

Regulatory peptides in the respiratory tract of *Macaca fascicularis*

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ABSTRACT The quantitative distribution and localisation of seven regulatory peptides (vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), calcitonin gene related peptide (CGRP), galanin, substance P, neuropeptide tyrosine (Y), and bombesin like peptides) were determined by radioimmunoassay and immunocytochemistry in six different regions of the respiratory tract of the cynomolgus monkey, *Macaca fascicularis*. In general, peptide concentrations were higher in the airways than in lung tissue itself. VIP and PHM were found in greatest abundance and in equimolar concentrations. Concentrations of substance P, neuropeptide Y, and bombesin were substantially lower. Immunocytochemistry localised all the peptides to nerve fibres, whose density generally paralleled the tissue concentrations by radioimmunoassay except in the case of bombesin, which was not detected. VIP, PHM, and galanin were mostly associated with glands of trachea and bronchus and with blood vessels and smooth muscle; CGRP and substance P were found principally beneath airway epithelium and around smooth muscle fibres and blood vessels; neuropeptide Y was found around blood vessels and seromucous glands only. The pattern of peptide distribution in the *Macaca fascicularis* respiratory tract is similar to that previously reported in human postmortem material, suggesting that the cynomolgus monkey may be a useful model for examining the pathophysiological role of peptides in human respiratory disease.

The presence of regulatory peptides in the mammalian respiratory tract has received increasing attention during the last decade.¹ Indeed, there is now accumulating evidence that peptides may have an important regulatory role, both in the normal respiratory tract and in the pathogenesis of some lung diseases.²⁻¹³

To identify disease related peptide changes, it is important to define the distribution of the various peptides throughout the normal respiratory tract. Most of the currently available data are based on fresh tissue obtained from rodents,²⁻⁷ cats,⁵⁻⁸ and dogs.⁹ In man only postmortem tissue has so far been examined.⁷⁻¹² The relevance of peptide changes extrapolated from these previous studies to human

disease states may be clouded by postmortem changes, or by differences between species.

The aim of this study was therefore to map the distribution of several regulatory peptides in freshly obtained tissue from the respiratory tract of a primate. We report here the distribution of immunoreactivities of vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), calcitonin gene related peptide (CGRP), galanin, substance P, neuropeptide tyrosine (neuropeptide Y), and bombesin like peptides in the cynomolgus monkey, *Macaca fascicularis*.

Methods

THE RESPIRATORY TRACT TISSUE

Respiratory tract tissue was obtained from five adult *Macaca fascicularis* monkeys (weight 4-5 kg), originally supplied by Charles River or Hazelton Primelab (USA). The animals were killed by intravenous pentobarbitone sodium. The complete respiratory tract

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of each animal was dissected out and divided into upper and lower trachea (full thickness), major bronchus (full thickness), inner lung zone containing medium sized bronchi, middle lung zone containing minor bronchi, and outer areas of lung comprising principally alveoli. Each specimen was subdivided for radioimmunoassay and immunocytochemistry.

Small portions (100–200 mg, including the full thickness of the airways) of each tissue were weighed, diced, and rapidly placed in 0.5 mol/l acetic acid (10 ml/g wet weight of tissue) at 100°C for 10 minutes. The extracts were then allowed to cool and stored at –20°C until they were assayed.

RADIOIMMUNOASSAY

The tissue extracts were thawed and duplicate aliquots of 10 µl were assayed for each of the seven peptides. All assays were performed in a total volume of 0.8 ml of 0.06 mol/l phosphate buffer, pH 7.4, containing 10 mmol/l EDTA, 45–450 µmol/l bovine serum albumin, and 20 KIU/ml aprotinin, and were incubated at 4°C for five days. Antibody bound label was separated from free label by adding to each tube 250 µl of a suspension containing 2–8 mg of charcoal (Norit GSX, Hopkin and Williams) coated with clinical grade dextran (1 g:10 g of charcoal, average molecular weight 70 000; Sigma). The tubes were centrifuged at 1600 g for 20 minutes at 4°C, followed by immediate separation of the supernatant. Details of each assay are summarised in table 1.

