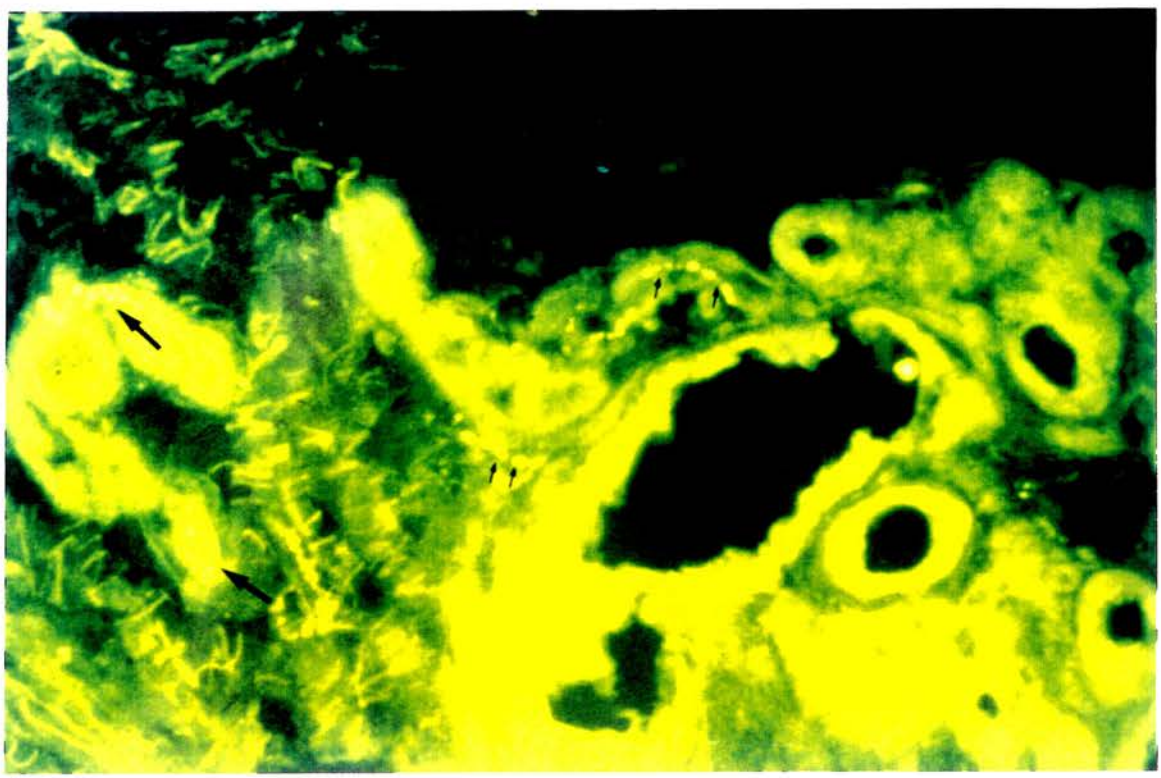

Figure 4. Section of the trachea showing blood vessels in the connective tissue layer underlying the trachealis muscle layer incubated with CGRP antiserum. There are several (small arrows) prominent immunoreactive nerve fibres close to blood vessels and two adjacent nerve bundles (large arrows) (x200).

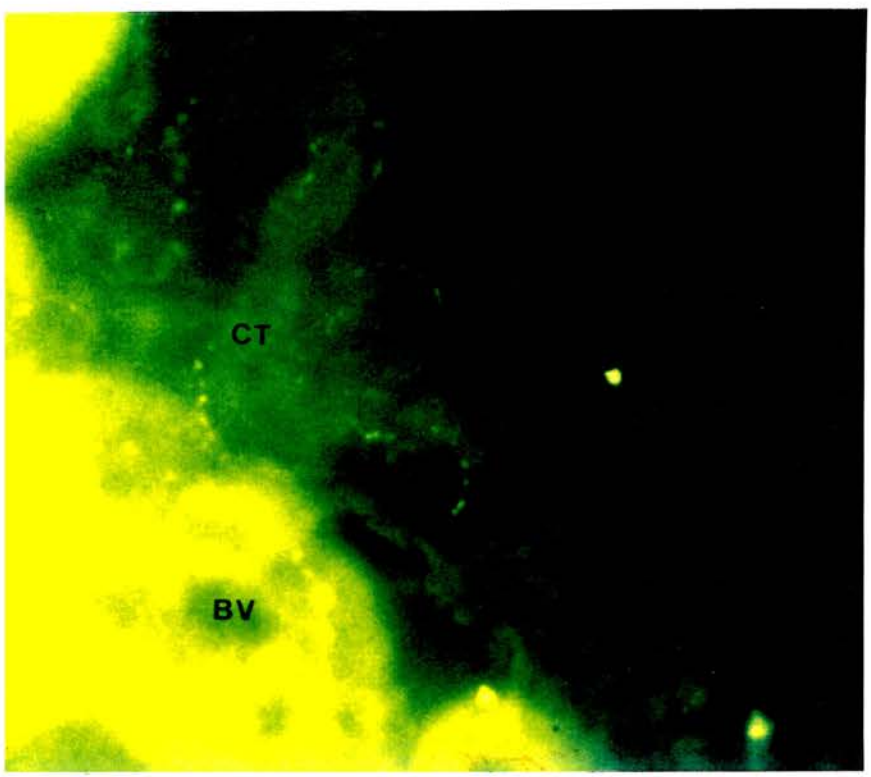

Figure 5. Enlargement of part of figure 4. In addition to the prominent CGRP-like imunoreactive nerve fibres around blood vessels (BV), there are less distinct fibres in the adjacent connective tissue (CT) (arrows) (x400).

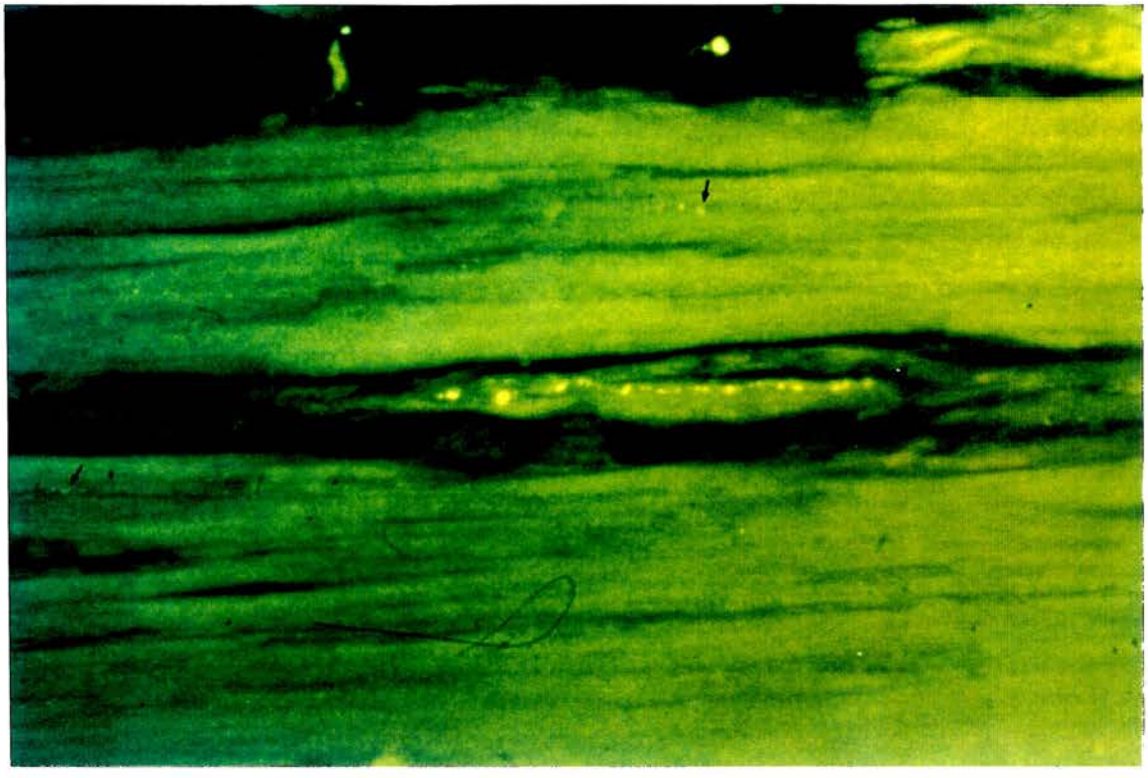

Figure 6. Single prominent CGRP-like immunoreactive nerve fibre and several less distinct fibres (arrows), in the trachealis smooth muscle layer. The prominent nerve fibre is located between muscle layers (x200).

