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Peptide-containing nerve fibers in the respiratory tract of the ferret

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Summary. The ferret is widely used in functional and neuromorphological studies on the respiratory tract. We have examined the occurrence and distribution of peptide-containing and adrenergic nerve fibers (using dopamine-β-hydroxylase as a marker). Adrenergic nerve fibers and fibers storing vasoactive intestinal peptide have a widespread distribution along the entire respiratory tract. Adrenergic nerve fibers were found in the lamina propria, as well as around blood vessels and glands and in smooth muscle. Nerve fibers storing vasoactive intestinal peptide occurred in the epithelium, the lamina propria, around blood vessels and glands, and among muscle bundles. Substance P-, neurokinin A- and calcitonin gene-related peptide-containing nerve fibers predominated beneath and within the epithelium along the entire respiratory tract. Neuropeptide Y-containing nerve fibers were prominent among smooth muscle bundles and around glands. The blood vessels in the wall of the airways were richly supplied with peptide-containing nerve fibers and adrenergic fibers. Ganglia located over the outer or dorsal surface of the tracheal wall harbored vasoactive intestinal peptide-containing nerve cell bodies. Substance P and neurokinin A invariably coexisted in the same nerve fibers. Further, coexistence of substance P/neurokinin A and calcitonin gene-related peptide was observed in the nerve fibers associated with the epithelium. Vasoactive intestinal peptide, neuropeptide Y and occasionally also substance P coexisted in the population of nerve fibers associated with blood vessels and smooth muscle. Many adrenergic nerve fibers contained neuropeptide Y.

Key words: Respiratory tract – Neuropeptides – Autonomic innervation – Coexistence, of neuropeptides/biogenic amines – Ferret

The airways are richly supplied with autonomic nerve fibers of both sympathetic and parasympathetic nature (Dahlström et al. 1966; Coburn 1984b; Baker et al. 1986). In addition, a dense innervation of the airways by sensory nerve fibers, identified by their sensitivity to the sensory neurotoxin capsaicin, has been demonstrated (Lundberg et al. 1984; Martling et al. 1988). Among the major neuropeptides in the airways as studied in the rat, guinea-pig, cat and man, are vasoactive intestinal peptide (VIP) (Uddman et al. 1978 and 1980; Dey et al. 1981; Håkanson et al. 1983; Barnes 1987), substance P (SP) (Sundler et al. 1977a; Wharton et al. 1979; Uddman et al. 1981; Laitinen et al. 1982; Polak and Bloom 1982; Lundberg et al. 1984), neurokinin A (NKA) (Sundler et al. 1985; Barnes 1987), calcitonin gene-related peptide (CGRP) (Uddman et al. 1985b; Barnes 1987; Martling et al. 1988), and neuropeptide Y (NPY) (Uddman et al. 1981; Sheppard et al. 1984; Sundler et al. 1986). Gastrin-releasing peptide (GRP) has also been described in nerve fibers, and galanin in nerve fibers and nerve cell bodies of the respiratory tract (Uddman et al. 1984a; Cheung et al. 1985).

So far, very little is known about the occurrence and distribution of peptide-containing nerve fibers in the airways of the ferret, although the general neuroanatomy has been extensively studied in this species (Cameron and Coburn 1984; Baker et al. 1986). Furthermore, the ferret is widely used in electrophysiological studies (Cameron and Coburn 1982, 1984; Baker et al. 1983; cf. Coburn 1984a) and as a model for the study of mucus secretion in vivo (Barber and Small 1977) and in vitro (Borson et al. 1980; Basbaum et al. 1981; Peatfield et al. 1983; Kyle and Widdicombe 1987). Additionally, the effects of peptides and other mediators on mucus secretion have been studied. Thus, in ferret trachea, VIP enhances glandular mucus secretion produced by phenylephrine, while it inhibits metacholine induced mucus volume output (Webber and Widdicombe 1987a, b). NPY has been reported to enhance both metacholine- and phenylephrine-induced secretion from mucus glands (Webber et al. 1987; Webber 1988). SP, eledoisin-related peptide (ERP) and histamine cause an increase in secretion and smooth muscle tone (Kyle and Widdicombe 1987). Recently, SP was demonstrated immunochemically in the ferret trachea (Borson et al. 1987). This study also showed that enkephalinase inhibitors augment SP-induced secretion from ferret tracheal glands. Immunocytochemical data on neuropeptides in the ferret airways are still lacking. We therefore found it interesting to outline the occurrence and distribution of peptide-containing nerve fibers along the respiratory tract of this species.

