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Calcitonin gene-related peptide immunoreactivity in adult mouse lung

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SUMMARY

Calcitonin gene-related peptide (CGRP) is a 37 aminoacid peptide coded by the calcitonin gene that is produced by thyroid C cells and medullary carcinoma. It is also widely distributed in neurons and endocrine cells throughout the body. The presence of CGRP in the lungs suggests that this peptide exerts important regulatory actions at this level, and it can act like a neuroregulator released both from nerve terminals and neuroendocrine (NE) cells. To understand the role of CGRP in the lung, it is important to explore its localization in different species. In this paper, we analyse the presence and localization of CGRP in the adult mouse lung using an immunocytochemical staining method. Our results show a widespread distribution of this peptide in isolated neuroendocrine cells and neuroepithelial bodies (NEBs), as well as in nerve fibres distributed in many areas of the lung, including bronchi and bronchioli. These fibres are in close contact with epithelium, neuroendocrine cells and smooth muscle. In addition, some immunostained nerve cell bodies and immunoreactive intrinsic ganglion cells can be shown. CGRP has been previously demonstrated in the mammalian lung using immunocytochemistry. To the best of our knowledge, this is the first time that CGRP has been immunocytochemically demonstrated in the mouse lung both in NE cells, NEBS, ganglion cells and in nerve fibres which are related to neuroendocrine cells.

INTRODUCTION

Since the discovery of a vast system of biologically active peptides localized both in endocrine cells and autonomic nerves, they have become established as important components of the peripheral regulatory system. Furthermore, since Wharton *et al.* (1978) demonstrated the presence of bombesin immunoreactivity (IR) in human lung, the polypeptides identified in the respiratory tract of several mammalian species have been very numerous. Some of them, such as bombesin, calcitonin and leuenkephalin, are located in endocrine cells (Becker *et al.*, 1980; Cutz *et al.*, 1981; Gosney *et al,* 1984), others (substance P, VIP, PHI, NPY and galanin) are demonstrated in nerve fibres (Uddman *et al.*, 1978; Wharton *et al.*, 1979; Christofides *et al.*, 1982; Lundberg *et al.*, 1982; Marcheusky *et al.*, 1984; Cheung *et al.*, 1985). However, the only peptide located up to now in both endocrine cells and nerve fibres in the respiratory system is CGRP (Cadieux *et al.*, 1986; Haller, 1992).

CGRP is a 37 aminoacid peptide coded by the calcitonin gene. This gene transcribes two mRNAs. One of them codes the calcitonin precursor protein. The other one is the MRNA coding for the CGRP precursor polypeptide (Rosendfeld *et al.*, 1983). Calcitonin gene-related peptide has a widespread central and peripheral distribution. In the respiratory tract, the presence of CGRP-IR has been reported in some mammalian species (Cadieux *et al.*,

1986; Lauweryns and Van Ranst, 1987; Scheuermann *et al.*, 1987; Lauweryns and Seldesiagh, 1991; Luts *et al.*, 1991; Nohr and Weihe, 1991; Haller, 1992). This was localized either in nerve fibres or in neuroendocrine cells. However, for the moment, no one has demonstrated the simultaneous localization and distribution of this peptide in the adult mouse lung in NE cells and in nerve fibres. For this reason, the aim of the present work is to provide the exact distribution of CGRP-IR in the adult mouse lung, and it is the scope of further light and electron microscopic studies to establish the coexistence of CGRP with other substances in the same pulmonary NE cells by comparing serial sections or by double-labelling techniques (Fakan, 1992; Scott, 1992).

On the one hand, we plan to describe the morphologic features of CGRP-IR distribution and to make further experiments to determine if these CGRP structures have an intrinsic or extrinsic origin.

MATERIALS AND METHODS

Animals and tissue treatment

Samples of pulmonary tissue were obtained from twenty adult male mice (*Mus musculus,* Swiss OF-1). The animals were anaesthetized with ether inhalation. The lungs were fixed by perfusion through the right ventricle with a fixative solution (4% phosphate-buffered paraformaidehyde pH 7.4). The samples were then postfixed by immersion in the same fixative for 2h at room temperature and haking. The fixed tissues were washed for two hours with 0.05 M TRIS-HCI buffer, pH 7.6 with 0.25% Triton X-1 00 (Sigma, St Louis, MO).

