Histol Histopathol (1997) 12: 909-918

Histology and Histopathology

Presence and distribution of 5HT-, VIP-, NPY-, and SP-immunoreactive structures in adult mouse lung

C. Verástegui, J. Fernández-Vivero, A. Prada, F. Rodríguez, A. Romero, M. González-Moreno and J.M. de Castro

Department of Morphological Sciences, Faculty of Medicine, University of Cádiz, Spain

Summary. A large number of biologically active substances have been identified and characterised in the respiratory tract of several mammals. These substances (amines and peptides) exert important regulatory influences on respiratory functions, and they act as neurotransmitters/neuromodulators, both being released from nerve terminals as neuroendocrine cells. However, these substances can also have other effects which suggest a paracrine action. Thus, to understand the role of amines and peptides in the lung, it is important to explore their localisation in different species. By using immunocytochemical staining methods we have studied the morphology and distribution of serotonin-, Substance P-, neuropeptide Y- and VIP-like immunoreactivity in the adult mouse lung. Moreover a pretreatment with colchicine, pargyline and 5-hydroxytryptophan as staining enlargement method was made. A widespread distribution of isolated endocrine cells and neuroepithelial bodies containing 5HT-like immunoreactivity was recorded within the lung. NPY-like immunoreactive nerve fibres were localised in the airway smooth muscle and surrounding the blood vessels. VIP-like immunoreactivity was revealed in single cells as well as in some nerve fibres and ganglia around the blood vessels and in the bronchial smooth muscle. SP-like IR was observed in nerve fibres located in the smooth muscle of the airways, surrounding bronchi and bronchioli but not next to the intrapulmonary blood vessels. Their localisation both in cells and nerve fibres of the respiratory system suggests that they play a role in the regulatory function of the mouse respiratory tract, exerting their influence by endocrine, paracrine, neurosecretory pathways or a combination of all of these.

Key words: Serotonin, Substance P, Neuropeptide Y, Vasoactive intestinal peptide, Lung

Offprint requests to: Cristina Verástegui, Departamento de Ciencias Morfológicas, Facultad de Medicina, Plaza Fragela s/n 11003 Cádiz, Spain

Introduction

The lung is supplied by the motor nerves comprising the sympathetic and parasympathetic autonomic nervous system and by sensory nerves that originate from sensory ganglia of the vagus nerve or from dorsal root ganglia (Richardson, 1979; Doidge and Satchell, 1982; Laitinen et al., 1985; Lundberg et al., 1988). It is now recognised that in addition to classic adrenergic and cholinergic innervation in airways, there is a "third nervous system" that is neither adrenergic nor cholinergic (NANC) (Campbell, 1971; Lundberg et al., 1988).

The function of the bronchial endocrine cells is to detect changes in the oxygen level of the airways. These cells respond to hypoxic conditions by releasing secretory substances. Some of these cells are connected with nerve fibres, whereas others are free of them. All these substances could act either via endocrine, paracrine or hemocrine pathways (Lauweryns and Peuskens, 1972; Lauweryns and Cokelaere, 1973a,b; Cutz et al., 1985a).

Chemical and immunocytochemical studies confirmed the simultaneous occurrence of neuropeptides and amines in neuroendocrine (NE) cells and led to the concept that the coexistence of different bioactive substances in these cells is the rule rather than the exception (Wharton et al., 1978; Cutz et al., 1981; Keith and Ekman, 1988).

In the respiratory tract, several biologically active substances (amines and peptides) have been identified in human and the other mammals. These substances are in relationships with adrenergic (Richardson, 1979; Doidge and Satchell, 1982; Laitinen et al., 1985), cholinergic (El-Bermani et al., 1982; Amenta et al., 1983), or sensory neural structures (Lauweryns and Van Lommel, 1983; Lundberg et al., 1988). Those neural structures could be both nerve fibres, isolated NE cells and neuroepithelial bodies (NEBs). These NEBs are cellular clusters described by Lauweryns and Peuskens in 1972 with a marked axonic confluence in their cellular basal pole. The storage of serotonin and peptide substances in epithelial NE cells may explain some mechanisms of

Table 1. Immunocytochemical localisation of biologically active substances described in the lungs of various species of adult mammals. The distribution and intensity of immunostaining for these substances varies between species with some showing relatively high levels of expression, and others intermediate or weak immunostaining.