Materials and methods

Fifteen adult ferrets were obtained from a local breeder. The animals were killed by an overdose of diethyl ether, and specimens from the nasal mucosa, the trachea, main

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Table 1. Details of the antibodies used

Antigen	Code	Raised against	Raised in	Working dilution	Source	Reference
VIP	7852	Unconjugated pure natural porcine VIP	Rabbit	1:640	Milab, Malmö, Sweden	Grunditz et al. 1986
VIP	8701	Unconjugated pure natural porcine VIP	Guinea-pig	1:1280	Milab, Malmö, Sweden	–
SP	SP 8	Protein-conjugated synthetic bovine SP	Rabbit	1:320	Dr PC Emson, MRC Cambridge UK	Sundler et al. 1985
CGRP	8427	Protein-conjugated synthetic rat CGRP	Rabbit	1:1280	Milab, Malmö, Sweden	Sundler et al. 1985
CGRP	8513	Protein-conjugated synthetic rat CGRP	Guinea-pig	1:1280	Milab, Malmö, Sweden	Sundler et al. 1985
NPY	8404	Protein-conjugated synthetic porcine NPY	Rabbit	1:320	Milab, Malmö, Sweden	Grunditz et al. 1984
GRP	6902	Protein-conjugated porcine GRP	Rabbit	1:640	N Yanaihara, Shizuoka Japan	Yanaihara et al. 1981
DBH	2012	Bovine adreno-medullary DBH	Rabbit	1:320	ETI Allendale NJ USA	–
Galanin	8416	Protein-conjugated synthetic rat galanin	Rabbit	1:320	Milab, Malmö, Sweden	Ekblad et al. 1985
Somatostatin	N-SOM	Protein-conjugated synthetic bovine somatostatin	Rabbit	1:200	Inkstar, Stillwater, USA	
Enkephalin	8107	Protein-conjugated synthetic metenk	Rabbit	1:320	Milab, Malmö, Sweden	Alumets et al. 1978
Neurotensin	HC 8	Synthetic bovine neurotensin	Rabbit	1:80	RE Carraway, Harward Med School, Boston MA, USA	Sundler et al. 1977b
NKA	NKA-2 (SK-2)	Protein-conjugated porcine NKA	Rabbit	1:320	Dr E Brodin, KI, Sthlm, Sweden	Sundler et al. 1985
Gastrin/CCK	4562	Protein-conjugated human gastrin 2–17	Rabbit	1:1280	Dr J Rehfeld, Rigshospitalet Copenh, Denmark	Lorén et al. 1979

bronchi and lung were taken for immunocytochemical analysis. The specimens were fixed by immersion in a mixture of 2% formaldehyde and 15% saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) overnight and thoroughly rinsed in Tyrode buffer containing 10% sucrose. They were frozen on dry ice and sectioned at 10 μm thickness in a cryostat.

The sections were processed for the immunocytochemical demonstration of VIP, SP, NKA, CGRP, NPY, GRP, galanin, cholecystokinin (CCK), somatostatin, enkephalin, and neurotensin. Details on the peptide antisera used are given in Table 1. The specificity of the immunostaining was tested by adding excess amounts of the antigen to the antibodies (10–100 μg synthetic or pure natural peptide per ml diluted antibody). Additionally, each of the peptide antisera was tested for cross reaction with the other peptides examined (10–100 μg peptide per ml diluted antiserum). No such cross reaction was found. However, cross reaction with still other peptides or protein-containing amino acid sequences recognized by the different antisera, cannot be excluded. It is appropriate, therefore, to refer to the immunoreactive material as VIP-like, SP-like and so on. For simplicity, however, the shorter terms are used henceforth.

As an immunocytochemical marker for adrenergic nerve fibers, we used an antibody against dopamine-β-hydroxylase (DBH) (Eugene Tech, Allendale, N.J. USA) diluted 1:320 (Rush and Geffen 1980).

In order to reveal coexistence of neuropeptides (or coexistence of DBH and neuropeptides), sequential or simultaneous double immunostaining techniques were used. In sequential immunostaining experiments sections were first stained for one peptide (or for DBH), examined in a fluorescence microscope and photographed. The antibodies were then eluted with acidic 2.5% potassium permanganate for 30 s (Tramu et al. 1978). The effectiveness of the elution was tested by the application of FITC-labelled second antibodies. Sections devoid of immunofluorescence were then processed for the immunocytochemical demonstration of another peptide. In simultaneous double immunostaining, a prerequisite is that the two primary antibodies are raised in different species and that the second antibodies are labelled with different fluorophores (Costa et al. 1986). In the present study, peptide antibodies raised in guinea-pigs or monoclonal antibodies were paired with antibodies raised in rabbits and the labels were fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate

(TRITC), respectively. Sections were first incubated with one of the pair of peptide antibodies and then with FITC-labelled second antibodies. The sections were then incubated with the other peptide antibody. The latter antibodies were visualized using TRITC-labelled second antibodies. The sections were examined in a fluorescence microscope fitted with appropriate filter settings for viewing FITC and TRITC fluorescence alternately (Zeiss No. 09 and 15, respectively).

Results

All the antisera used, except those against GRP, gastrin, CCK, somatostain, enkephalin and neurotensin, demonstrated neuronal elements in the ferret airways. The results are summarized in Table 2.