Immunohistochemistry

After rinsing, serial sections (50-100 μm) were obtained by cryostat sectioning and processed by the streptavidin-biotin immunoperoxidase method (Bonnard *et al.*, 1984). After treatment with peroxidase blocking reagent and nonspecific blocking reagent (pig normal serum), sections were exposed to a rabbit anti-CGRP (to synthetic rat 1-37) polyclonal commercial antiserum (Cambridge Research Biochemicals, U.K.) diluted 1-2000 in a moisture chamber overnight at 4°C.

After rinsing in the same buffer, sections were incubated sequentially with biotinylated IgG (Sigma) diluted 1:50 for 30 min at room temperature and then with extravidinbiotin-peroxidase complex (Sigma) diluted 1:50 for 20 min at room temperature. The sections were revealed by immersion in a 0.05% diaminobenzidine solution in the presence of 0.01% H_2O_2 in the washing buffer, under microscopic control. Negative control procedures were carried out omitting one or more steps of the method and by the use of sections incubated with antiserum inactivated by the addition of excess antigen. Antiserum was preincubated with 1 -1 0 nmol of CGRP (1 -37) and absorbed antiserum was used to confirm the immunocytochemical specificity.

RESULTS

Numerous nerve fibres, solitary epithelial cells and neuroepithelial bodies displaying CGRP-IR were observed in the lungs of all the studied animals. The largest number of solitary immunoreactive cells were found in the bronchial and bronchiolar epithelium in close contact with the basal membrane. Their shapes were pyramidal, exhibiting a uniform cytoplasmic staining with a large nucleus and without lumen contact (Fig. 1).

The immunoreactive NEBs were located in the bronchial and bronchiolar epithelium, fundamentally in close proximity to bronchial bifurcations and parts of bronchial branchings. An average NEB possessed 3-10 columnar CGRP immunoreactive cells extended from the basal membrane to the airway lumen (Fig. 2). Occasionally, the number of immunoreactive cells were larger than 10. In contrast with the single NE cells, the NEBs contacted with the airway lumen and they were characteristically found at bronchial bifurcations.

Numerous immunoreactive nerve fibres were seen throughout the lung fundamentally around the bronchi and bronchioles where they were in close contact with the epithelium, the smooth muscle and blood vessels (Fig. 3). The fibres located in the epithelium were large, varicous and generally with a longitudinal arrangement. They were in contact with the epithelial basal membrane. Frequently they had fine ramifications distributed radially in the epithelium, reaching occasionally the airway lumen (Fig. 3). Some of the immunoreactive nerve fibres were in close contact with the basal aspect of the immunoreactive single and clustered cells, suggesting a synaptic contact (Fig. 4).