	BB	NPY	CAL	SP	LEU-E	GAL	CGRP	VIP	CCK	5HT	REFERENCES
Human	C	F	C	F	F, C	F	F	F	C	C	Wharton et al., 1978; Polak and Bloom, 1982; Lundberg et al., 1984; Sheppard et al., 1984; Lauweryns and Van Rast, 1987; Cutz et al., 1989; Dey and Zhu, 1993; Wangand Cutz, 1993
Monkey					C, F				C	C	Polak and Bloom, 1982; Lauweryns and Van Ranst, 1987; Wang and Cutz, 1993
Pig		F		F	F, C	F	F	F	C	C	Wharton et al., 1979; Polak and Bloom, 1982; Cheung et al., 1985; Lauweryns and Van Rast, 1987; Martling et al., 1990; Wang and Cutz, 1993
Dog						F		F	C	C	Dey et al., 1981a; Polak and Bloom, 1982; Cheung et al., 1985; Wang and Cutz, 1993
Cat		F		F		F, G		F			Dey et al., 1981a; Polak and Bloom, 1982; Said, 1982; Lundberg et al., 1984; Sheppard et al., 1984; Uddman et al., 1984; Dey and Zhu, 1993
Guinea pig	F	F		F	F	F	F	F		C	Wharton et al., 1979; Polak and Bloom, 1982; Lundberg et al., 1984; Sheppard et al., 1984; Uddman et al., 1984; Cheung et al., 1985; Shimosegawa et al., 1989
Rabbit					F			F	C	C	Lauweryns et al., 1973; Said, 1982; Shimosegawa et al., 1989; Wang and Cutz, 1993
Hamster							C		C	C	Polak and Bloom, 1982; Wang and Cutz, 1993
Rat		F	C	F	F	F	F, C	F	C		Polak and Bloom, 1982; Said, 1982; Lundberg et al., 1984; Sheppard et al., 1984; Uddman et al., 1994; Cheung et al., 1985; Cadieux et al., 1986; Shimosegawa et al., 1989; Wang and Cutz, 1993
Mouse		F		F							Sheppard et al., 1984; Uddman et al., 1984

BB: bombesin; NPY: neuropeptide Y; CAL: calcitonin; SP: substance P; LEU-E: leu-enkephalin; GAL: galanin; CGRP: calcitonin gene-related peptide; VIP: vasoactive intestinal peptide; CCK: cholecistokinin; 5HT: serotonin; F: fibres; C: neuroendocrine cells; G: ganglion cells.

local or paracrine regulation mediated by them (e.g. mucous secretion, smooth muscle tone, vasomotor function). In a wide sense, these amines and peptides may act as the integration of the pulmonary activity, and may play a role as neuroendocrine regulators and neurotransmitters (Wasano and Yamamoto, 1978; Wharton et al, 1978; Cutz et al., 1981; Uddman et al., 1984; Dayer et al., 1985).

For the NEBs, a paracrine function has been suggested, but they also serve as functional neuroreceptor organs based on their strategic localisation (Lauweryns and Peuskens, 1972; Lauweryns and Cokelaere, 1973a). However, the exact function of the NEBs remains unknown. They may respond to numerous stimuli as baroreceptors or chemoreceptors. This possible role has been studied in various animal species (Lauweryns and Cokelaere, 1973a,b; Lauweryns and Goddeeris, 1975; Lauweryns et al., 1977; Wasano and Yamamoto, 1981; Keith and Will, 1982).

In the lung, the localisation and concentration of several neurotransmitters-neuromodulators are different. According to the mammal species and also in the same species these concentrations and localisations are age-dependent. Table 1 provides data on the immunocytochemical localisation of biologically active substances which have been described in the lung of various adult mammals. This interspecies variability suggests that functions and roles of the neurotransmitters-neuromodulators probably differ between the different species. This points out the importance to determine the presence, localisation and distribution of these substances at pulmonary level in each animal. For this reason, we have chosen as experimental animal the adult mouse. Although the mouse has widely been used for studying the control of the intrinsic neuroendocrine system in many organs, little is known about the distribution at the pulmonary level of the substances acting in this control system.

The existence of serotonin in the mammalian respiratory tract has been well documented (Lauweryns et al., 1973, 1982; Cutz et al., 1975; Eaton and Fedde, 1977; Dayer et al., 1985; Bock de et al., 1986).

In addition to serotonin, VIP and substance (SP) are the two peptides so far demonstrated by immunocytochemistry in the mammalian pulmonary nervous system (Uddman et al., 1978; Wharton et al., 1979; Dey et al., 1981a; Ghatei et al., 1982). VIP-immunoreactivity (-IR), though present in several species, is found more abundantly in the cat (Dey et al., 1981a; Said, 1982), and it is the most abundant peptide isolated from human lung (Polak and Bloom, 1986). There is accumulating evidence that VIP may be the neurotransmitter in NANC nerves, but its role cannot be confirmed until specific blockers are developed.

SP was initially detected in nerves of the respiratory tract of the guinea-pig (Nilsson et al., 1977; Terenghi et al., 1983; Saria et al., 1987; Shimosegawa et al., 1989), and this peptide has been to also exist in the respiratory

tract of humans (Lundberg et al., 1984).

Neuropeptide Y (NPY) has been found in the lungs of several mammals, including human (Sheppard et al., 1984). This neurotransmitter has been demonstrated in sympathetic adrenergic nerves, mostly in relation to blood vessels (Lundberg et al., 1982).

It is now clear that the neuropeptides have a wide influence on the pathophysiological aspects of the lung. SP is a potent endogenous bronchoconstrictor, whereas VIP is a potent endogenous bronchodilator (Said, 1982; Lilly et al., 1993), and NPY may act as a vasoconstrictor. There is abundant evidence that these neuropeptides are released in the lung in a variable conditions and that they have the capacity to modulate the bronchoactivity of the same stimuli that release them. So, VIP seems to be a good candidate as neurotransmitter in airway NANC nerves and the sensory neuropeptide SP may make an important contribution to the physiopathological features of asthma. The recent development of the potential and selective tachykinin receptor antagonists opens new possibilities for therapy of airway diseases. However, the lack of specific and potent antagonists against most of the neuropeptides makes it difficult to define their physiological role in the control of the respiratory tract.