Distribution of individual neuropeptides. VIP-containing fibers were demonstrated in the epithelium, lamina propria and in association with muscle bundles, blood vessels and glands (Figs. 1a, 2a, b, 5a). The fibers were strongly immunoreactive in the nasal mucosa, trachea and bronchi. In the lung, they were few and occurred scattered in the septa and close to the blood vessels. Small ganglia located over the outer or dorsal surface of the tracheal wall regularly contained VIP-containing nerve cell bodies.

SP-containing nerve fibers were numerous in the nasal epithelium and the lamina propria (Figs. 1b, 5c). A few fibers occurred around the blood vessels and glands. They were distributed in the epithelium, lamina propria and smooth muscle of the trachea and main bronchi. Within the epithelium some fibers seemed to penetrate to the luminal surface. Moderate numbers of SP-containing fibers were seen around the blood vessels and surrounding the glands of the trachea and main bronchi. In the lung only a few fibers occurred around blood vessels.

NKA-containing fibers were richly distributed in the nasal epithelium and the lamina propria, as well as around the blood vessels and glands. In the trachea and main bronchi, moderate numbers of NKA-containing fibers were seen in the epithelium, around the blood vessels and glands in the trachea and main bronchi (Fig. 1c). In the lungs a few scattered fibers occurred around blood vessels.

CGRP-containing fibers were observed in the nasal epithelium and lamina propria, as well as in the trachea and main bronchi (Figs. 1d, 2d, 5b). Generally, CGRP-containing fibers were more numerous in the nasal mucosa than in the trachea and main bronchi. CGRP-containing fibers were moderate in number in the smooth muscle of the trachea and the main bronchi. A few fibers were also seen around blood vessels and seromucous glands along the entire respiratory tract.

NPY-containing fibers were predominantly found in the smooth muscle of the trachea and main bronchi (Fig. 1e). They occurred also as single scattered fibers around glands. A few fibers were seen around blood vessels. NPY-containing fibers could not be detected in association with the epithelium and lamina propria of any part of the respiratory tract.

Galanin-containing fibers were found sparsely in the distal parts of the trachea where they were distributed mainly in the smooth muscle. Some fibers also occurred around blood vessels and glands in the trachea and main bronchi. The nasal mucosa and the lung seemed to lack galanin-containing fibers.

Table 2. Distribution and relative frequency of peptide-containing and DBH immunoreactive nerve fibers in various locations along the respiratory tract

	Epithelium	Lamina propria	Muscle	Blood vessels	Glands
Nasal mucosa					
SP	++	++		+	+
VIP	+	++		+	+++
CGRP	+	++		+	+
NPY	0	0		+	0
Galanin	0	0		0	0
DBH	0	0		++	++
Proximal trachea					
SP	++	+	+	+	+
VIP	+	++	++	++	+++
CGRP	++	+	+	+	0
NPY	+	0	++	++	+
Galanin	0	0	0	+	+
DBH	0	0	+++	++	++
Distal trachea					
SP	++	+	+	++	+
VIP	+	+	++	++	+++
CGRP	++	++	0	+	0
NPY	+	0	+	+	0
Galanin	0	0	+	+	+
DBH	0	0	+++	+++	++
Main bronchi					
SP	+	+	+	0	++
VIP	+	0	+++	++	+++
CGRP	+	0	+	+	+
NPY	0	0	++	++	++
Galanin	+	0	0	+	+
DBH	0	0	+++	+++	++
Lung	Epithelium			Septa	
SP	0			+	
VIP	+			+	
CGRP	+			+	
NPY	+			+	
Galanin	0			0	
DBH	0			+	

+++ numerous nerve fibers; ++ moderate number of fibers; + few fibers; 0 no fibers detected. *SP* substance P; *VIP* vasoactive intestinal polypeptide; *CGRP* calcitonin gene-related peptide; *NPY* neuropeptide Y; *DBH* dopamine-β-hydroxylase

Distribution of adrenergic nerve fibers (Figs. 1f, 2c). DBH-containing fibers were richly distributed in the smooth muscle in the trachea and bronchi. They were also seen in great numbers around the seromucous glands and blood vessels. The epithelium and the lamina propria were virtually devoid of DBH-containing fibers. In the nasal mucosa they occurred in moderate numbers around blood vessels and glands.

Coexistence of neuropeptides. SP and NKA invariably coexisted in the same nerve fibers along the entire respiratory tract.