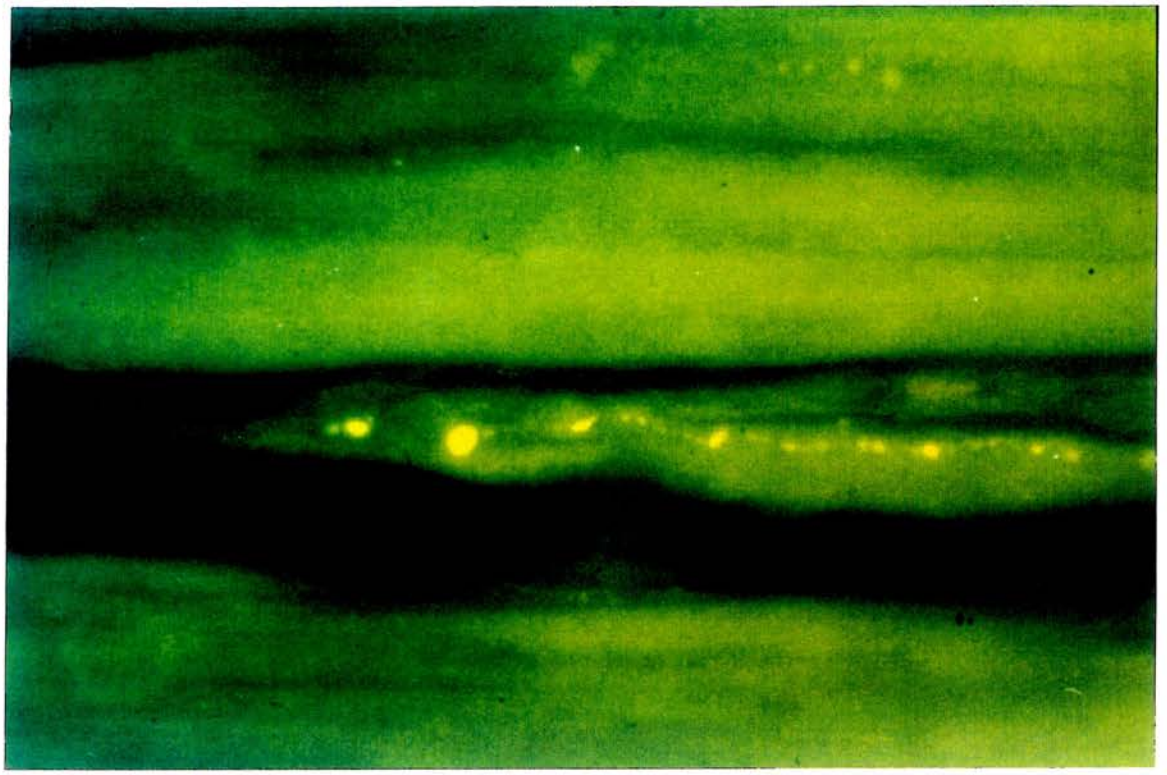

Figure 7. Enlargement of the prominent CGRP-like immunoreactive fibre in figure 6 demonstrating the distinct varicosities along the fibre length.

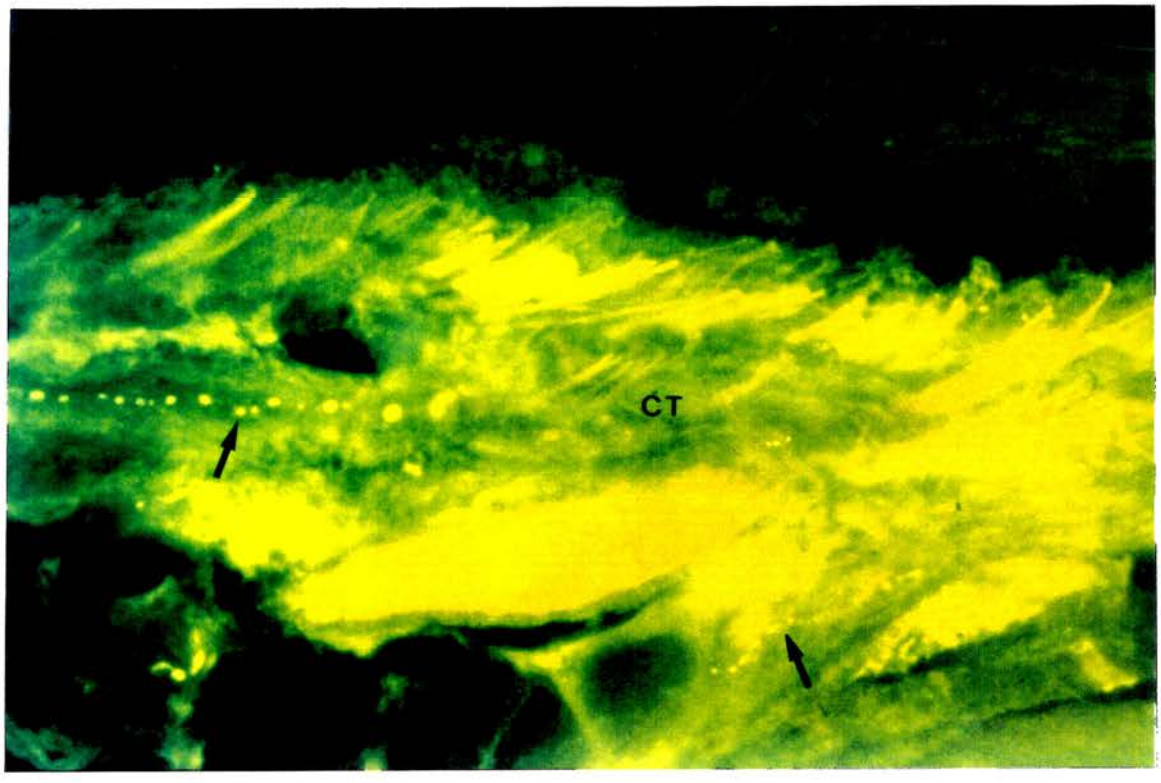

Figure 8. A section of the trachea showing smooth muscle (SM) and connective tissue (CT) incubated with SP antiserum. There are prominent SP-like immunoreactive nerve fibres in the connective tissue layer (arrows), but no fibres visible in the smooth muscle layer (x200).