In this report we use several drugs to enlarge the immunostaining. In some samples we used colchicine to inhibit the fast axonal flow at neural cells which causes an unphysiological accumulation of many substances including transmitters (Hökfelt et al., 1978). Moreover, the animal pretreatment with the precursor 5-hydroxytryptophan and with pargyline, the inhibitor of monoamine-oxidase, increased the amount and the intensity at the 5HT-immunoreactive cells.

This work contributes to establish the morphological basis of the mouse pulmonary innervation for the selected neuroregulators, and the point of the scope of light microscopic studies is to establish the coexistence of these substances in the same nerve fibres or NE cells by comparing serial sections or by double-labelling techniques.

Materials and methods

Animals and tissue treatment

Twenty adult male mice (*Mus musculus*, Swiss OF-1) were studied in our experiments.

Ten animals received a total dose of 10 mg/kg (i.p.) of colchicine, 24 h before being killed. The other ten did not receive this treatment. In both groups, two animals were used for immunohistochemical detection of 5HT and these animals received a total dose of 100 mg/kg (i.p.) of 5-hydroxytrytophan and pargyline (100 mg/kg), respectively 60 and 90 min before sacrifice. Animals were anaesthetized with ether inhalation. The lungs were fixed by perfusion through the right ventricle with a fixative solution (4% phosphate-buffered paraformaldehyde, pH 7.4). The samples were then postfixed by immersion in the same fixative for 2h at room temperature. The fixed tissues were then washed for two hours in 0.05 M TRIS-HCl buffer, pH 7.6, with 0.25% Triton X-100 (Sigma, St Louis, MO).

Immunohistochemistry

After rinsing, serial sections (50-100 μm) were obtained by cryostat sectioning and processed by using the streptavidin-biotin immunoperoxidase method (Bonnard et al., 1984). After treatment with peroxidase blocking reagent and the nonspecific blocking reagent (pig normal serum), sections were exposed to a rabbit anti-5HT, anti-VIP, anti-NPY and anti-SP polyclonal commercial antisera (Cambridge Research Biochemicals, U.K.) diluted 1:1000, 1:2000, 1:500 and 1:1000 respectively in a moisture chamber overnight at 4 °C. The second step was developed by using the avidin-biotin-peroxidase complex (Sigma) diluted 1:50 for 20 min at room temperature. The sections were revealed by rising in a 0.05% diaminobenzidine solution in presence of 0.01% H_2O_2 in the washing buffer under microscopic control. The negative control procedures were carried out by omitting one or more steps of the method and by using sections incubated with inactivated antisera with added excess antigen.

Results

We noted in all animals which received an intraperitoneal dose of colchicine 24 h before killing, the amount and the intensity of the immunoreactive structures was increased.

Serotonin

A widespread distribution of isolated endocrine cells and neuroepithelial bodies with 5HT-like IR was recorded in the lung. The immunoreactive isolated NE cells were located in the epithelium of large bronchi and bronchioles, next to the basal lamina, but they did not establish any contact with the airway lumen (Fig. 1). Their shape was pyramidal and they had a voluminous nucleus. Furthermore, we identified serotonin immunoreactive NEBs located preferentially in the bronchial and bronchiolar bifurcations (Fig. 2). The NEBs were composed of a variable number of serotoninergic cells (3 to 50) that were in close contact with the basal lamina, reaching the airway lumen, and which nuclei were round, pale and with a basal disposition.

Our results showed that in the animals which received the pretreatment with the precursor 5-hydroxytryptophan and with the inhibitor of monoamine oxidase pargyline, the amount and the intensity of the 5HT-immunoreactive cell level was increased.

Neuropeptide Y

A rich supply of fine, varicose nerve fibres for this

mediator were identified in the lung of the adult mouse. These fibres were preferentially located in the smooth muscle of the airways, in the subepithelial layer and around bronchi and bronchioles (Fig. 3). Furthermore, we observed, the presence of NPY-immunoreactive nerves at the vascular level. Those immunoreactive