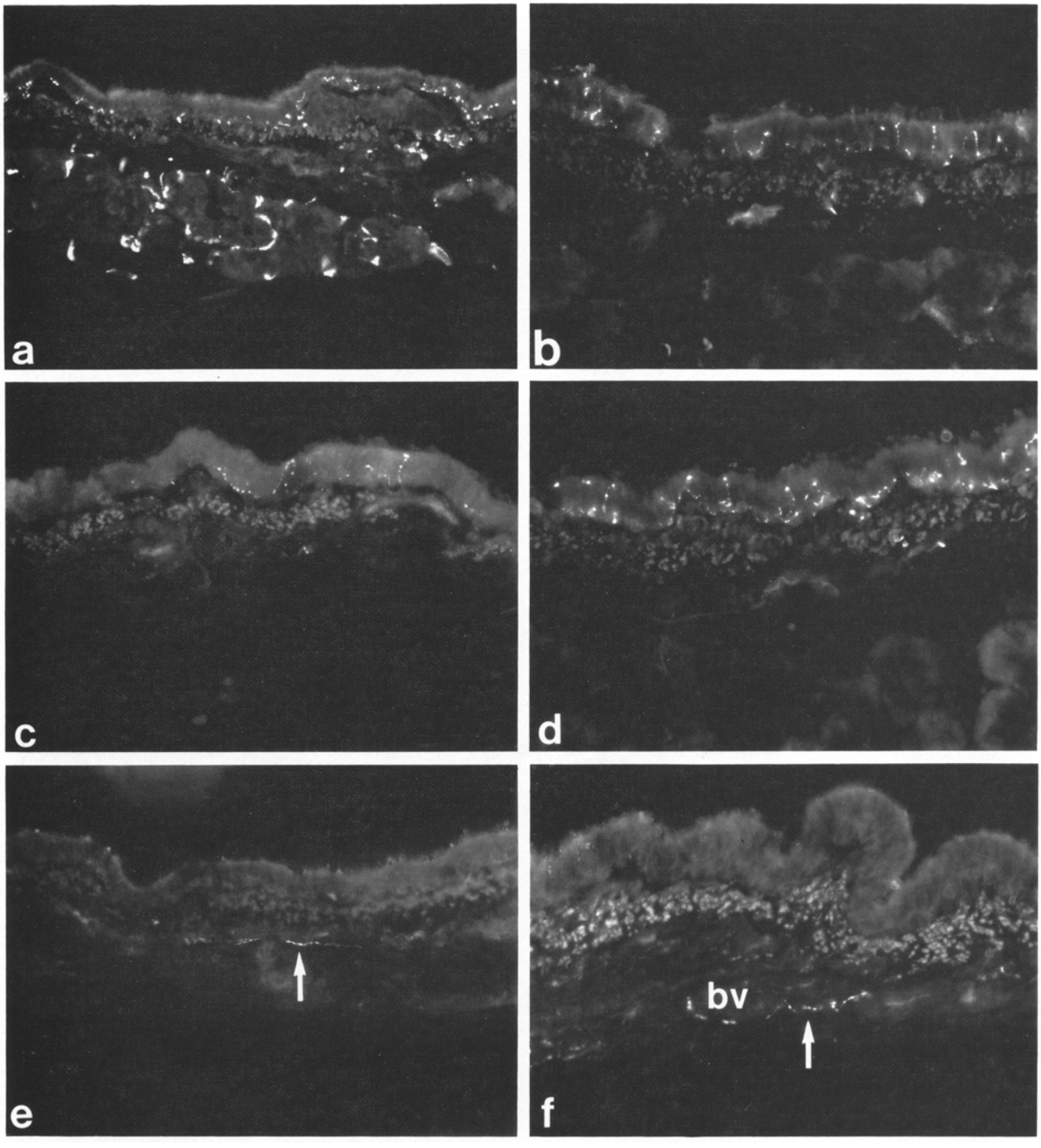

Fig. 1a–f. Ferret tracheal wall. Sections immunostained for VIP (**a**), SP (**b**), NKA (**c**), CGRP (**d**), NPY (**e**) and DBH (**f**). Numerous VIP-immunofluorescent fibers in association with glandular acini and in the lamina propria layer. SP-, NKA- and CGRP-containing fibers predominate within and beneath the epithelium with some fibers apparently penetrating the epithelium to reach the luminal surface. NPY- and DBH-containing fibers (*arrows*) occur among bundles of smooth muscle and around blood vessels (*bv*). × 270

A subpopulation of the DBH-containing fibers was also found to contain NPY, indicating that NPY is stored in a subset of adrenergic nerves (Fig. 3e, f). An additional population of NPY-containing fibers lacked DBH.

SP and VIP were found to coexist in nerve fibers in the lamina propria, smooth muscle and around glands (Fig. 3a–d). However, a subset of SP-containing fibers lacked VIP and some VIP-containing fibers lacked SP. In the epithelium and the lamina propria SP and CGRP were