ANTISERA

The antisera for radioimmunoassay and immunocytochemistry were raised by multiple subcutaneous injections of peptide conjugates in rabbits. Details of conjugates and antiserum specificities are given in table 1. The antisera to VIP,¹⁴ PHM,¹⁵ CGRP,¹⁶ galanin,¹⁷ substance P,¹⁸ and neuropeptide Y¹⁹ showed no cross reaction with any other gut-brain peptides. Antiserum to bombesin²⁰ cross reacted fully with mammalian bombesin like peptide (96% with gastrin releasing peptide (GRP) and 98% with the C terminal decapeptide of GRP) and 0.2% with substance P.

GEL PERMEATION CHROMATOGRAPHY

To study the various molecular forms of each peptide immunoreactivity present in the respiratory tract, two extracts of trachea were subjected to gel permeation chromatography. Samples (total volume 0.8 ml) were applied to a column (1.6 × 100 cm) containing Sephadex G-50 superfine (Pharmacia) and eluted at a flow rate of 3 ml/h at 4°C with 0.06 mol/l phosphate buffer, pH 7.4, containing 10 mmol/l EDTA, 0.2 mol/l sodium chloride, and 50 µmol/l bovine serum albumin. The column was precalibrated with Dextran blue 2000 (mol wt 2 × 10⁶), cytochrome C (mol wt 12 384) and ¹²⁵I Na. Individual pure standard peptides were chromatographed separately to determine their respective elution coefficients (K_{av}). The marker substances were included in all column runs and fractions representing an elution volume of up to twice the bed volume were assayed for all peptides.

Table 1 Summary of antigen conjugation and antiserum characteristics used in radioimmunoassay and immunocytochemistry

Peptide	Bombesin	CGRP	Galanin	NPY	PHM	Substance P	VIP
Antigen conjugate	[Lys ³¹]-bombesin BSA	Human CGRP BSA	Porcine galanin —	Porcine NPY BSA	Human PHM BSA	Substance P BSA	Porcine VIP BSA
Method of conjugation	Glut	CDI	Unconjugated	Glut	Glut	Glut	CDI
Antibody specificity	C-terminal	Unknown	N-terminal	N-terminal	N-terminal	C-terminal	Mid to C-terminal
¹²⁵ I label	[Tyr ⁴]-bombesin	[His] CGRP	Porcine galanin	Porcine NPY	PHM	[Tyr ⁸]-substance P	Porcine VIP
Method of iodination	Chloramine T	Chloramine T	Chloramine T	Chloramine T	Chloramine T	Chloramine T	Bolton and Hunter
Final antibody dilution for radioimmunoassay	1:640 000	1:200 000	1:480 000	1:120 000	1:240 000	1:8000	1:320 000
Sensitivity* fmol/tube	0.2	1.0	1.0	0.6	0.8	0.3	0.3
Antibody dilution for immunocytochemistry	1:400	1:200	1:1000	1:400	1:400	1:500	1:2000
nmol of antigen/ml diluted antibody to abolish immunostaining	Not done	0.1	1.0	1.0	1.0	0.5	0.5

*95% confidence limit.

CGRP—calcitonin gene related peptide; NPY—neuropeptide tyrosine; PHM—peptide histidine methionine; VIP—vasoactive intestinal peptide; BSA—bovine serum albumin; Glut—glutaraldehyde; CDI—carbodiimide.