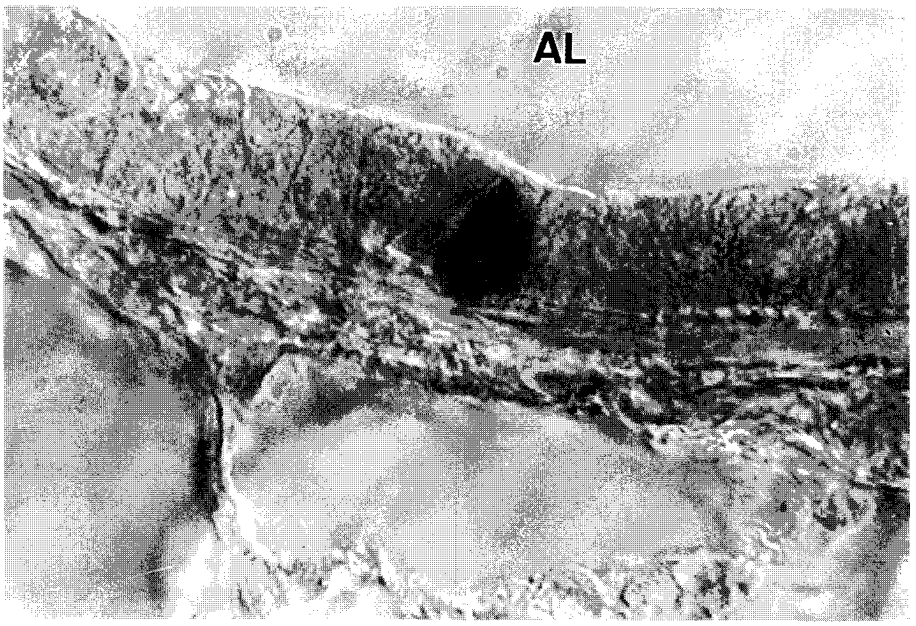

Fig. 1 - Isolated CGRP-immunoreactive cell in adult mouse bronchial epithelium, displaying a pyramidal shape, in close contact to the basement membrane, exhibiting an uniform cytoplasmic staining with a large nucleus and without luminal contact. Streptavidin-biotin-peroxidase (SBP) method. Original magnification (OM) x 1000. AL: airway lumen.

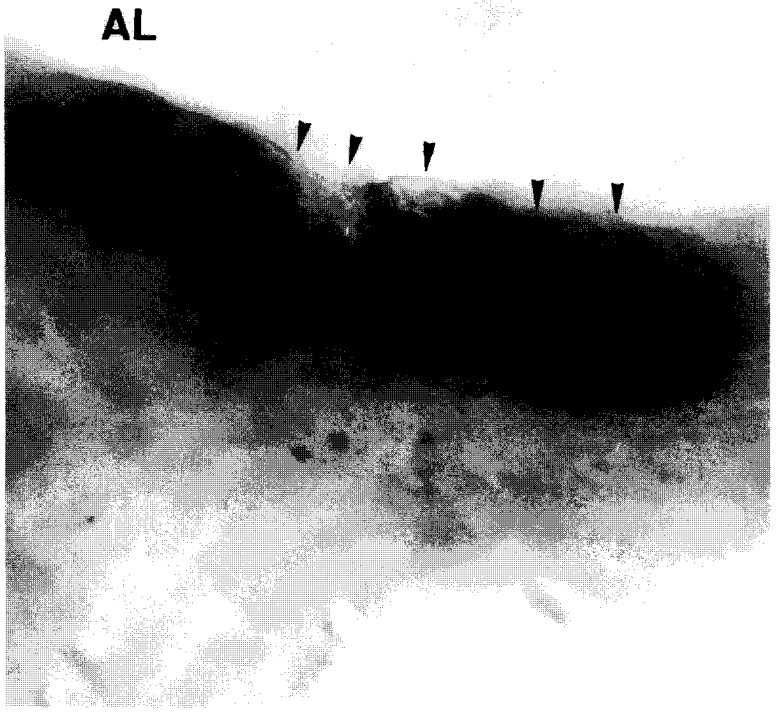

Fig. 2 - Adult mouse bronchial epithelium with a NEB revealing CGRP-IR. The immunoreactive cells were intercalated with non-immunoreactive cells (arrowheads). Some of the immunoreactive cells extend from the basement membrane to the airway lumen (AL). SBP method. OM x 1 000.

The CGRP immunoreactivity was also observed either in large nerve cells in the pulmonary parenchyme or in the smooth muscle (Fig. 5). In addition, some immunoreactive cells could be detected in the intrinsic ganglia next to bronchial bifurcations, at subepithelial level (Fig. 6).

Non immunoreactivity was detected in the control sections incubated with inactivated serum or when some step of the method was omitted.

Discussion

Our results show the widespread distribution of CGRP-IR in the adult mouse lung, the peptide being localized to both endocrine cells and nerve fibres.

The respiratory system has become re-established as an important component of the neuroendocrine regulatory system since the discovery of a vast system of biologically active substances (amines and peptides) which are localized in both endocrine cells and nerve structures of the lung. These substances possess potentially far-reaching implications for pulmonary physiology and physiopathology.

The pioneering studies of Lauweryns *et al.* (1970, 1973a, 1973b), showed that pulmonary argyrophilic cells contained a fluorogenic amine, later identified as serotonin. They provided the first description of innervated groups of these cells, called neuroepithelial bodies (Lauweryns and Peuskens, 1972). They were based on a series of experiments in newborn rabbits. Lauweryns *et al.* proposed that NEBs acted like hypoxia-sensitive intrapulmonary receptors (Lauweryns *et al.*, 1972; Lauweryns and Cokelaere, 1973a, 1973b). Up to now, several biologically active substances have been identified in the respiratory tract of several species both in nerve fibres, in isolated NE cells and NEBS. Recently, Polak (1993) has compiled a very important body of information which provides data on the immunocytochemical localization of specific substances (endocrine cell markers, peptides and amines) in pulmonary neuroendocrine cells of many species.