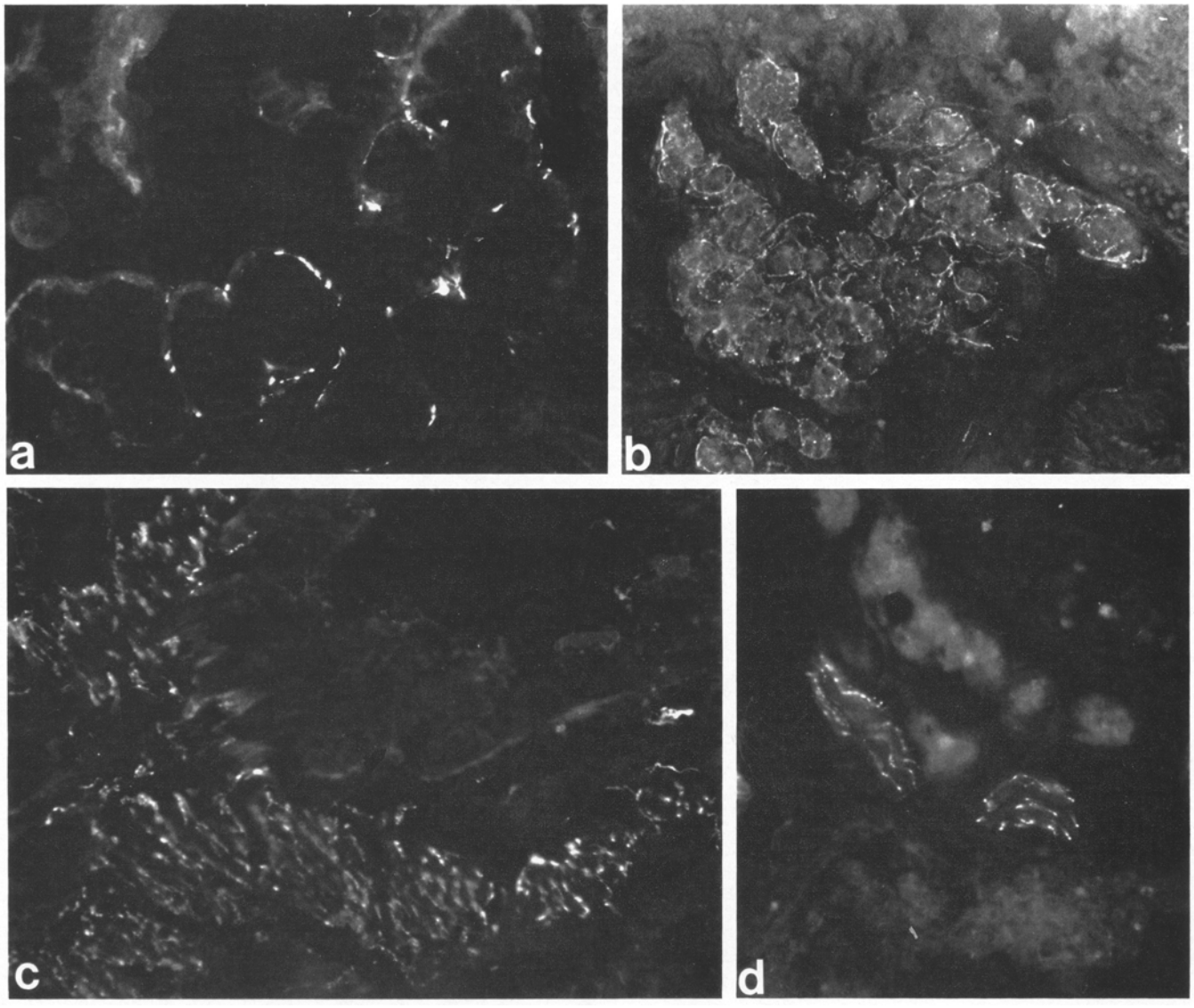

Fig. 2a–d. Ferret tracheal and bronchial wall, showing VIP-containing fibers around seromucous glands in the trachea (**a**) and in main bronchus (**b**), and DBH-containing fibers richly distributed among bundles of smooth muscle and around blood vessels and glands of a transversally cut, small bronchus (**c**). CGRP-containing fibers in nerve trunks in the tracheal wall (**d**). **a, d** ×380; **b** ×160; **c** ×320

found to coexist in one population of nerve fibers (Fig. 4a, b). A few CGRP-containing fibers seemed to lack SP. In the smooth muscle and around glands most of the SP-containing fibers lacked CGRP.

In the tracheobronchial smooth muscle, the majority of VIP-containing fibers also appeared to store NPY (Fig. 4c, d). Additional NPY-containing fibers in this location lacked VIP. In other locations, the vast majority of VIP-containing fibers seemed to lack NPY.

Discussion

On the whole, the distributional pattern of peptide-containing nerve fibers in the ferret was found to resemble that in other mammals (Polak and Bloom 1982; Håkanson et al. 1983; Barnes 1987). However, compared to, e.g., the rat and guinea-pig, the ferret seems to possess fewer SP- and CGRP-containing fibers within and beneath the epithelium (Håkanson et al. 1983; Lundberg et al. 1984; Sundler et al. 1985; Uddman et al. 1985b; Barnes 1987; Martling et al. 1988). These juxta- and intraepithelial SP/CGRP-containing fibers are known to be sensitive to capsaicin (Lundberg et al. 1984; Martling et al. 1988) and thus thought to represent the peripheral ramifications of primary sensory neurons. Another notable finding was the very rich innervation of seromucous glands by VIP-containing fibers. This distribution fits with the results of recent studies showing potent effects of VIP upon mucus secretion in the ferret (Peatfield et al. 1983; Webber and Widdicombe 1987a, b). NPY and SP have also been reported to affect mucus secretion (Web-