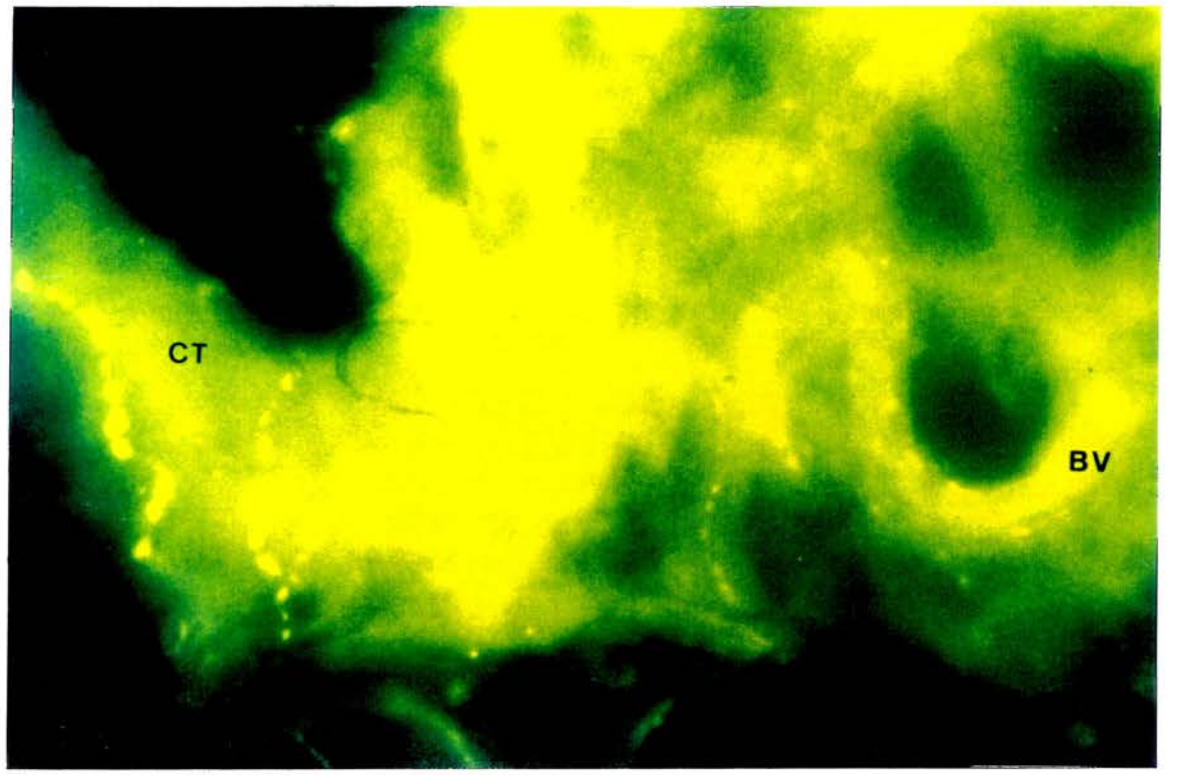

Figure 9. Prominent SP-like immunoreactive nerve fibres in connective tissue (CT) and close to blood vessels (BV) (x400).

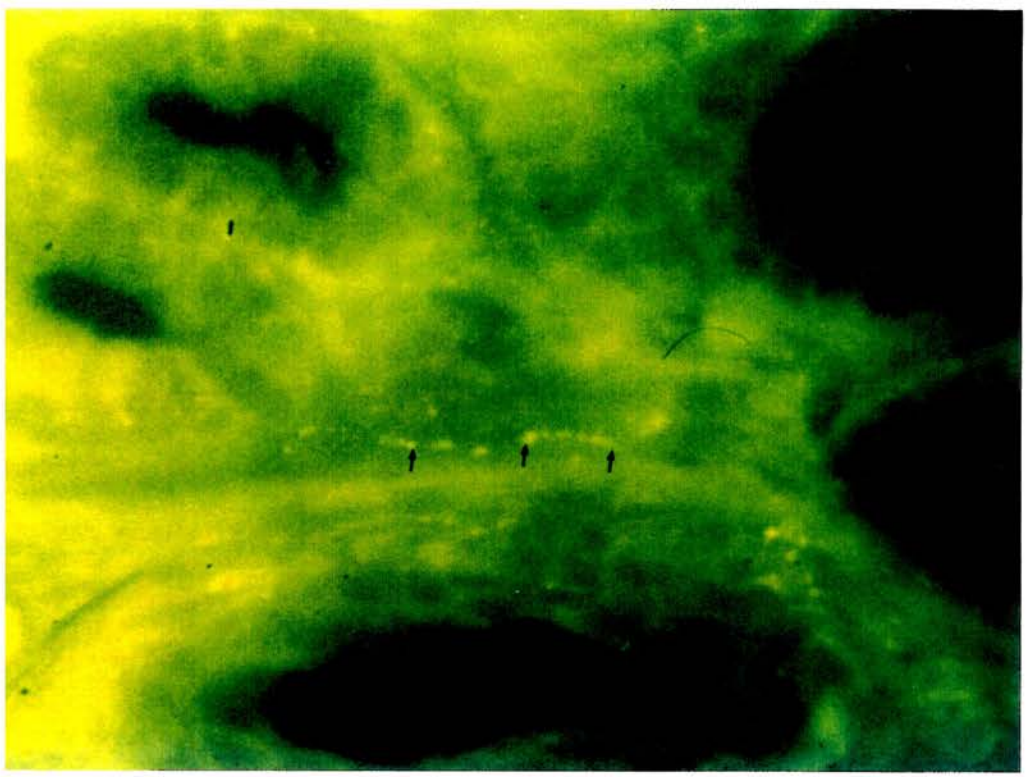

Figure 10. Section of the trachea showing submucosal mucous glands processed for SP-like immunoreactivity. There is a single faint SP-like immunoreactive nerve fibre coursing between glands (arrow) (x400).

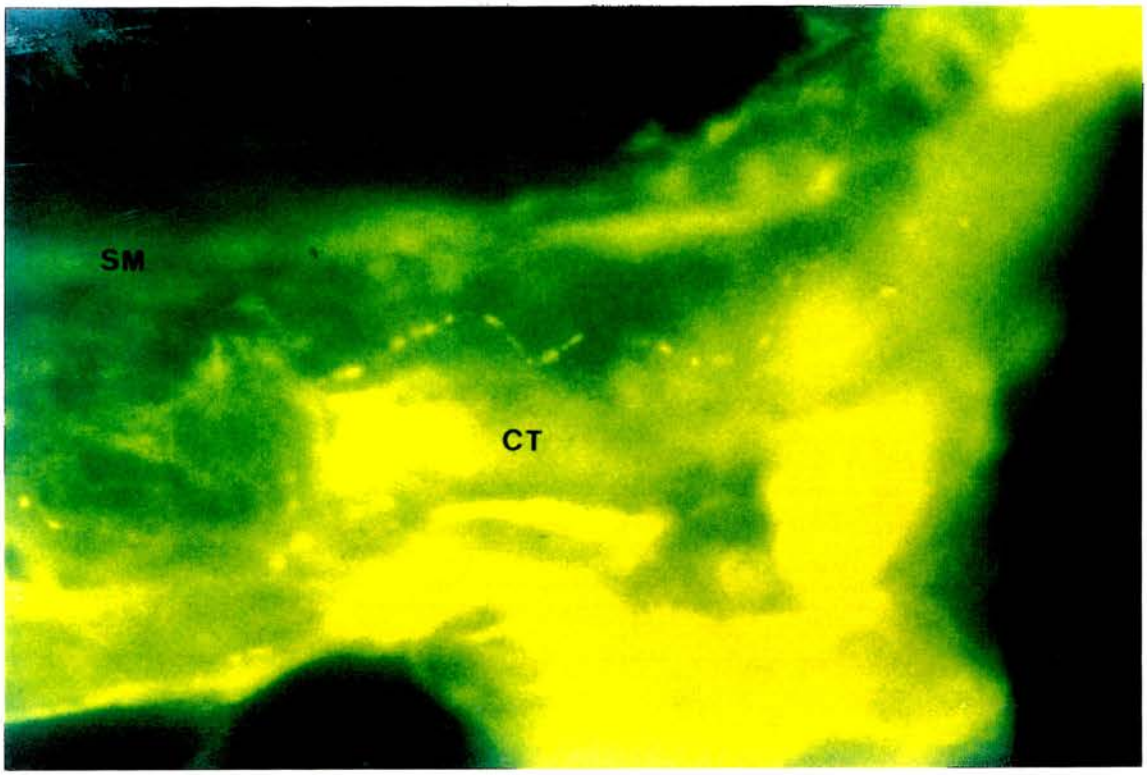

Figure 11. Section of trachea including smooth muscle (SM) and connective tissue (CT) incubated with SP antiserum. There are SP-like immunoreactive nerve fibres coursing between the smooth muscle and connective tissue layers (x400).

APPENDIX II

1. REAGENTS

Information includes the familar and scientific name of the reagent, it's molecular weight and manufacturer/supplier.