TREATMENT OF DATA

The concentrations of immunoreactive peptides were expressed as pmol/g wet weight of tissue and quoted as means with standard errors in parentheses; the significance of the difference between means was calculated by Student's unpaired *t* test. For comparison of the elution position of the molecular forms of the seven peptides, *K_{av}* values were calculated according to the method of Laurent and Killander.²¹

IMMUNOCYTOCHEMISTRY

Tissue samples were fixed by immersion in a 0.4% solution of parabenzoquinone²² in 0.05 mol/l phosphate buffer saline (PBS), pH 7.6, for two hours, then rinsed and stored at 4°C in PBS containing 15% sucrose and 0.01% sodium azide. Cryostat blocks were prepared and sections 10 µm thick were cut from each of the selected areas and immunostained by a modified indirect immunofluorescence method.²³

Briefly, sections were immersed in PBS containing 0.2% Triton X-100 for 60 minutes before application of primary antibody for 16 hours followed by washing in PBS and reapplication of the antibody for four hours. Antibody dilutions are given in table 1. Fluorescein conjugated goat antirabbit immunoglobulin (Miles Laboratories) was used at a dilution of 1:100 for 30 minutes. All washes were performed in PBS. Sections were mounted in glycerol:PBS (9:1) and examined with a Leitz fluorescence microscope. The specificity of the immunostaining results was verified in each case by absorbing the diluted primary antiserum overnight at 4°C with the homologous antigen before applying it to tissue sections for immunostaining as above.

Results

For each peptide radioimmunoassay, the within and between assay variation was less than 10% and dilution curves of tissue extracts or column peak fractions

were parallel to those of pure peptide standards. Table 2 shows the regional distribution of seven peptide immunoreactivities as determined by radioimmunoassay of the tissue extracts. VIP and PHM were found in greatest abundance and in equimolar concentrations in the airways, CGRP, galanin, substance P, neuropeptide Y, and bombesin like immunoreactivity were found at lower concentrations. In general, all the peptides were most abundant in the upper airways except for bombesin, which showed a more even distribution. Neuropeptide Y, substance P, and CGRP concentrations were below the detection limit of the assay (table 2).

GEL PERMEATION CHROMATOGRAPHY

Gel permeation chromatography was performed on two tracheal and bronchial extracts as these areas contained the greatest amounts of peptides; the profiles of galanin, neuropeptide Y, PHM, and CGRP immunoreactivities are shown in figure 1.

The profiles obtained for each peptide showed no significant variation in the elution position of the molecular forms between the different chromatographic analyses. VIP, substance P, galanin, and neuropeptide Y like immunoreactivities emerged as major peaks coinciding with pure porcine VIP (*K_{av}* 0.52), substance P (*K_{av}* = 0.92), galanin (*K_{av}* 0.68), and neuropeptide Y (*K_{av}* 0.55) respectively. PHM like immunoreactivity was found to be present in three molecular forms. The first and second peaks (*K_{av}* 0.12 and 0.33) represented the *N*-terminal extended forms of PHM,¹⁵ while the third peak (*K_{av}* 0.53) eluted exactly in the position of synthetic PHM. Most of the CGRP like immunoreactivity was eluted in a similar position to that of synthetic human CGRP, *K_{av}* 0.38; and a smaller amount was eluted with a *K_{av}* of 0.85. The nature of this immunoreactive peak is unknown, as synthetic fragments of human CGRP are not commercially available to determine the specificity of the antibody.

Table 2 Distribution of peptides in monkey respiratory tract (pmol/g wet weight of tissue, means with standard errors in parentheses)

Peptide	Trachea		Bronchus	Lung		
	Upper	Lower		Inner	Middle	Outer
VIP	5.7 (1.1)	6.1 (0.5)	6.5 (0.7)	1.2 (0.2)*	1.8 (0.2)**	0.8 (0.2)†
PHM	7.6 (1.1)	7.9 (0.8)	8.0 (0.7)	2.5 (0.3)‡	2.5 (0.5)††	2.1 (0.4)§
CGRP	2.9 (0.9)	3.1 (1.2)	4.7 (1.6)	<0.3	<0.3	<0.3
Galanin	2.5 (0.4)	2.8 (0.1)	2.7 (0.3)	1.3 (0.2)	1.2 (0.1)	1.3 (0.1)
Substance P	1.7 (0.4)	2.9 (0.5)	3.6 (1.0)	<0.3	<0.3	<0.3
NPY	0.9 (0.4)	1.0 (0.5)	2.5 (0.7)	<0.4	<0.4	<0.4
Bombesin	1.1 (0.1)	1.5 (0.4)	1.5 (0.3)	0.9 (0.2)	1.3 (0.2)	0.9 (0.3)