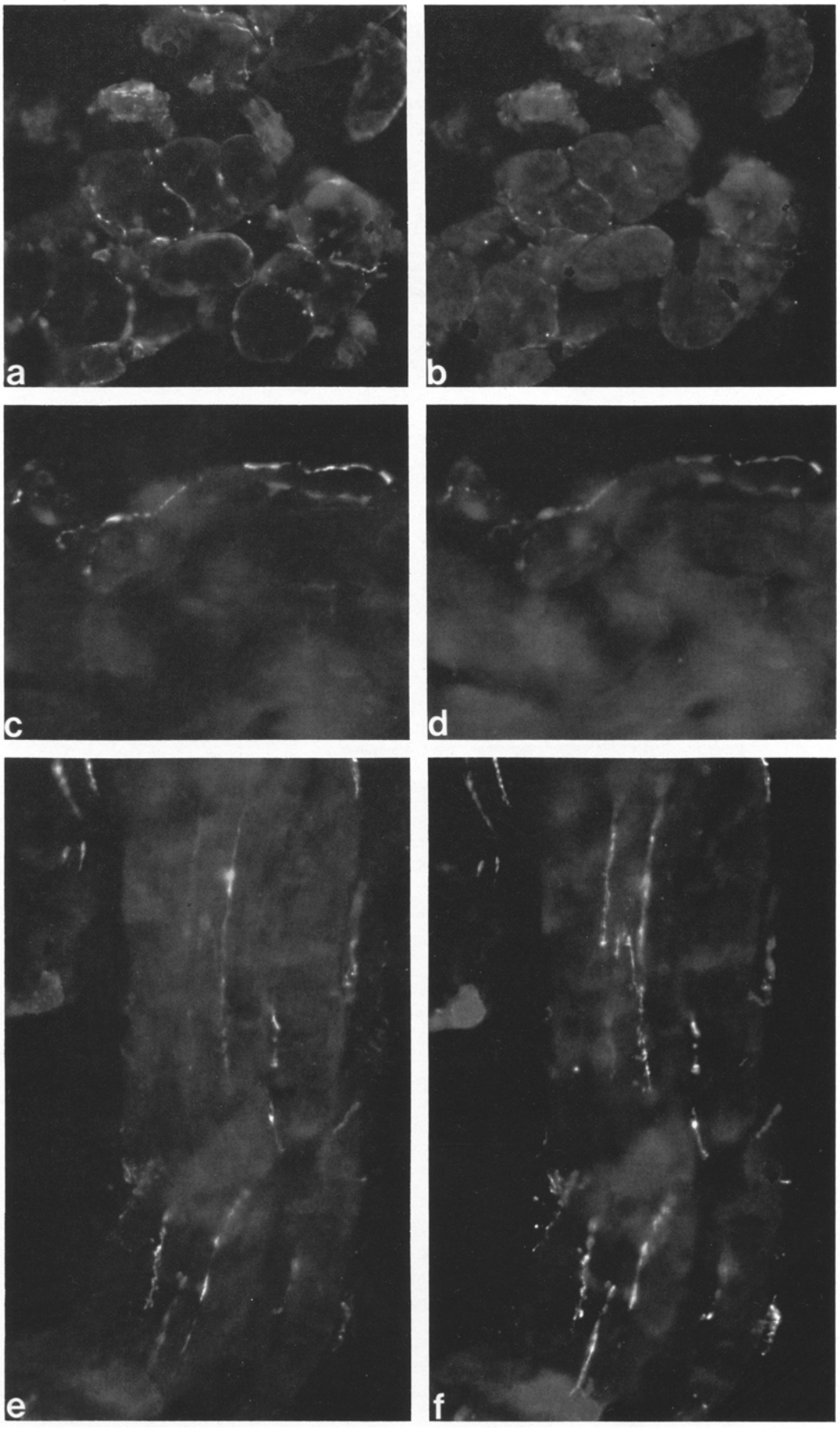

Fig. 3a–f. Ferret tracheal wall. Double immunostaining for VIP (**a, c**) and SP (**b, d**), as well as for DBH (**e**) and NPY. (**f**). **a, b** Sections from seromucous glands; **c–f** sections from smooth muscle. The SP-containing fibers also contain VIP. A minor population of the VIP-containing fibers in the seromucous gland appears to lack SP. Virtually all DBH-immunoreactive- and presumably noradrenaline-containing nerve fibers also contain NPY (**e, f**). **a, b** ×250; **c–f** ×350