The mammalian respiratory tract is innervated by sensory and autonomic nerves. It is now well recognised that numerous biologically active peptides are present in these nerve fibres. One of them is CGRP, which has been described in pulmonary sensory fibres of rats (Cadieux *et al.*, 1986; Lauweryns and Van Ranst, 1987; Springall *et al.*, 1988; Van Ranst and Lauweryns, 1990), Guinea pigs

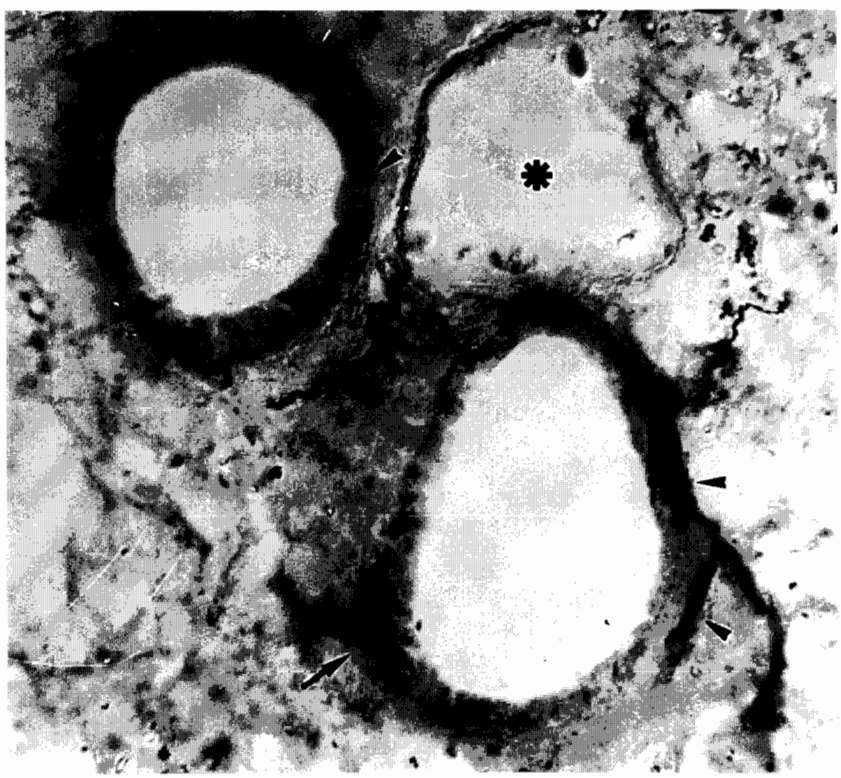

Fig. 3 - CGRP-immunoreactive nerve fibres distributed around the adult mouse bronchi, in close contact with the epithelium (arrow), the smooth muscle (arrowheads) and blood vessels. It is possible see fine ramifications distributed radially in the epithelium. Blood vessel: (asterisk). SBP method. OM x 250.