p* < 0.01, upper trachea vs inner lung; *p* < 0.01, upper trachea vs middle lung; †*p* < 0.01, upper trachea vs outer lung; ‡*p* < 0.01, upper trachea vs inner lung; ††*p* < 0.01, upper trachea vs middle lung; §*p* < 0.01, upper trachea vs outer lung. Abbreviations as in table 1.

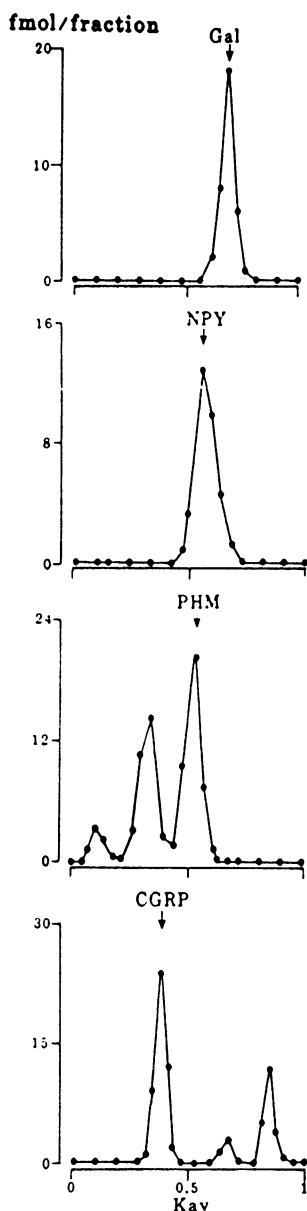


Fig 1 Gel permeation chromatographic pattern profiles of trachea extracts on a column (1.6 × 100 cm) of Sephadex G-50 superfine. Gal—galanin; NPY—neuropeptide Y; PHM—peptide histidine methionine; CGRP—calcitonin gene related peptide.

IMMUNOCYTOCHEMISTRY

VIP, PHM, CGRP, galanin, substance P, and neuropeptide Y were identified in the tracheobronchial wall of each of the five monkeys examined. The peptides were localised only in nerve fibres, which were

more numerous in the upper respiratory tract. The nerves most frequently identified were those containing VIP, followed by lesser populations of fibres containing PHM, CGRP, substance P, galanin, and neuropeptide Y. According to their distribution, the peptides could be divided into three different groups: firstly, VIP, PHM, and galanin; secondly, CGRP and substance P; and thirdly, neuropeptide Y. Bombesin immunoreactivity was not detected.

Nerve fibres immunoreactive with VIP, PHM, and galanin were localised abundantly around the acini of the seromucous glands in the submucosa of both trachea and bronchus. They formed a complex network, which occasionally encompassed almost every individual cell of the acini, and were also seen in great numbers around the smooth muscle of the airway and in the adventitia (figs 2–4). The three peptides were also detected in the nerve fibres surrounding blood vessels (both arteries and veins), including those of the subepithelial layer of the trachea, although less frequently than fibres containing CGRP and substance P.

In the trachea, CGRP and substance P fibres were seen in the subepithelium (fig 5), mainly associated with small blood vessels, and rarely penetrating the epithelium (fig 6). They were also found in the adventitia, around smooth muscle fibres and in large nerve bundles (fig 6). The number of these fibres decreased in the bronchi and was negligible in the intrapulmonary small airways. Scattered positive nerve fibres were, however, observed between the media and the adventitia of blood vessels (mainly arteries) from the trachea to the lung parenchyma.