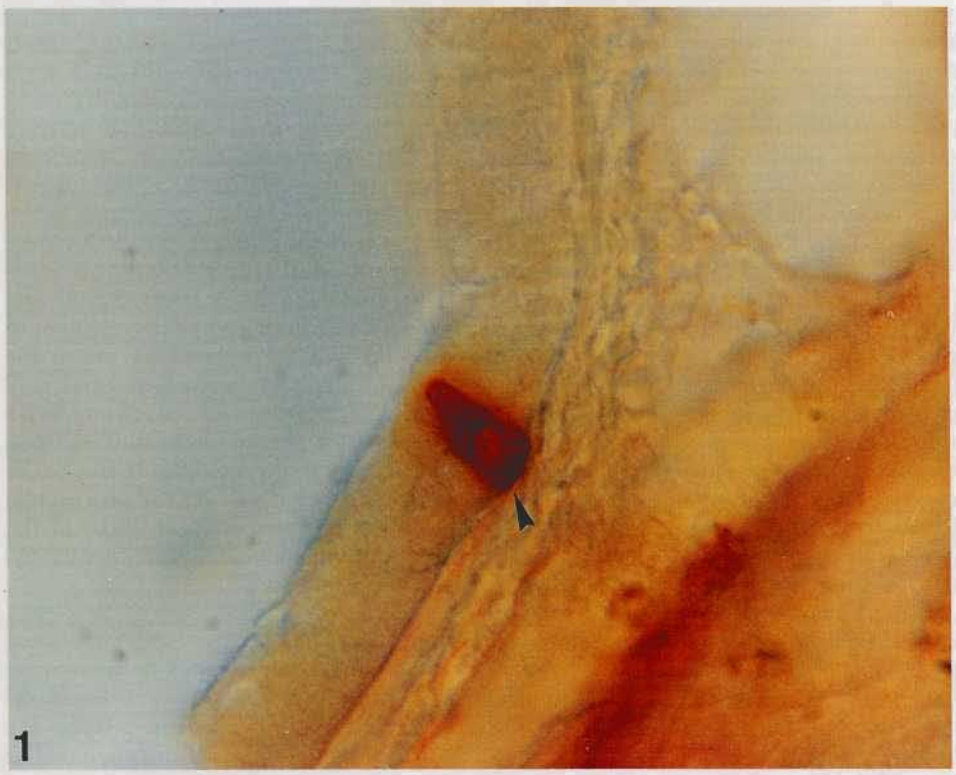

Fig. 1. An isolated 5HT-immunoreactive cell displaying a pyramidal shape at bronchial epithelium level in contact with the basal lamina (arrowhead). x 1,000

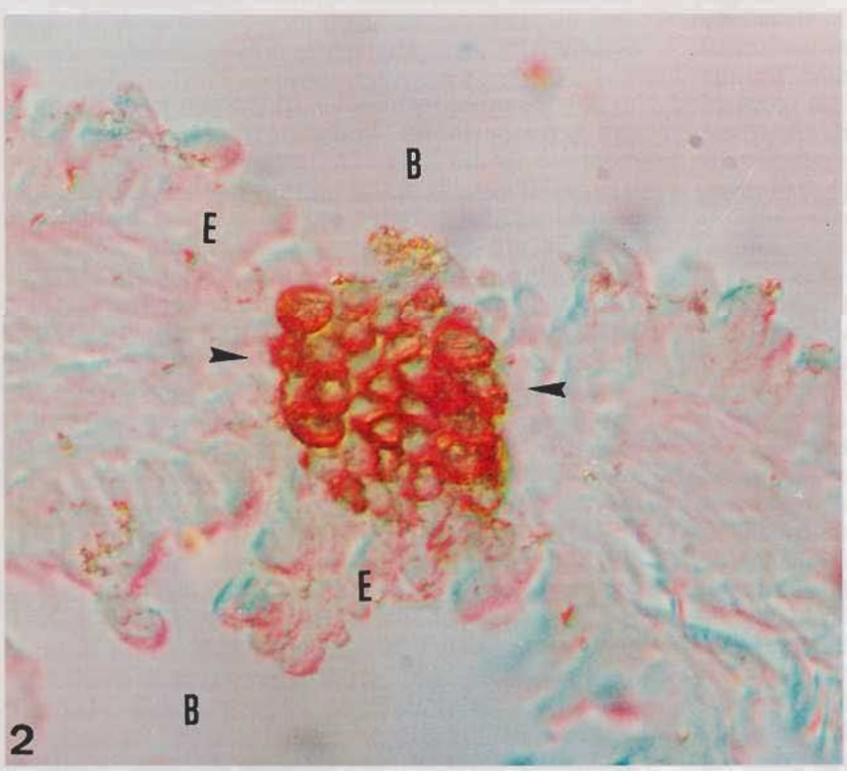

Fig. 2. Adult mouse bronchial bifurcation epithelium revealing 5HT-IR in a NEB which is extending from the basement membrane to the bronchial airway lumen (arrowheads). E: Epithelium; B: Bronchus. x 1,000

fibres of large calibre had a sinuous tract and penetrated through the vascular wall, reaching the endothelium (Fig. 4). Some of them ended in the vascular lumen forming a bulk that was constituted by the reunion of different converging fibres at this level.