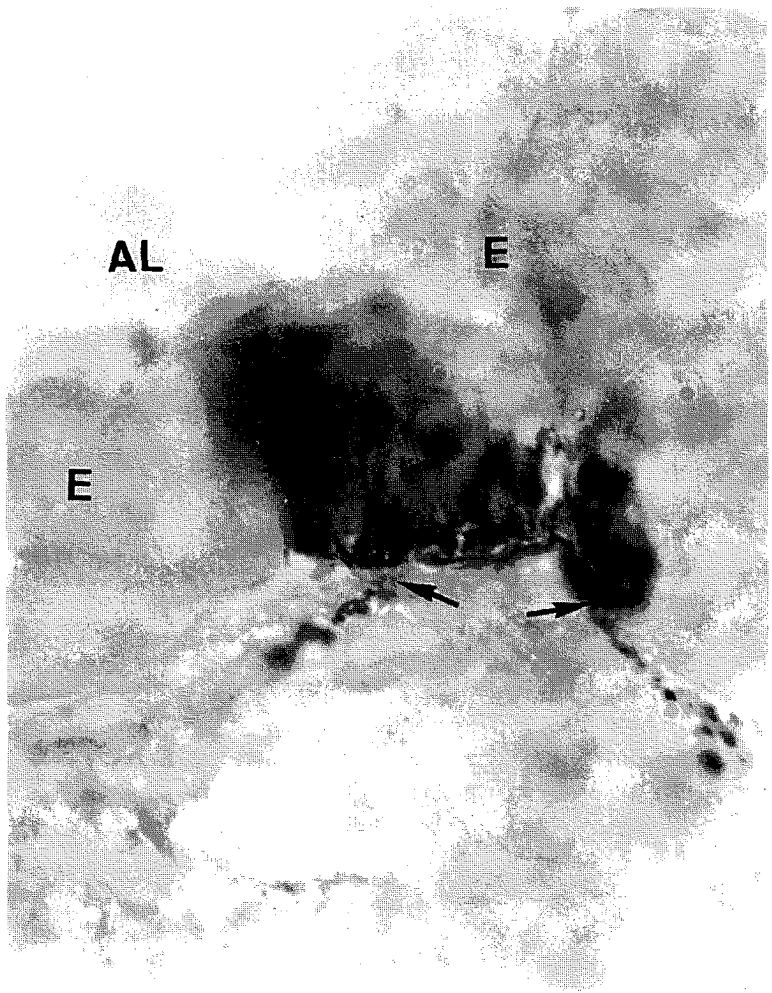

Fig. 4 - Adult mouse bronchial epithelium (E) revealing CGRP-immunoreactive nerve fibres in close contact with the basal aspect of the immunoreactive clustered cells, suggesting a synaptic contact (arrows). SBP method. AL: Airway Lumen OM x 1 000.

(Lundberg *et al.*, 1985; Mak and Barnes, 1988), cats (Scheuermann *et al.*, 1987; Lauweryns and Seldeslagh, 1991) and humans (Palmer *et al.*, 1987; Mak and Barnes, 1988).

In human lung, the CGRP-IR has been restrictively described to nerve fibres associated with vascular and bronchial smooth muscle (Palmer *et al.*, 1987; Komatsu *et al.*, 1991). However in the rat (Cadieux *et al.*, 1986; Lauweryns and Van Ranst, 1987) and in the newborn cat (Scheuermann *et al.*, 1987; Lauweryns and Seldeslagh, 1991) the immunoreactivity was located both in endocrine cells and sensory nerve fibres distributed throughout the bronchial epithelium. These immunoreactive nerve fibres were detected close to the epithelial basal membrane, sometimes forming varicosities below the NEBS.

In the airway of the mouse, Luts *et al.* (1991) described the presence of NEBs in the lungs which possessed a small number of elements displaying CGRP immunostaining. Nevertheless, there are no papers which describe the presence and distribution of CGRP-IR in nerve fibres of the adult mouse lung. To the best of our knowledge, this is the first study to show the simultaneous presence of CGRP-IR in NE epithelial cells (singly or clustered) and nerve fibres of the adult mouse lung allowing a best analysis of their distribution by the use of thick sections. This interspecies variability suggests that functions and roles of the CGRP in the lungs possibly differ in the different species. This points out the importance to determine in each species the presence, localization and distribution of CGRP at the pulmonary level. For this reason, we have chosen the adult mouse as the experimental animal. Although the mouse has been widely used for studying the control of the intrinsic neuroendocrine system in many organs, little is known about the distribution of the

substances acting in this control system at the pulmonary level.

The precise function of CGRP in the lung has not been elucidated yet and, at present, is largely speculative. Its appearance during fetal life (Cadieux *et al.*, 1986; Johnson *et al.*, 1988), and the fact that there is a higher density of CGRP-containing endocrine cells in the lungs of fetal and newborn animals than in the lungs of older ones (Luts *et al.*, 1994), suggests that CGRP-structures may be functional in the fetus, and may be involved in the pulmonary adaptation at the moment of birth, during the rapid vasodilatation upon aeration of the lung.