Neuropeptide Y was the peptide with the most distinctive distribution. It was found only in nerve fibres circumscribing blood vessels (both arteries and veins) and seromucous glands. Nerves containing neuropeptide Y (fig 7) were associated with the blood vessels of the entire tracheobronchial tree, from the upper trachea to peripheral bronchi.

Discussion

In this study seven immunoreactive peptides—VIP, PHM, CGRP, galanin, substance P, neuropeptide Y, and bombesin—have been detected in the respiratory tract of the cynomolgus monkey, *Macaca fascicularis*. All the peptides were localised exclusively to the nerve fibres and were generally more abundant in the upper airways.

While most of the peptides, such as neuropeptide tyrosine,⁷ peptide histidine isoleucine (PHI),⁵ substance P, and vasoactive intestinal peptide, are present in nerve fibres^{3,6,8} others, such as adrenocorticotrophic hormone,⁴ the bombesin like peptides,⁹ calcitonin,¹⁰ and enkephalins,¹⁰ are found in

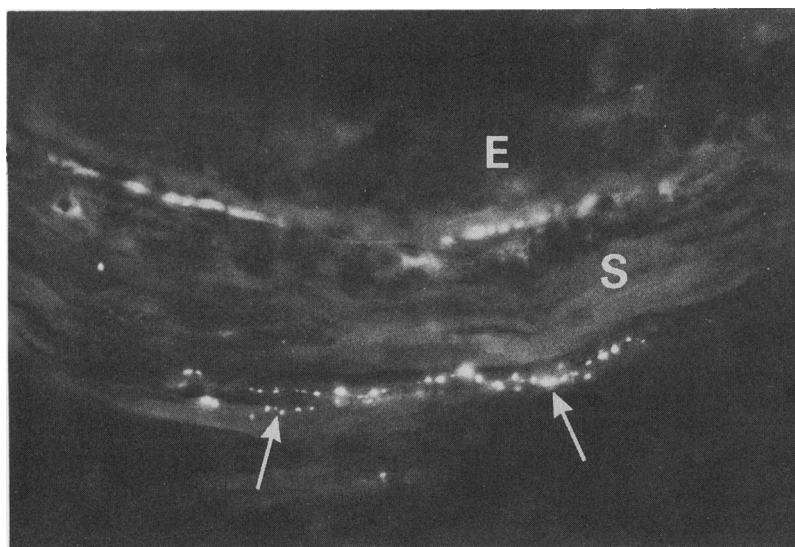


Fig 2 Nerve fibres (arrows) showing vasoactive peptide like immunoreactivity in smooth muscle (S) of stem bronchus (E—epithelium). Indirect immunofluorescence method, 10 µm section of tissue fixed in benzoquinone solution.

the endocrine cells. Calcitonin gene related peptide is found in both nerve fibres and mucosal endocrine cells of the rat respiratory tract.²

The presence of bombesin like peptides in the endocrine cells of the fetal human bronchial mucosa has been known since 1978.⁹ The low concentration in the adult monkey respiratory tract observed here is consistent with findings in adult rat, guinea pig, and cat.⁶ Although the pattern of distribution in monkey respiratory tract was very similar to that in normal adult human lung obtained at necropsy, the concentrations previously reported in the man are more than three times higher.¹¹ Concentrations of bombesin like pep-

tides change markedly during growth and development of the human lung,^{9 11 24} and bombesin acts as a growth factor for established small cell bronchial carcinoma.²⁵ This group of peptides may therefore have an important role in growth and in tumour promotion in the respiratory tract.