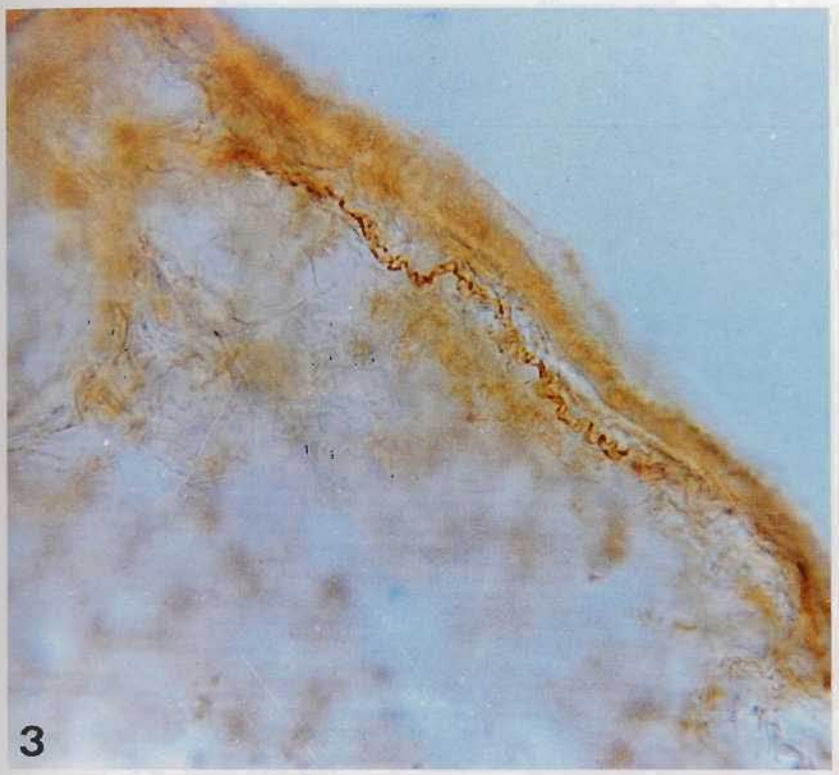

Fig.3. NPY immunoreactive nerve fibres beneath the smooth muscle of the mouse lung. x 1,000

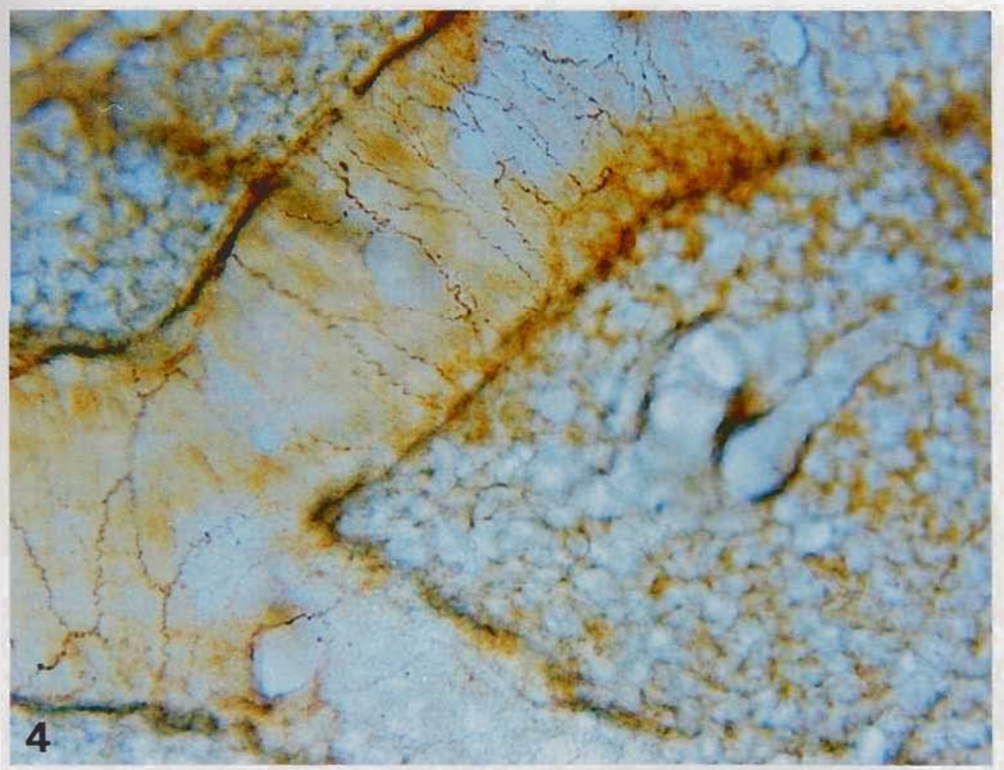

Fig.4. A network of NPY immunoreactive nerve fibres surrounds a blood vessel. x 1,000

Substance P

Nerve fibres displaying SP-IR were observed distributed within bronchi and bronchioli walls. The immunoreactive fibres showed different morphologies (e.g. varicose, filiform). Different calibre and sometimes multiple branching was also observed. In some nerves, SP-immunoreactive fibres coexisted with non-immuno-