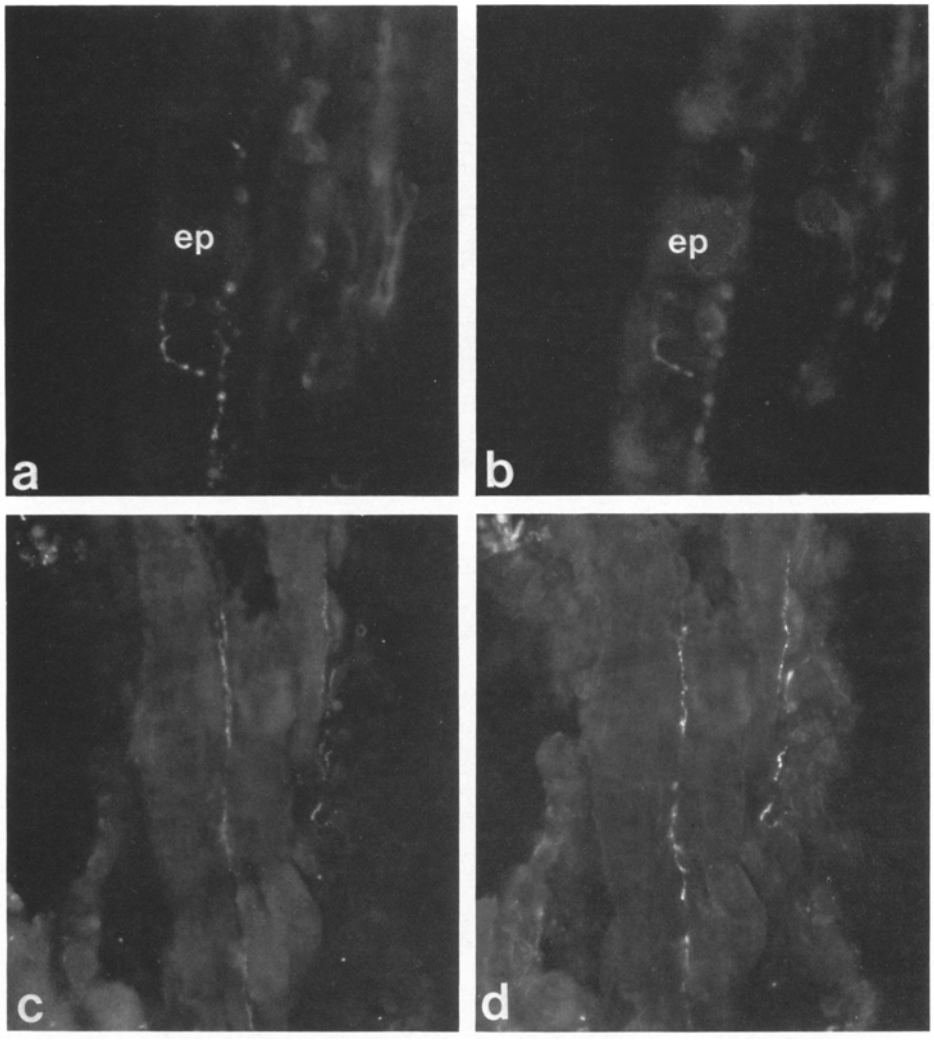

Fig. 4a–d. Ferret tracheal wall. Double immunostaining for SP (**a**) and CGRP (**b**), as well as for NPY (**c**) and VIP (**d**). All the SP-containing fibers in the mucosa also appear to contain CGRP (**a, b**). In this figure all VIP-containing fibers also display NPY (**c, d**); *ep* epithelium. ×250

ber et al. 1987; Kyle and Widdicombe 1987; Webber 1988), and our present results demonstrate nerve fibers containing these peptides around glandular acini. Galanin-containing fibers have recently been described in the respiratory tract of the pig, guinea-pig, rat, dog and chicken (Cheung et al. 1985). The present results indicate that such fibers also occur in the ferret trachea, albeit in small numbers. On the whole little is known about the source of the different nerve fiber populations in the airways (cf. Gabella 1987). The presence of VIP-containing nerve cell bodies in the local ganglia of the tracheal wall was also observed in the cat and man (Uddman et al. 1978; Dey et al. 1981). The occurrence of VIP-containing nerve cell bodies in the tracheal ganglia suggests that the VIP-containing fibers of the mucosa have a local origin.

Numerous studies have revealed a complicated pattern of colocalization of putative transmitters in the airways. The occurrence of NPY in adrenergic (DBH-positive) nerve fibers was entirely predictable from results obtained in other species (cf. Lundberg et al. 1982; Uddman et al. 1984b; Sundler et al. 1986). The coexistence of SP and CGRP in mucosal nerve fibers of presumably sensory nature was also to be expected from previous observations (cf. Sundler et al. 1985; Lundberg et al. 1985; Costa et al. 1986; Gibbins et al. 1987). NPY and VIP were found to coexist in a certain population of nerve fibers in the airways of the ferret. In fact, also NPY/VIP-containing (non-adrenergic) fibers have been described in other tissues, such as the gut and the thyroid gland of several species (Ekblad et al. 1984; Morris et al. 1985; Grunditz et al. 1988) and the salivary glands of the rat (Sundler et al. 1989). The observed coexistence of SP and VIP in a population of nerve fibers in the ferret airways parallels the situation recently described in nerve fibers and nerve cell bodies in the cat trachea (Dey et al. 1988), in lumbo-sacral dorsal root ganglia of the cat (Leah et al. 1985), and in the parotid gland of the rat (Al-Hadithi et al. 1988; Sundler et al. 1989).