Calcitonin gene related peptide is a recently discovered peptide formed by alternative processing of the calcitonin gene in neural and thyroid tissues.^{26 27} CGRP like immunoreactivity is seen throughout the central nervous system and in neurones supplying many peripheral tissues.^{26–29} In rodent respiratory tract considerable amounts have been found in both

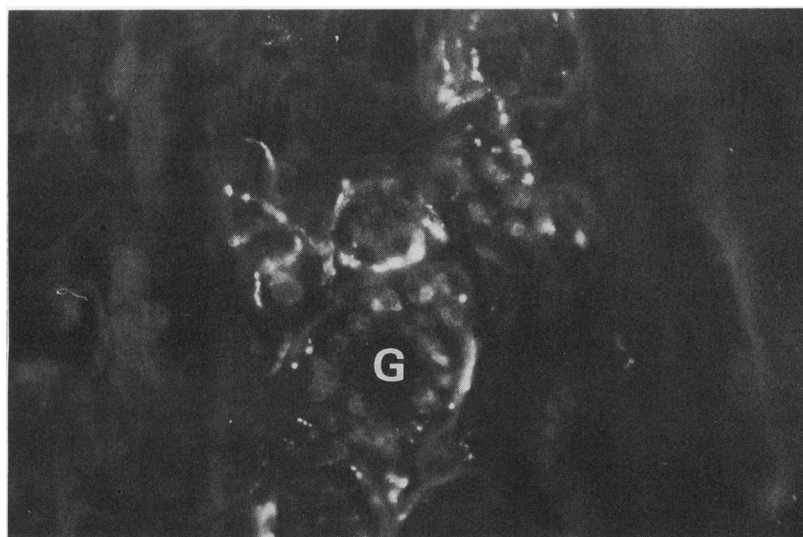


Fig 3 Section of trachea showing nerve fibres immunoreactive for peptide histidine methionine surrounding seromucous glands (G) in the adventitia. Indirect immunofluorescence method, 10 µm section of tissue fixed in benzoquinone solution.

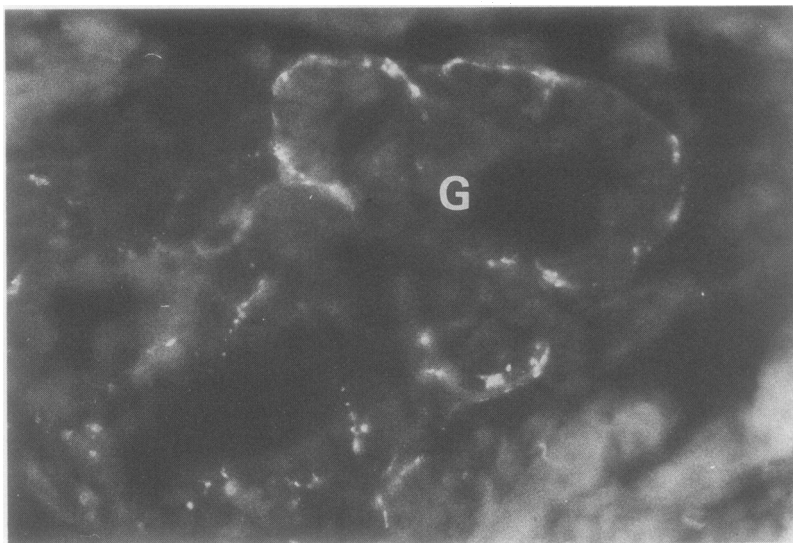


Fig 4 Galanin immunoreactive nerve fibres surrounding seromucous glands (G) in the submucosa of trachea. Indirect immunofluorescence method, 10 μ m section of tissue fixed in benzoquinone solution.

mucosal endocrine cells and sensory nerve fibres.² The concentration of CGRP in the monkey respiratory tract, however, is at least 30 times less than that reported in the rat and guinea pig, and is comparable to the concentrations found in human upper airways (unpublished observation).