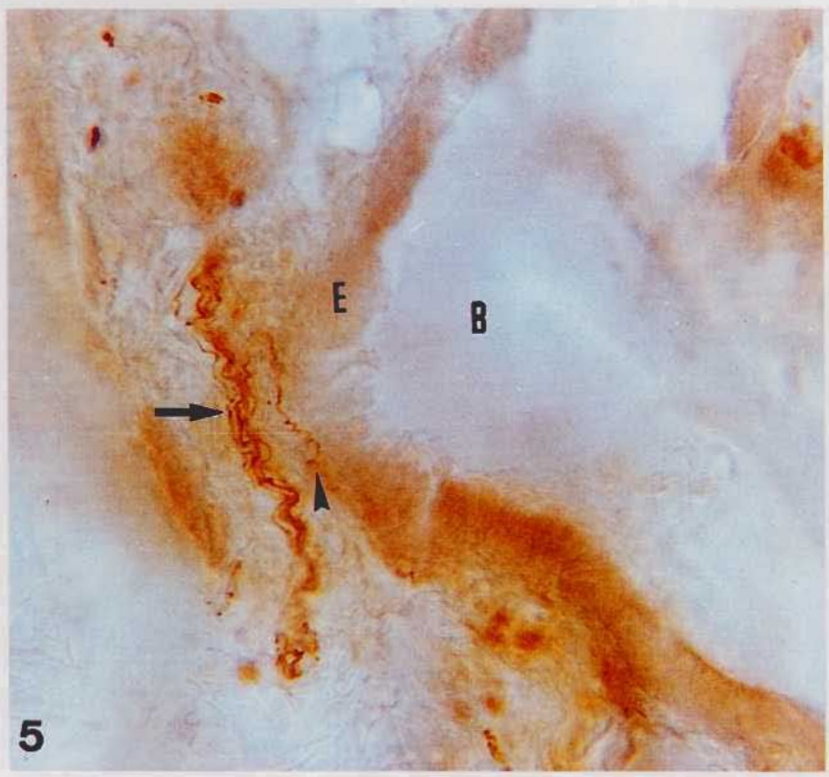

Fig. 5. SP immunoreactive nerve fibres with a peribronchial trajectory (arrow). A nerve fibre can be seen at subepithelial level (arrowhead). x 1,000

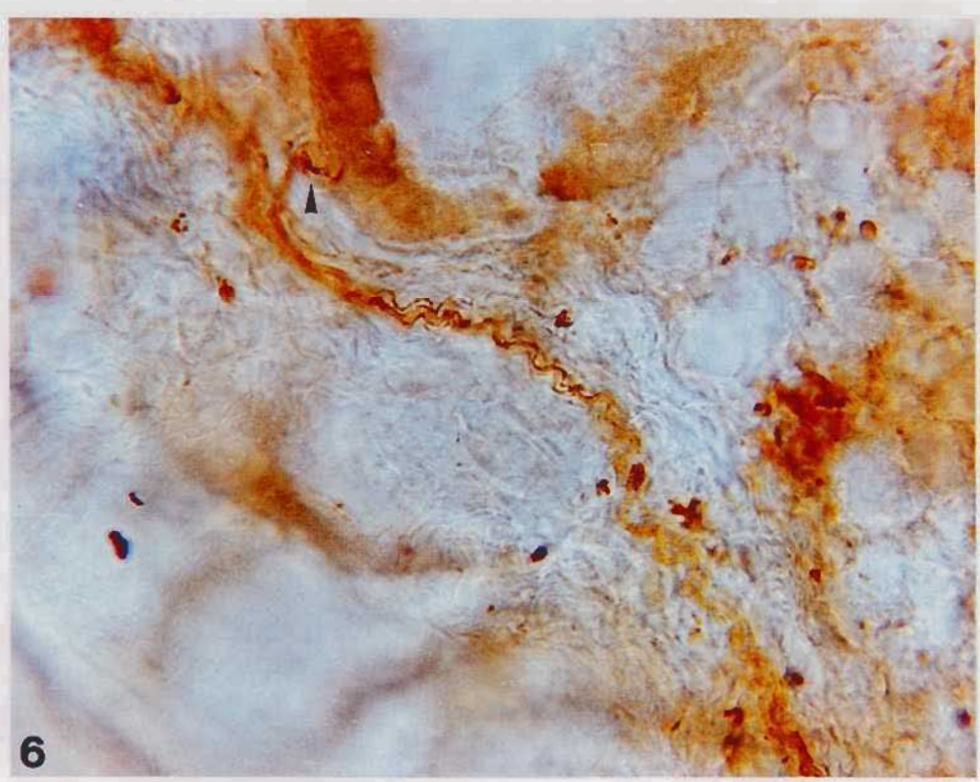

Fig. 6. SP immunoreactive nerve fibres with different calibers which give branchets penetrating the bronchial epithelium (arrowhead). x 1,000

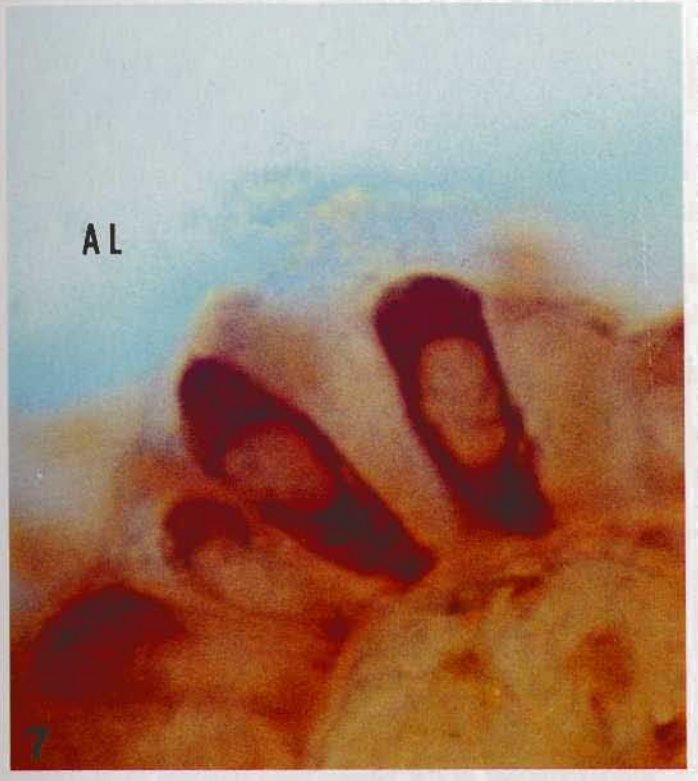

Fig. 7. VIP immunoreactive cells at bronchial epithelium in close contact with the basal lamina, where they are arranged between the cells of the epithelium. AL: airway lumen. x 1,000

reactive fibres (Fig. 5). The nervous fibres possessed a peribronchial distribution giving branches which penetrated the bronchial epithelium into the epithelial cells (Fig. 6). We also identified immunoreactive fibres with a long course next to the major bronchi, and these emitted branches at the bifurcation levels. These branches followed the course of the bronchi and bronchioles. No immunoreactivity was observed next to the intrapulmonay blood vessels.