In conclusion, the ferret airways are richly supplied with peptide-containing nerve fibers. The functional roles of the neuropeptides demonstrated in the airways are far from clear, although the effects have been studied to some extent (for references, see "Introduction"). The complex chemical coding of many neurons (coexistence of multiple putative transmitters) supplying the airways opens the possibility for various kinds of interactions between the messengers delivered.

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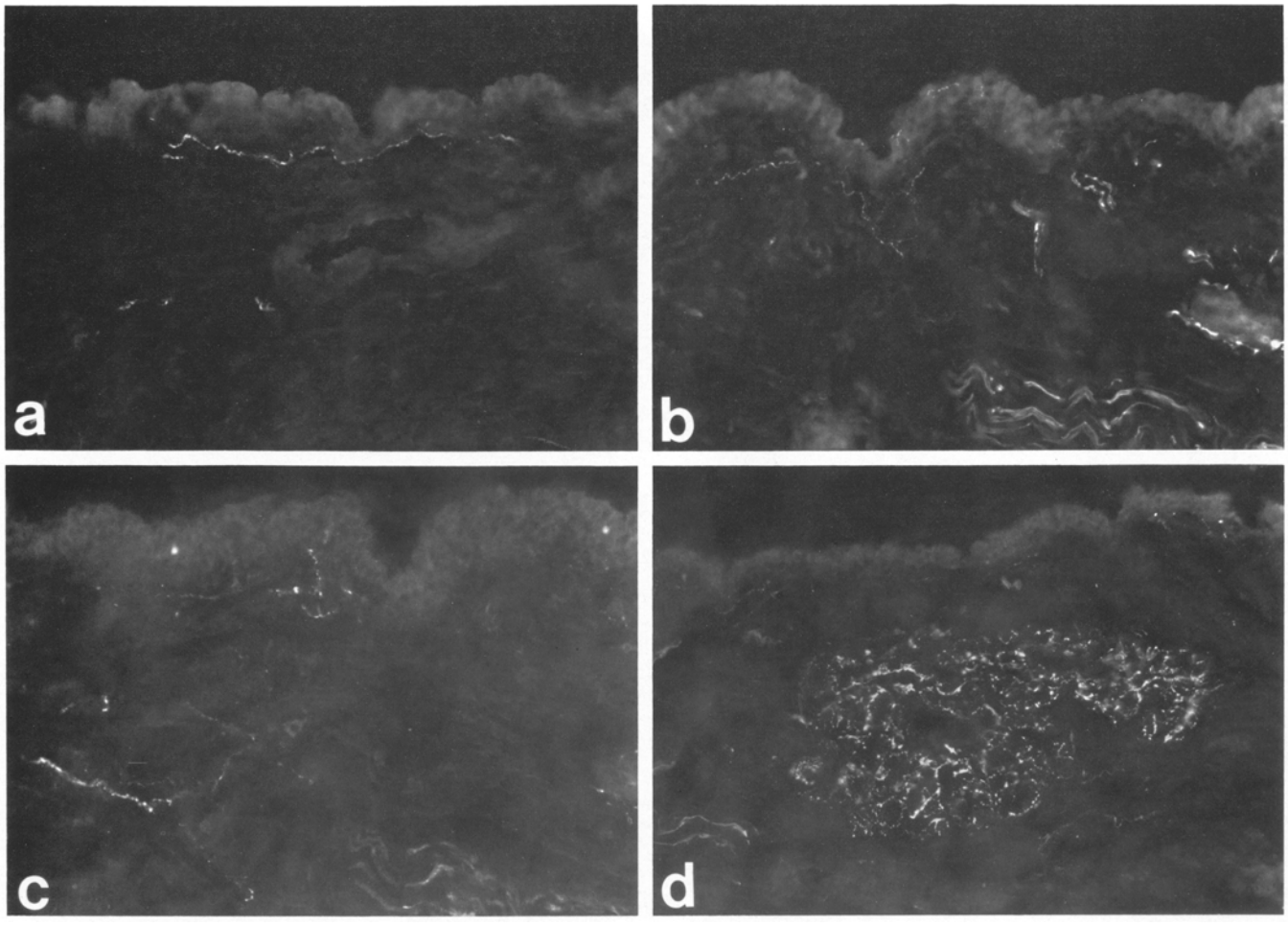

Fig. 5a–d. Ferret nasal mucosa. Sections immunostained for VIP (**a**), CGRP (**b**), SP (**c**), and DBH (**d**). VIP-containing fibers occur in moderate numbers in the lamina propria and around blood vessels and glands. CGRP- and SP-containing fibers are seen both within the epithelium and around blood vessels and glands. DBH-containing fibers are densely accumulated around the acini of a gland. × 270

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