1. Agar powder (High Gel Strength); BDH Laboratory Supplies Ltd, UK
2. Calcium Chloride; $CaCl_2$; MW 58.44; BDH Laboratory Supplies Ltd UK
3. (+/-)-Chlorpheniramine Maleate; $C_{16}H_{19}ClN_2.C_4H_4O_4$; MW 390.9 Sigma Chemical Company Ltd
4. Chrome Alum; Raymond A Lamb, UK
5. CP96,345-1; ({2S,3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1 azabicyclo[2.2.2]octan-3-amine); MW 485.0; Pfizer Inc, USA (gift)
6. Decon 90; Decon Laboratories Ltd, UK
7. Di-sodium Hydrogen Orthophosphate 2-Hydrate; $Na_2HPO_4.2H_2O$; MW 177.99; BDH Laboratory Supplies Ltd, UK
8. Formaldehyde; HCHO; MW 30.03; BDH Laboratory Supplies Ltd
9. Gelatin; BDH Laboratory Supplies
10. D-Glucose; $C_6H_{12}O_6$; MW 180.16; BDH Laboratory Supplies
11. Heparin Sodium; Leo Laboratories Ltd, UK
12. Hexamethonium Bromide (Hexane-1,6-bis[trimethylammonium bromide]; $C_{12}H_{30}N_2Br_2$; MW 362.2; Sigma Chemical Company Ltd, UK
13. Isopentane (2-methylbutane); $(CH_3)_2CH.CH_2.CH_3$; MW 72.15; BDH Laboratory Supplies Ltd, UK
14. L-659874; Ac-Leu-Met-Gln-Trp-Phe-Gly-NH_2; MW 822.0; Cambridge Research Biochemicals, UK
15. Lidocaine Hydrochloride Anhydrous (2-Diethylamino-N-[2,6-dimethylphenyl]acetamide; lignocaine); $C_{14}H_{22}N_2O.HCl$; 270.8; Astra Pharmaceuticals Ltd, UK
16. Magnesium Sulphate 7-Hydrate; $MgSO_4.7H_2O$; MW 246.47; BDH Laboratory Supplies Ltd, UK
17. Methacholine Chloride (Acetyl-B-methylcholine chloride); $C_8H_{18}ClNO_2$; MW 195.7; Sigma Chemical Company Ltd, UK
18. Nedocromil Sodium (Disodium 6,9-dihydro-9-ethyl-4,6-dioxo-10-propyl-4H-pyrano[3,2-g]quinoline-2,8-dicarboxylate); $C_{19}H_{15}NNa_2O_7.1.5H_2O$; MW 442.33; Fisons PLC, UK (gift)
19. Neurokinin A; His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2; MW 1297.0; Cambridge Research Biochemicals,UK
20. Neurokinin B; Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2; MW 1210.45; Cambidge Research Biochemicals, UK

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THE EFFECT OF TACHYKININS ON SHEEP BRONCHOMOTOR TONE

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SUMMARY

The mammalian tachykinin neuropeptides substance P (SP) and neurokinin A (NKA) are present in the airways of several species and are involved in control of bronchomotor tone. We investigated the effect of SP and NKA on various respiratory and cardiovascular parameters in anaesthetized sheep. Dose-dependent decrease in dynamic compliance (C_{dyn}) and increase in respiratory resistance (R_L) occurred with intravenous administration of SP. The predominant effect of NKA was on C_{dyn} with little or no effect on R_L. Consequently SP is a more potent bronchoconstrictor than NKA in the sheep and affects both central and peripheral airway tone. The sensitivity to SP and NKA and the order of potency found suggests the NK-1 receptor predominates in sheep airways. Atropine and the ganglion blocker hexamethonium markedly reduced the bronchoconstriction caused by SP. SP and NKA were equipotent in causing a significant reduction in respiratory rate. SP caused a fall in mean blood pressure while NKA caused mild vasoconstriction. Neither peptide affected heart rate. We concluded that SP is a more potent bronchoconstrictor than NKA in the sheep and that the mechanism of action is mainly indirect involving modulation of postganglionic cholinergic nerve endings.

INTRODUCTION

Substance P (SP) and neurokinin A (NKA) are two closely related tachykinin neuropeptides that have been identified in sensory nerves, particularly C fibre sensory afferents, in the airways of several mammalian species (Lundberg, Brodin, Hua & Saria, 1984; Frigo, Barbareschi, Mariscotti, Motta, Felisati, Pignataro & Manghisi, 1989). SP and NKA cause contraction of airway smooth muscle *in vivo* (Evans, Dixon, Clarke, Conradson & Barnes, 1988; Shore & Drazen, 1989) and *in vitro* in a variety of species (Tanaka & Grunstein, 1986; Advenier, Naline, Drapeau & Regoli, 1987) and it has been suggested that the tachykinin neuropeptides are involved in airway inflammation and in the pathogenesis of asthma in man (Barnes, 1986). In addition to causing contraction of airway smooth muscle they can also activate mast cells and alveolar macrophages (Foreman & Jordan, 1983; Hartung & Toyka, 1983) and increase vascular permeability and protein leakage in lung tissue as part of the pulmonary inflammatory response (Hua, Lundberg, Theodorsson-Norheim & Brodin, 1984; Lundberg *et al.* 1984).

The bronchoconstrictor mechanism of action of the tachykinin neuropeptides is not completely understood. SP and NKA can act directly on NK-1 and NK-2 receptors in airway smooth muscle in guinea-pigs (Gerard, 1987; Kroll, Karlsson, Lundberg & Persson, 1990) and man (Advenier *et al.* 1987). In rabbits and rats SP-induced bronchoconstriction is atropine-sensitive, suggesting SP indirectly affects bronchomotor tone by modulating acetylcholine release from cholinergic nerves in these species (Tanaka & Grunstein, 1986; Joos, Pauwels & Van Der Straeten, 1988). SP can also cause the release of histamine and prostaglandins from mast cells and macrophages which could induce contraction of airway smooth muscle. However, in normal guinea-pig airways histamine is not involved in the

bronchomotor response to SP (Gerard, 1987; Shore & Drazen, 1989), but endogenous cyclo-oxygenase-derived metabolites of arachidonic acid mediate the contractile effect of SP in guinea-pig peripheral airways (Gerard, 1987) and also enhance the response to repeated SP administration in this species (Shore & Drazen, 1989). The effect of SP on mast cells and histamine release in sheep is not known.

Recently the sheep has attracted attention as a potential animal model for bronchial asthma in man (Wanner, Ahmed, Abraham, Phipps & Long, 1988), necessitating a greater understanding of its respiratory pharmacology and physiology. Abraham, Ahmed, Cortes, Soler, Farmer, Baugh & Harbeson (1991) have recently reported that inhaled SP and NKA have little effect on bronchomotor tone in conscious allergic adult sheep, but a response with SP can be obtained if the sheep are pretreated with the neutral endopeptidase inhibitor thiorphan. In the present study we have examined the respiratory and cardiovascular effects of injected SP and NKA in normal sheep and present evidence that SP causes bronchoconstriction by indirect mechanisms, modulating the activity of cholinergic nerves.

METHODS

Suffolk-cross sheep were obtained from commercial livestock sources. All sheep were female, between 6 and 12 months of age and weighed 30–40 kg. The animals were kept in a conventional animal house for at least 1 week before experiments and fed *ad lib.* hay and water. Food was withdrawn 24 h before experiments.

The sheep were anaesthetized with an intravenous injection of pentobarbitone at 25 mg (kg body weight)$^{-1}$. An in-dwelling catheter was placed in the right cephalic vein and anaesthesia was maintained with a constant infusion of 0·54% pentobarbitone in 0·15 M-NaCl adjusted to deliver 2 mg kg^{-1} h^{-1} of pentobarbitone. The sheep were intubated with a cuffed endotracheal tube and placed in the prone position with the head supported in a sling. An oesophageal balloon, constructed from a condom sealed over a length of rigid polyethylene tubing, was positioned in the lower oesophagus and a similar tube was connected to the opening of the endotracheal tube. Transpulmonary pressure was measured as the difference between oesophageal and airway pressure using a differential pressure transducer (UP2, Pioden Controls, Canterbury, Kent). Airflow at the endotracheal tube opening was measured with a pneumotachograph (F100L, Mercury Electronics, Glasgow) connected to another differential pressure transducer (UP1, Pioden Controls). Blood pressure and heart rate were monitored via a femoral artery catheter connected to a pressure transducer (4-327 I, Transamerica Deval, Pasadena, CA, USA). Signals from all transducers were amplified and recorded on heat-sensitive paper, and flow signals were integrated to give tidal volume measurements (MX 19, Devices, Welwyn Garden City, Herts). Respiratory pressure and pneumotachograph transducers were phase matched to a frequency of 6 Hz.