Vasoactive intestinal peptide

We observed VIP-IR in the adult mouse lung both in nerve fibres and in ganglion cells. The IR could also be observed in NE cells of the bronchial epithelium. These cells were mainly located at the bronchial bifurcation and they were in close contact with the airway lumen and with the basal lamina of the epithelium. The cellular morphology was pyramidal with a basal pole bigger than the apical one. They had a large nucleus and a granular cytoplasmatic staining. They were arranged in the cells of the epithelium (Fig. 7). Furthermore, it was possible to identify some immunoreactive nervous fibres, basically in the vascular walls and with a perivascular disposition (Fig. 8). The VIP-IR was also observed in the intrinsic ganglia located under the bronchial epithelium and next to blood vessels. They were formed by a variable number of cells, immuno-reactive and non-immunoreactive cells coexisting. We also observed VIP-immunoreactive nerve fibres, some of which contact with the ganglion cells in the vicinity of these structures

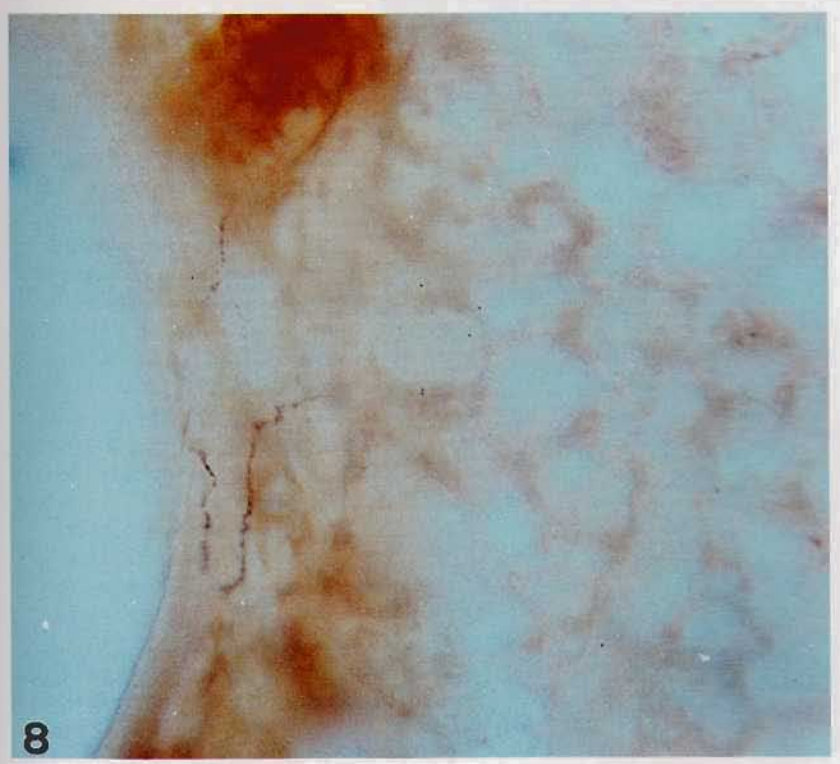

Fig. 8. VIP immunoreactive nerve fibres at vascular level. x 1,000

(Fig. 9).

Discussion

This work shows that immunoreactive SP, NPY and VIP are widely distributed in nerve fibres surrounding bronchi, bronchioli and blood vessels and immunoreactive 5HT and VIP are presented in NE cells and NEBs at the level of bronchial bifurcation in the adult mouse lung. In agreement with our results, some drugs, such as colchicine, have been used extensively to enhance the detectability of monoamines and peptides in the immunocytochemical techniques.

The understanding of the action of these neurotransmitters-neuromodulators is difficult. Until a few years ago, most of the known peptide hormones were thought to be produced exclusively by endocrine glands. The peptides of the lung are diffusely distributed, and it is likely that the principal tissue upon which these hormones act is the lung itself. Serotonin has been reported to have a contractile effect on smooth muscles of the digestive tract in rats, guinea pigs and rabbits (Kedzierska, 1970). In the breathing lung, it has been proposed that the role of serotonin is to control the mucus production or ciliary function (Dey et al., 1981b). Our results show that the distribution of 5HT NE cells and NEBs at the bronchial epithelium are in agreement with previous data in other mammals (Lauweryns et al., 1973, 1974, 1982; Eaton and Fedde, 1977; Sonstegard et al., 1982). Even though the distribution and amount of 5-HT cells in the respiratory epithelium in fetus and newborns was bigger than adults (Eaton and Fedde, 1977; Sonstegard et al., 1982; Redick and Hung, 1984; Cutz et al., 1985b; Bock de et al., 1986), the intensity of the immunocytochemical staining and the amount of the immunoreactive cells can be increased in fetal animals following injection of 5-L-hidroxytryptophane, the amine precursor, to the mother during pregnancy (Hage, 1974; Lauweryns et al., 1982), or directly to the specimen for study (Ericson et al., 1972; Cutz et al., 1974, 1975; Lauweryns et al., 1977; Marcheusky and Kleinerman, 1983; Carabba et al., 1985).