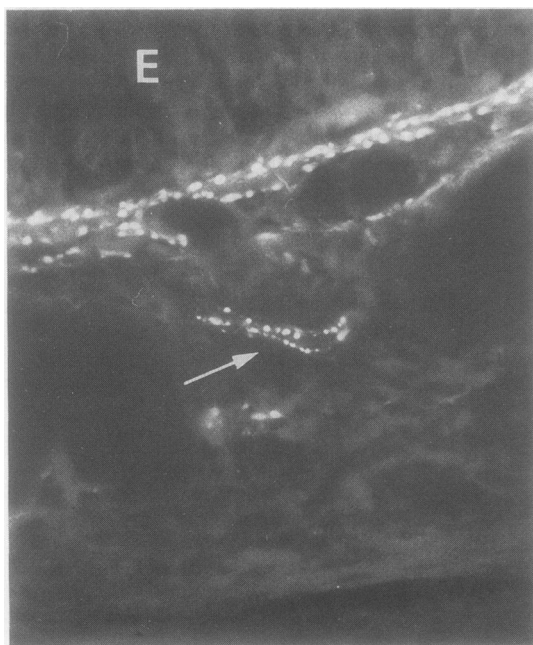


Fig 5 Section of trachea with nerve fibres immunoreactive for substance P (arrow) running below the epithelium (E). Indirect immunofluorescence method, 10 μ m section of tissue fixed in benzoquinone solution.

The recently discovered peptide histidine methionine (27 amino acid residues) is encoded with human vasoactive intestinal peptide messenger RNA³⁰ and differs only by two amino acids from its porcine counterpart, peptide histidine isoleucine.³¹ Both PHI and VIP like immunoreactivities have been found in equimolar concentrations in most of the tissues examined,³² including the mammalian respiratory tract. The present study extends these findings to the cynomolgus monkey.

Galanin was first isolated from porcine intestine³³ and was subsequently found in the central nervous system³⁴ and peripheral tissue.^{34,35} It has recently been localised in the nerve fibres of the mammalian respiratory tract.³⁶ In the monkey lung the distribution pattern of galanin like immunoreactivity is very similar to that reported in other species.³⁶

The pattern of distribution of these regulatory peptides in the respiratory tract of *Macaca fascicularis* is very similar to that observed in man. In both species VIP and PHM nerves are the most abundant,^{8,32,37} followed by lesser populations of fibres containing CGRP, substance P,³⁸ and neuropeptide Y^{7,39}; airway smooth muscle is supplied by nerves containing galanin and VIP. In the rat, on the other hand, CGRP is the most widely distributed regulatory peptide and is present in both nerves and mucosal endocrine cells,² while VIP and PHI-PHM nerve fibres are concentrated around the seromucous glands of the major airways. Moreover, substance P immunoreactive nerve fibres are very abundant in the rat respiratory tract and have a distribution similar to that of CGRP. Substance P, however, is not thought to be present in the endocrine cells.