Inspiratory and expiratory respiratory resistance (R_L; $cmH_2O\,l^{-1}\,s$) and dynamic compliance (C_{dyn}; $ml\,cmH_2O^{-1}$) were calculated graphically from pressure, flow and volume measurements (Frank, Mead & Ferris, 1957). Inspiratory and expiratory values were used to calculate an 'average' C_{dyn} and R_L for the entire respiratory cycle. Systolic (S) and diastolic (D) pressure were measured from which mean blood pressure (MBP) was calculated ($MBP = D + (S - D/3)$).

Each experiment began with a dose–response curve to intravenous SP ($n = 10$). In some sheep ($n = 7$) this was followed 1 h later with a similar dose–response curve for NKA. With preliminary studies we found that the airway response to SP and NKA was not affected by the order in which the two peptides were administered, but as SP is more rapidly metabolized than NKA (Shore & Drazen, 1989) it was felt that SP should be administered prior to NKA. During each dose–response curve at least 3 min elapsed between peptide doses. Where there was marked bronchoconstriction at higher dose levels the subsequent dose was not administered until transpulmonary pressure had returned to baseline levels. Occasionally forced lung inflation was necessary to return transpulmonary pressure levels to baseline. C_{dyn} and R_L values were measured 20–30 s after peptide administration as this was when the most pronounced airway changes occurred. Changes in respiratory parameters usually began to return to baseline levels 40–50 s after peptide administration. Respiratory rate was also measured during this period. Values of C_{dyn}, R_L and respiratory rate were compared to those occurring immediately prior to each dose.

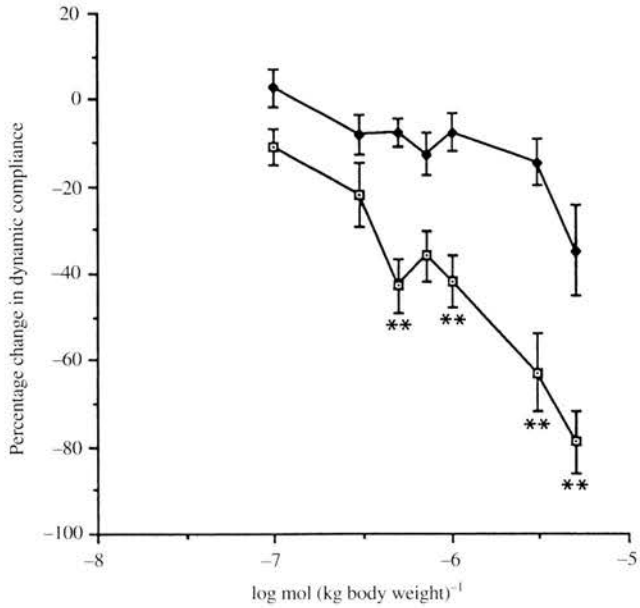

Fig. 1. Percentage decrease (means ± S.E.M.) in dynamic compliance after intravenous administration of substance P (□; $n = 10$) and neurokinin A (◆; $n = 7$). Significance of difference between SP and NKA, ** $P < 0.01$.

Mean blood pressure and heart rate (HR) changes were measured for SP ($n = 7$) and NKA ($n = 4$) 10 15 s after injection. Values were compared to those at the beginning of each dose response curve as changes in MBP and HR, in contrast to respiratory parameters, did not always return to baseline values between doses.

The effect of atropine ($n = 6$) and hexamethonium ($n = 3$) on the response to SP was also investigated. Sixty minutes were allowed to elapse between the last peptide dose–response curve and either the atropine or hexamethonium studies. A submaximal dose of SP was administered and this was followed 10 min later with an intravenous dose of atropine or hexamethonium. After a further 10 min the SP dose was repeated.

Substance P and neurokinin A (Cambridge Research Biochemicals) were dissolved in 0·15 M -NaCl to a concentration of 10^{-3} mol ml^{-1} and stored as 1 ml aliquots in Eppindorp tubes at −40 °C. Prior to each experiment the peptides were further diluted with 0·15 M-NaCl to a working concentration of 10^{-4} mol ml^{-1} and stored on ice. The peptides were administered intravenously by rapid bolus injection, in the dose range 10^{-7}–5×10^{-6} mol (kg body weight)$^{-1}$. Atropine sulphate and hexamethonium (Sigma) were dissolved in 0·15 M-NaCl immediately prior to use and administered intravenously at 1 and 20 mg (kg body weight)$^{-1}$ respectively over a period of 1 min.

Data are presented as means (±S.E.M.). The significance of any difference between SP and NKA and between the peptides and baseline measurements and the effects of atropine and hexamethonium were determined using one-way analysis of variance (ANOVA) and Student's paired t test where appropriate. $P < 0.05$ was taken as significant.

RESULTS

The bronchoconstrictor effects of intravenous substance P (SP) and neurokinin A (NKA) were examined in anaesthetized sheep. There was no significant different between baseline values of R_L and C_{dyn} prior to SP or NKA administration. SP and NKA caused a dose-dependent reduction in C_{dyn} with a maximal change of 79 ± 7 % with SP and of 34 ± 10 % with NKA (Fig. 1). While both peptides had a similar effect on C_{dyn}, SP was more potent than NKA at all dose levels except 10^{-7}, 3×10^{-7} and 7×10^{-7} mol kg^{-1} ($P < 0.01$). In direct

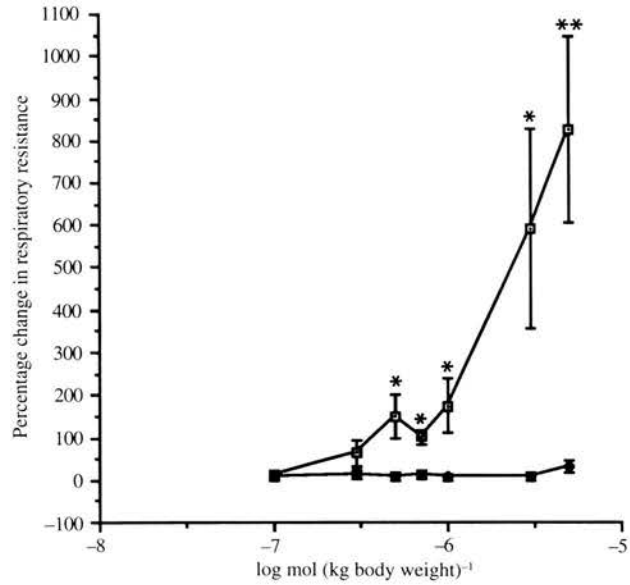

Fig. 2. Percentage increase (means ± S.E.M.) in respiratory resistance after intravenous administration of substance P (□; $n = 10$) and neurokinin A (◆; $n = 7$). Significance of difference between SP and NKA, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

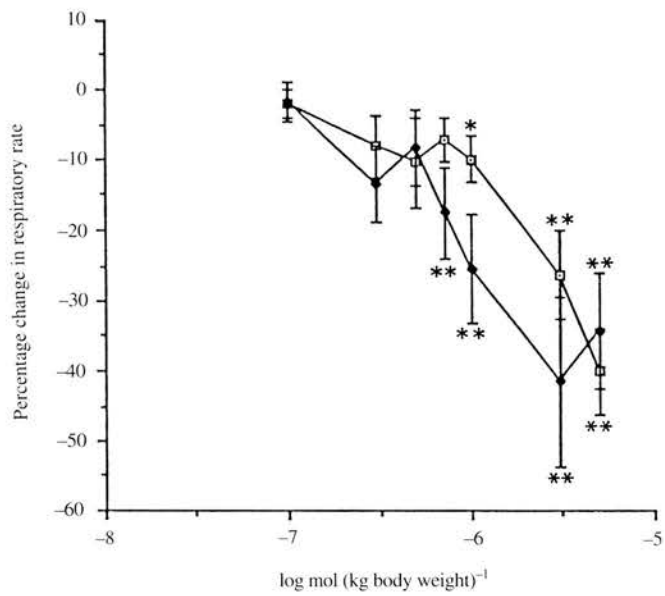

Fig. 3. Percentage decrease (means ± S.E.M.) in respiratory rate after intravenous administration of substance P (□; $n = 10$) and neurokinin A (◆; $n = 7$). * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

contrast, SP had a marked effect on R_L, while the effect of NKA on R_L was minimal and only significantly different from baseline at the highest dose of 5×10^{-6} mol kg^{-1} (Fig. 2). The difference between the effect of SP and NKA on R_L was statistically significant at doses of 5×10^{-7} mol kg^{-1} or greater ($P < 0{\cdot}05$).