On the other hand, a rich supply of nerve fibres containing VIP were previously seen close to blood vessels, glands and nonvascular smooth muscle (Luts et al., 1993). However, an interesting finding was the presence of VIP-IR in NE cells of the bronchial epithelium. The placement of endocrine cells throughout the airways suggests that they are involved in the maintenance of important lung functions. The presence of VIP-IR in some local ganglia located under the bronchial epithelium and next to blood vessels suggests that VIP nerves come from these local parasympathetic ganglia.

Substance P-IR was found within nerve fibres, in the airway muscle, around blood vessels and beneath the bronchial epithelium (Luts et al., 1993); this IR being most abundant in the guinea pig (Terenghi et al.,1983; Saria et al., 1987; Shimosegawa et al., 1989). The distribution of the SP-IR fibres in the mouse lung was

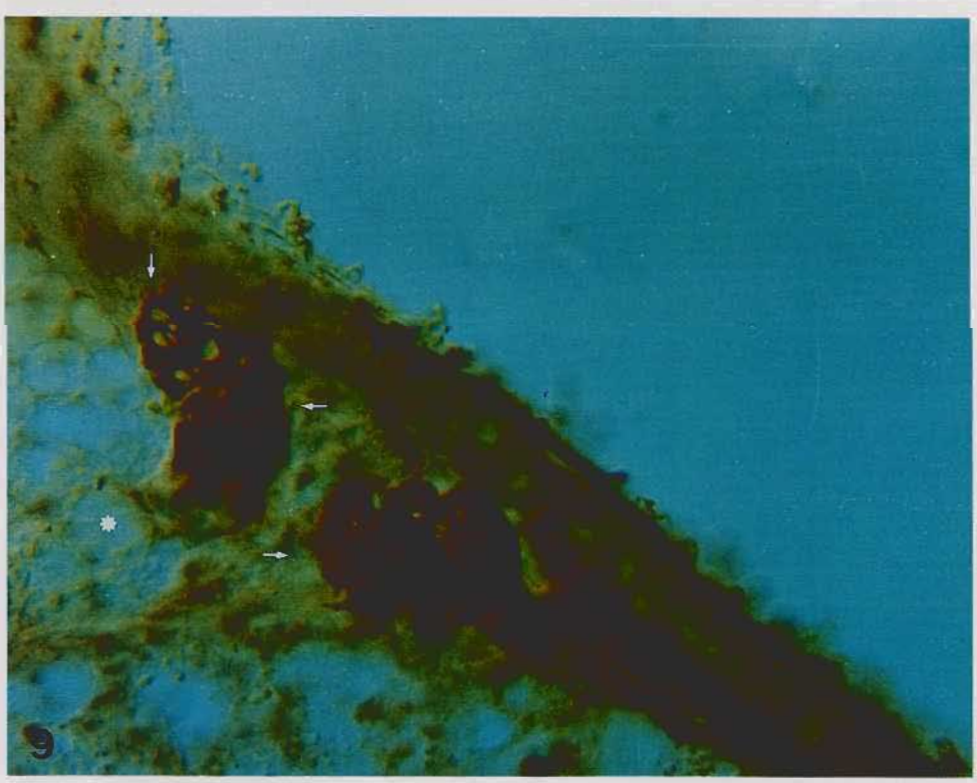

Fig. 9. VIP intrapulmonar ganglia immunoreactive (arrows) located under the bronchial epithelium, and next to blood vessels (asterisk). Original magnification x 1,000

different from described in other species. Thus, the SP innervation is dense in the guinea pig and rat tracheobronchial smooth muscle (Lundberg et al., 1988) but only a few SP immunoreactive fibres were found at peripheral airways level. This is in disagreement with our results since we found a rich SP immunoreactive supply at bronchial and bronchiolar epithelium.

NPY has been found in several mammalian lungs, and it is primarily localised in blood vessels (Sheppard et al., 1984). The distribution of NPY is similar to the sympathetic nerves, and it is probably a co-transmitter of norepinephrine and a potent constrictor of vascular smooth muscle (Lundberg et al., 1982). NPY may also play an important role in regulating pulmonary and bronchial vessels, but it probably has less important influence on the airway tone. In conclusion, the dense innervation by NPY-containing fibres (smooth muscle and vascular innervation) suggests that the sympathetic control of airways may not only involve noradrenaline as transmitter.

A comparison with the known localisation of these neuroregulators in the lung of various species revealed that the distribution of NPY-, SP-, VIP-, and 5HT-IR does not correspond to that studied to date in mouse lung. Only NPY and SP have been reported in mouse lung (Sheppard et al., 1984; Uddman et al., 1984). However, to the best of our knowledge, this is the first time that VIP and 5HT have been immunocytochemically demonstrated in the mouse lung in NE cells (VIP and 5HT), NEBs (5HT), nerve fibres (VIP) and ganglia (VIP).

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Accepted February 19, 1997