These observations highlight two important points

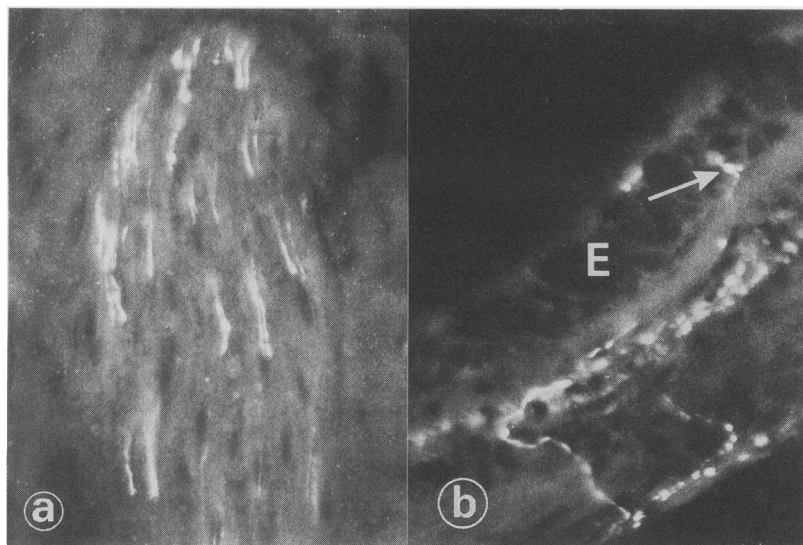


Fig 6 Calcitonin gene related peptide immunoreactive nerve fibres running (a) in a nerve bundle in the adventitia of trachea, and (b) below the epithelium (E) of a stem bronchus. A fine nerve fibre (arrow) is also seen in the bronchial epithelium in (b). Indirect immunofluorescence method, 10 μ m section of tissue fixed in benzoquinone solution.

Firstly, peptide distribution in the respiratory tract of primates differs substantially from that of lower mammals such as rodents and, secondly, the distribution in fresh monkey tissue is closely similar to that of human postmortem material. This suggests that deterioration in peptide immunoreactivity in the respiratory tract is relatively trivial for at least several hours after death. An important corollary is that the cynomolgus monkey may be a useful model for examining the role of regulatory peptides in human disease.

There is now extensive evidence that peptides play an important regulatory role in the pathophysiology of asthma. Fibres containing VIP, for example, inner-

vate the airways in monkeys (present study) and man.⁸⁻¹¹ Inhalation of VIP protects against histamine induced bronchoconstriction in man¹³ and may be the transmitter mediating the nonadrenergic inhibitory neuronal responses that have been found in baboons⁴⁰ and in human lung tissue.⁴¹⁻⁴² Other peptides, such as substance P, may perform an excitatory function and exacerbate asthma.⁴³⁻⁴⁵ The distribution of peptides in the rodent appears to differ appreciably from that in man, and rodent models of asthma are therefore unlikely to reflect human disease.⁴⁶⁻⁴⁷ Non-human primates have been extensively used for the study of human asthma.⁴⁸⁻⁴⁹ The similarities in the distribution of regulatory peptides in the monkey

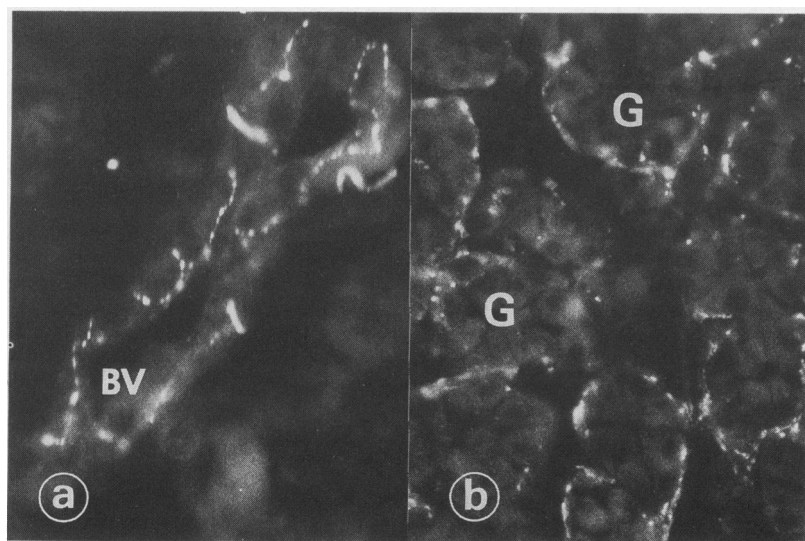


Fig 7 Nerve fibres immunoreactive for neuropeptide Y (a) running along the wall of a blood vessel (BV) in lung, and (b) around seromucous glands (G) in the adventitia of trachea. Indirect immunofluorescence method, 10 μ m section of tissue fixed in benzoquinone solution.

and human respiratory tract described in this study further support their use as models of human pulmonary disease.

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