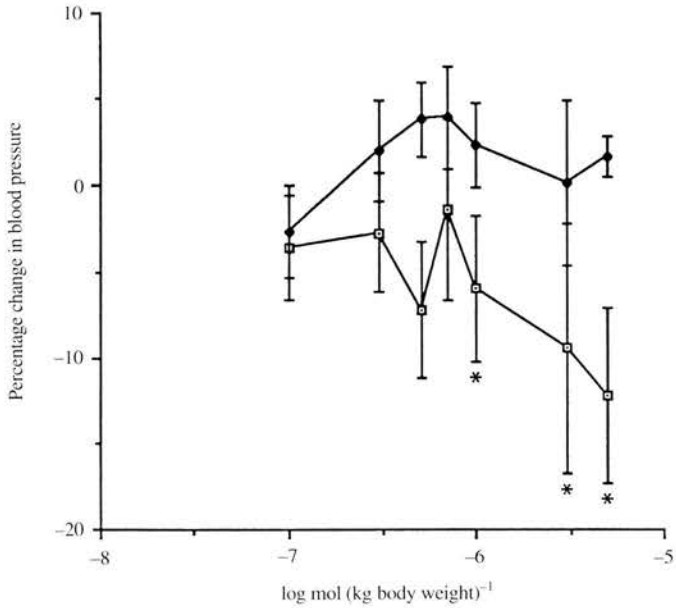

Fig. 4. Percentage change (means ± S.E.M.) in mean blood pressure after intravenous administration of substance P (□; $n = 7$) and neurokinin A (◆; $n = 4$). Significance of difference from baseline, * $P < 0{\cdot}05$.

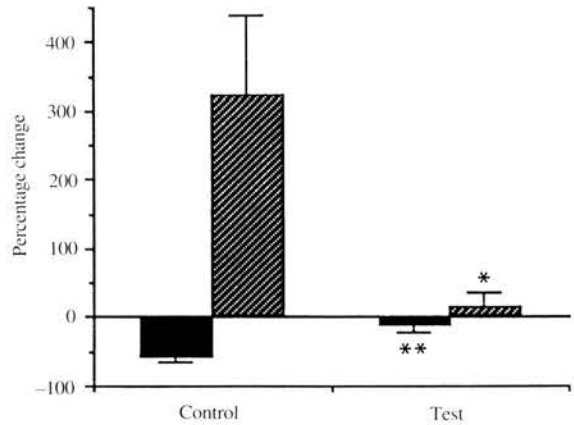

Fig. 5. The effect of intravenous pretreatment with atropine (1 mg kg^{-1}) on percentage change (means ± S.E.M.) in dynamic compliance (filled columns) and respiratory resistance (hatched columns) after intravenous administration of substance P ($n = 6$). Control and Test represent the SP response before and after atropine, respectively. Significance of difference between control and test, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

There was a dose-dependent reduction in respiratory rate with SP and NKA and this was significantly different from baseline values at doses of 7×10^{-7} mol kg^{-1} or greater ($P < 0{\cdot}05$; Fig. 3). NKA had a more marked effect on respiratory rate, but the difference between the two neuropeptides was not significant. SP caused a dose-dependent reduction in MBP while NKA caused a marginal increase (Fig. 4). With both peptides, changes in MBP were due to changes in the same direction in both systolic and diastolic pressures. The change in MBP with SP was significantly different from baseline at doses of 10^{-6} mol (kg body weight)$^{-1}$ or greater.

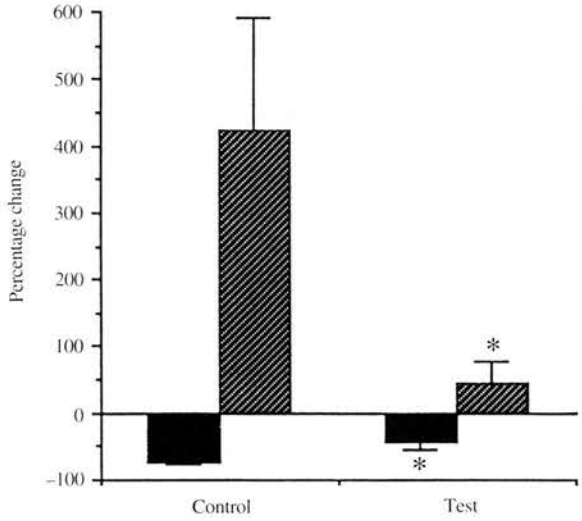

Fig. 6. The effect of intravenous pretreatment with hexamethonium (20 mg kg^{-1}) on percentage change (mean ± S.E.M.) in dynamic compliance (filled columns) and respiratory resistance (hatched columns) after intravenous administration of substance P ($n = 3$). Control and Test represent the SP response before and after hexamethonium, respectively. Significance of any difference, * $P < 0.05$.

Atropine ($n = 6$) had no significant effect on basal bronchomotor tone, but reduced the effect of a submaximal dose of SP (Fig. 5). SP caused a mean fall in C_{dyn} of 57 ± 9% and an increase in R_L of 321 ± 115%. After atropine pretreatment the changes in C_{dyn} and R_L were −13 ±11 and +15 ±18% respectively ($P < 0.05$). Hexamethonium ($n = 3$) also reduced the change in C_{dyn} and R_L to SP (Fig. 6) from a control value of −71 ± −4 to −43 ± 10% for C_{dyn} ($P < 0.05$) and from 425 ± 168 to 44 ± 33% for R_L ($P < 0.05$). Hexamethonium also markedly reduce mean blood pressure from 98 ± 10 to 67 ± 14 mmHg ($P < 0.05$).

DISCUSSION

This study demonstrates that SP is a potent bronchoconstrictor agent in the sheep and has a marked effect on both central (R_L) and peripheral (C_{dyn}) bronchomotor tone. NKA has some effect on C_{dyn} but has little or no effect on R_L. This is an unusual finding, and while NKA might preferentially constrict the peripheral airways, it is probable that the decrease in C_{dyn} with NKA is due to pulmonary vascular congestion and subsequent alveolar collapse. Our results compare with inhalation studies where SP, in allergic sheep pretreated with the neutral endopeptidase inhibitor thiorphan, is a more potent bronchoconstrictor than NKA (Abraham *et al.* 1991). However, the bronchoconstrictor effect of inhaled SP in allergic sheep is only modest and is far less than that achieved with bradykinin. The effect of injected SP and NKA in allergic sheep has not been reported. The bronchoconstrictor order of potency for SP and NKA in sheep contrasts with that reported for other species. NKA is more potent than SP in rats (Joos *et al.* 1988), in guinea-pigs (Hua *et al.* 1984; Uchida, Nomura, Ohtsuka, Hasegawa, Goto, Kimura, Sugita & Uchiyama, 1987) and in isolated airway tissue preparations in man (Advenier *et al.* 1987; Naline, Devillier, Drapeau, Toty, Bakdach, Regoli & Advenier, 1989). This suggests that the NK-2 receptor is the predominant tachykinin receptor in the airways of these species. In both asthmatic and normal humans inhaled and infused SP (NK-1 receptor agonist) has little or no effect

on bronchomotor tone (Joos, Pauwels & Van Der Straeten, 1987; Evans *et al.* 1988), while NKA is an effective bronchoconstrictor agent in asthma patients (Joos *et al.* 1987). On the basis of our results it would appear that the NK-1 receptor predominates in sheep airways. The recent development of specific tachykinin receptor antagonists for NK-1 and NK-2 receptors will facilitate characterization of this division, but their present use is constrained by cost. However, as with rabbits and rats, the bronchoconstrictor response to SP in the sheep was largely inhibited by atropine pretreatment (Tanaka & Grunstein, 1984, 1986; Joos *et al.* 1988), suggesting that SP acts indirectly causing bronchoconstriction by prejunctional modulation of acetylcholine release from vagal cholinergic nerve endings. In allergic sheep bradykinin-induced bronchoconstriction, which results from stimulation of C fibre afferent endings, can also be inhibited by atropine. It is possible that several bronchoactive neuropeptides, including SP, have a similar mechanism of action when administered exogenously to sheep (Abrahams *et al.* 1991). In the guinea-pig atropine has no effect on SP-induced bronchoconstriction (Shore & Drazen, 1989) and SP has a direct effect on smooth muscle (Gerard, 1987). There is evidence that peptides associated with non-adrenergic non-cholinergic neurotransmission in airways can act by a combination of direct and indirect mechanisms. Vasoactive intestinal peptide (VIP) relaxes airway smooth muscle by inhibiting excitatory neurotransmission prejunctionally and has a direct action on smooth muscle (Hiroyuki & Ito, 1990). We have also found that SP causes contraction of isolated sheep trachealis muscle *in vitro* suggesting an additional direct action for SP (B. M. Corcoran & A. L. Haigh, unpublished observation).

The cardiovascular effects of SP and NKA could indirectly affect bronchomotor tone through arterial baroreceptor and chemoreceptor reflexes. Baroreceptor activation results in reflex inhibition of respiration and bronchodilatation (Grunstein, Derenne & Milic-Emili, 1975). The marginal pressor response with NKA may have activated this reflex, but this is unlikely. Chemoreceptor activation by arterial hypoxaemia will cause bronchoconstriction and this could have augmented the bronchoconstriction caused by SP. However, we did not monitor blood gases.

The reduction of the bronchoconstrictor response to SP by hexamethonium, although not as marked as with atropine, contrasts with previous reports in other species. Hexamethonium enhances the SP-induced bronchoconstriction in the rat (Joos *et al.* 1988) and guinea-pig, and in the latter case this is due to inhibition of sympathetic innervation to the airways (Stewart & Fennessy, 1986). Our results suggest that SP-induced bronchoconstriction in the sheep involves ganglionic neurotransmission and might be of reflex origin, involving activation of pulmonary vagal afferent nerves. The marked vasodilatation that occurred with hexamethonium was systemic evidence of ganglion blockade, but we have not assessed the role, if any, of vasodilatation in the SP response.

The significant fall in respiratory rate with SP and NKA has not been reported previously. The reduction in respiratory rate did not involve a reduction in tidal volume suggesting the baroreceptor reflex was not activated. Furthermore, the bronchoconstriction preceded or coincided with the reduction in respiratory rate suggesting pulmonary stretch receptors or blood gas changes are not involved in the bronchomotor response. In man slow infusion of SP results in an increase in ventilation and in the ventilatory response to hypoxia, but with no change in the respiratory rate (Fuller, Maxwell, Dixon, McGregor, Barnes, Bloom & Barnes, 1987; Maxwell, Fuller, Dixon, Cuss & Barnes, 1990). Intra-atrial (pulmonary artery) injection of SP in rabbits increases the respiratory rate by activating C fibre bronchopulmonary afferent nerve endings (Prabhakar, Runold, Yamamoto, Lagercrantz, Cherniack & Von Euler, 1987). However, Green, Schmidt, Schultz, Roberts,

Coleridge & Coleridge (1984) demonstrated that stimulation of bronchopulmonary C fibre afferents by capsaicin, which releases neuropeptides from sensory nerve endings, can also result in transient apnoea and have suggested co-existence of both depressant and stimulant divisions of the pulmonary chemoreflex.

The reduction in mean blood pressure (MBP) with intravenous SP compares with previous reports from other species including man (Hua *et al.* 1984; Fuller *et al.* 1987; Joos *et al.* 1988) indicating that SP is a potent vasodilator. Joos *et al.* (1988) have speculated that the reduction in C_{dyn} with SP may be secondary to vasodilatation as SP can relax precontracted pulmonary vessels. However, we have shown that NKA causes mild vasoconstriction and so can reduce C_{dyn} without causing a reduction in blood pressure. Neither peptide had a significant or consistent effect on heart rate.

In summary, we have demonstrated that the tachykinin neuropeptides SP and NKA cause a dose-dependent bronchoconstriction in sheep. SP is a more potent bronchoconstrictor than NKA. While the exact mode and site of action of SP and NKA is not known the response to SP may be indirect, vagally mediated and possibly of reflex origin involving prejunctional release of acetylcholine from postganglionic cholinergic nerve endings.

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Age-related respiratory responses to substance P in normal sheep

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The in vivo effects of substance P (SP) on respiratory parameters in four different age groups of sheep were examined. Intravenous SP (10^{-8} to 5×10^{-6} mol kg^{-1} bodyweight) caused a dose-dependent reduction in dynamic compliance and increase in respiratory resistance in all four groups. The bronchoconstrictor response was age-related, with the greatest response occurring in the youngest age group (four to six months). In the oldest group (over four years) there was minimal bronchomotor response to SP, but a dose-dependent apnoea was present. These findings indicate that there is an age-related alteration in the respiratory response to SP in sheep.

SUBSTANCE P (SP) is an undecapeptide member of the tachykinin family of neuropeptides which has been identified in the respiratory tract of several mammalian species and is present in sensory nerves including unmyelinated C-fibre afferents (Lundberg et al 1984). In most species SP is a potent bronchoconstrictor and vasodilator (Corcoran and Haigh 1992), but in adult humans its main effect is on ventilation (Fuller et al 1987, Maxwell et al 1990). SP has also been identified in the carotid bodies and is believed to be a mediator in the hypoxic ventilatory response (Scheibner et al 1988). The bronchomotor and ventilatory responses to SP also appear to change with age (Tanaka and Grunstein 1990) and the increase in the hypoxia response coincides with an age-related increase in carotid body glomus cell SP-like immunoreactivity (Scheibner et al 1988). The present study was undertaken to investigate the bronchomotor response to SP in normal sheep and also to determine if there is any alteration in this response with age.

Experiments were carried out on 11 Suffolk-cross and four Scottish Blackface female sheep obtained from commercial sources. The sheep weighed 30 to 60 kg and were divided into four age groups: group 1, four to six months (n=3); group 2, six to 12 months (n=4); group 3, approximately 18 months (n=4); group 4, approximately four years (n=4). Group 4 were Scottish Blackface as these were the only aged sheep available from commercial sources.

The sheep were anaesthetised with an intravenous injection of 30 mg kg^{-1} bodyweight and maintained with a constant infusion of 0·54 per cent pentobarbitone in normal saline (0·15 M) and adjusted to deliver 2 mg $kg^{-1}h^{-1}$ of pentobarbitone. The sheep were intubated with a cuffed endotracheal tube, placed in a prone position with the head supported by a sling, and allowed to breath room air. Dynamic compliance (Cdyn) and respiratory resistance (RL) were calculated graphically from transpulmonary pressure, respiratory flow and tidal volume using methods described elsewhere (Corcoran and Haigh 1992). Periodic forced lung inflation with 100 per cent oxygen was used to reverse changes in Cdyn associated with anaesthesia. The presence of hypoxia or hypercapnia, however, was not assessed.

SP (Cambridge Research Biochemicals) was dissolved in normal saline (0·15 M) and administered by rapid intravenous injection via the right cephalic vein in the dose range of 10^{-8} to 5×10^{-6} mol kg^{-1} bodyweight. An injection of 1 ml normal saline was used as control and had no effect on basal bronchomotor tone. Dose-response curves for SP were recorded within one hour of the induction of anaesthesia.

Cdyn, RL and respiratory rate were measured 20 to 30 seconds after SP injection. The presence of apnoea (seconds) was also noted and included periods of complete cessation of respiration and, or, concurrent reduced pressure and flow. Typically apnoea appeared 25 to 30 seconds after injection.

Data are presented as the mean (±SEM). The significance of any difference between groups was determined by one-way analysis of variance. $P<0·05$ was taken as significant.

There was a dose-dependent decrease in Cdyn and increase in RL with SP in all sheep which was most pronounced in the youngest group (Fig 1). This group also achieved a greater maximum bronchoconstriction, as measured by Cdyn and RL, compared to the other groups. The changes in RL and Cdyn in groups 1 and 2 were significantly different from those in groups 3 and 4, while the increase in RL in group 1 was significantly different from those in group 2. Group 3 had a significantly greater decline in Cdyn than group 4.

There was a dose-dependent reduction in respiratory

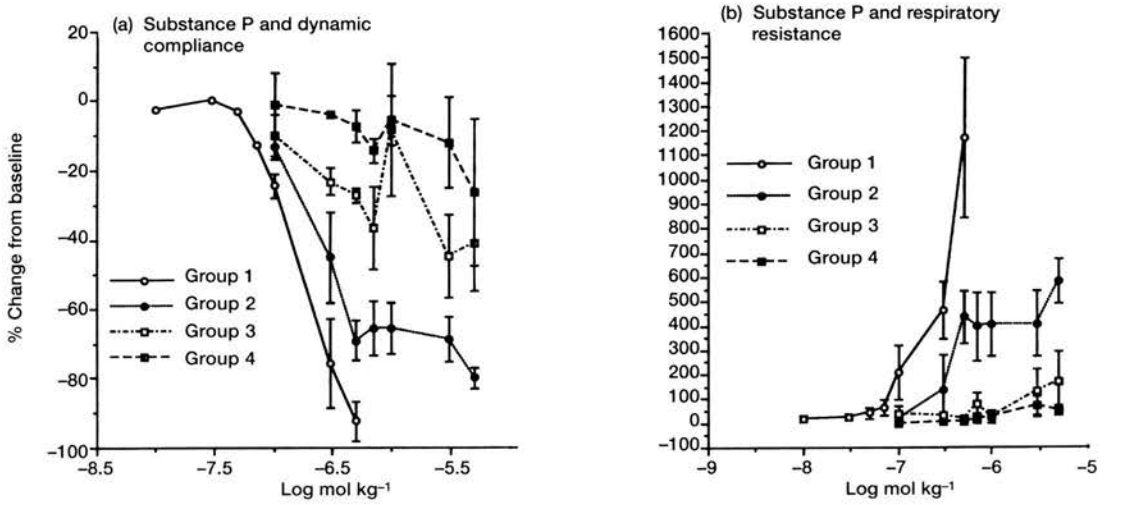

FIG 1: Percentage change in dynamic compliance and respiratory resistance after intravenous injection of substance P (10^{-8} to 5×10^{-6} mol kg^{-1}). Group 1, four to six months; group 2, six to 12 months; group 3, 12 to 18 months; group 4, more than four years. Values are mean ± SEM (vertical bars)

rate in all four groups (Fig 2a). Groups 1 to 4 had mean (±SEM) basal respiratory rates of 30, 35 (±2·9), 36·2 (±8) and 29 (±3·3) breaths per minutes, respectively. There was no statistically significant difference in basal respiratory rates in groups. The decrease in respiratory rate was similar in groups 1 and 4 and was significantly different from group 3. Three sheep in group 4 also had marked apnoeic periods (Fig 2b), which were dose dependent (data not shown). However, one sheep in group 4 did not develop apnoea, but had the most pronounced bronchoconstrictor response of that group. At no time did group 1 sheep demonstrate apnoea and in groups 2 and 3 apnoeic periods tended to be minor or inconsistent.

The authors have demonstrated that SP is a potent bronchoconstrictor in sheep, that the response decreases with age and that in aged sheep SP causes apnoea. They have not assessed the affect of breed differences or possible subclinical respiratory disease in the aged sheep on the results. SP is recognised as a potent bronchoconstrictor in a variety of species including sheep (Corcoran and Haigh 1992), but has little effect on bronchomotor tone in adult humans, where the predominant effect is to increase ventilation and improve

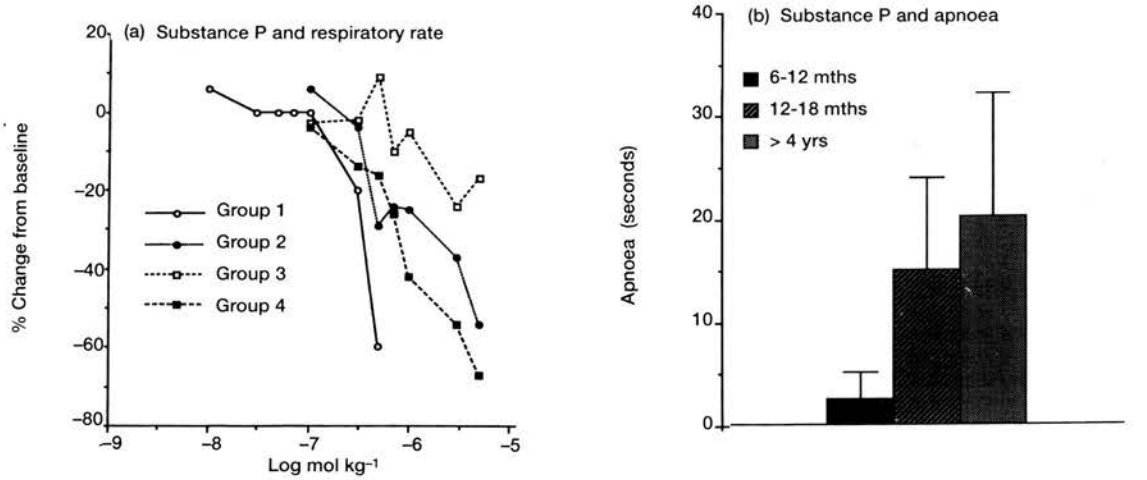

FIG 2: (a) Percentage change in respiratory rate after intravenous injection of substance P (10^{-8} to 5×10^{-6} mol kg^{-1}). Age groups as for Fig 1. Error bars have been omitted for clarity. (b) Apnoea (seconds) after intravenous injection of substance P (5×10^{-6} mol kg^{-1}) in three different age groups. Values are mean ± SEM (vertical bars)

the hypoxic respiratory drive (Fuller et al 1987). While the increased ventilatory response in man is due to direct action of SP on peripheral chemoreceptors in the carotid bodies (Maxwell et al 1990), SP is also believed to be involved in the pulmonary chemoreflex (rapid shallow breathing). Activation of pulmonary C-fibre afferents by both SP and capsaicin stimulates rapid shallow breathing (Green et al 1984, Prabhakar et al 1987). Injection of capsaicin as a bolus into the right atrium, but not the systemic arterial circulation, can also result in apnoea (Green et al 1984) and therefore the rapid injection of SP into the systemic venous circulation may activate this apnoeic part of the pulmonary chemoreflex.

The change with age of the sheep response to SP contrasts with other species and the reaction of sheep to other spasmogenic agents. Sheep have been shown to demonstrate an age-related increase in the bronchomotor response to both histamine and carbachol (Saunders et al 1984). In rabbits, the neuromodulatory action of SP on cholinergic neurotransmission and control of bronchomotor tone increases with age (Tanaka and Grunstein 1990), but this is due to an increase in intrinsic bronchoreactivity rather than an increase in the contractile response to SP. In the cat carotid body there is an age-related increase in SP-like immunoreactivity coinciding with development of the hypoxic ventilatory response (Scheibner et al 1988), while there is an age-related decrease in SP-containing nerves in normal human airways (Hislop et al 1990). The change in human neuronal expression of peptides and the sheep bronchomotor response to SP roughly coincides with the onset of maturity.

The apnoeic response in aged sheep is probably due to activation of pulmonary C-fibre afferent nerve endings and while the functional significance of the findings is not known, these preliminary results suggest a limited role for SP in the control of bronchomotor tone in adult sheep.

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