The storage of CGRP in NE epithelial cells, and their relation with sensory nerve fibres, may explain some mechanisms of local or paracrine regulation or neuroendocrine intrinsic pulmonary regulation mediated by this neuropeptide. Furthermore, its distribution in the lung suggests a release from sensory nerves through an axon reflex where it may play a role in the regulation of the smooth muscle tone and in the calibre regulation of pulmonary vessels (Carstairs, 1987). Nevertheless, the afferent or efferent nature of the CGRP-IR nerves in the lung, as well as their origin, are not well established. The presence of CGRP nerves next to local ganglion cells suggests that CGRP may modulate ganglionic transmission, or that CGRP has an intrinsic origin.

On the other hand, it is well recognized that CGRP and substance P coexist in some human sensory nerve endings (Lundberg *et al.*, 1985; Martling *et al.*, 1988), acting as co-transmitters in the respiratory system.

Recently, it has been demonstrated that the CGRP and other substances such as calcitonin coexist in NE cells of several species (Lauweryns and Seldesiagh, 1991; Stahlman and Gray, 1993; Luts *et al.*, 1994). CGRP also coexist with serotonin in some neuroepithelial bodies (Keith and Ekman, 1988; Springall *et al.*, 1988; Adriaensen *et al.*, 1991; Luts *et al.*, 1991; Haller, 1992); this may be related to the local pulmonary response to several stimuli, such as the composition of inhaled air.

Finally, the presence of CGRP in the lungs suggests a role as an authentic neurotransmitter with local effects, and its implication in the intrapulmonary chemoreceptor system acting by the integration of the pulmonary activity.

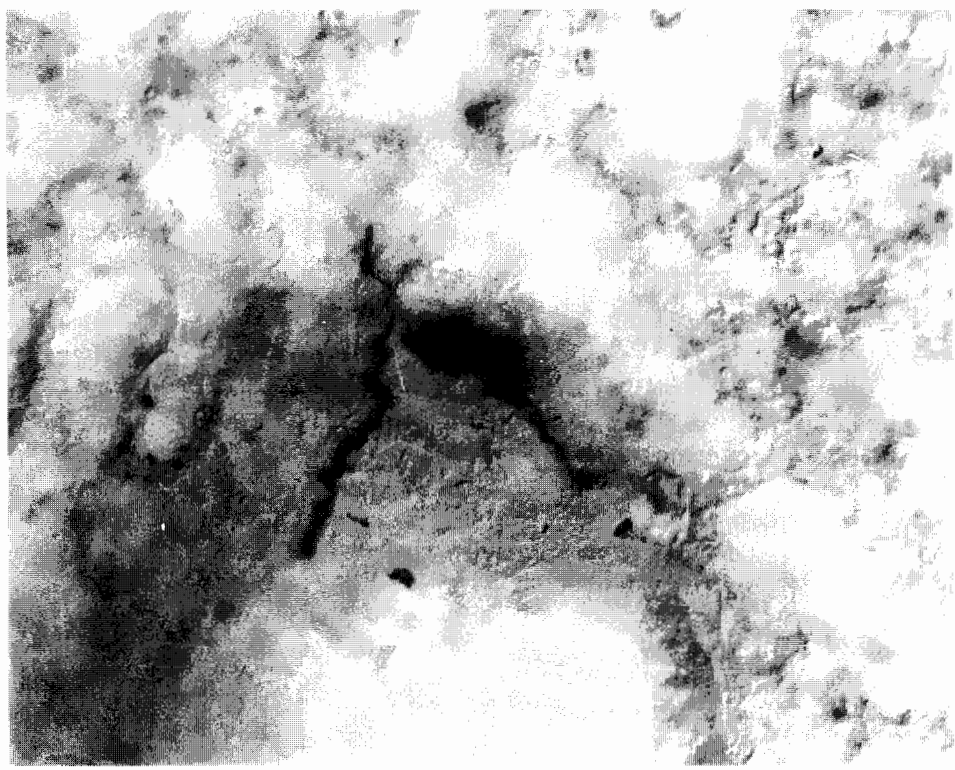

Fig. 5 - CGRP immunoreactive nerve cell in the vicinity of a bronchus. Streptavidin-biotin- peroxidase method. OM x 250.

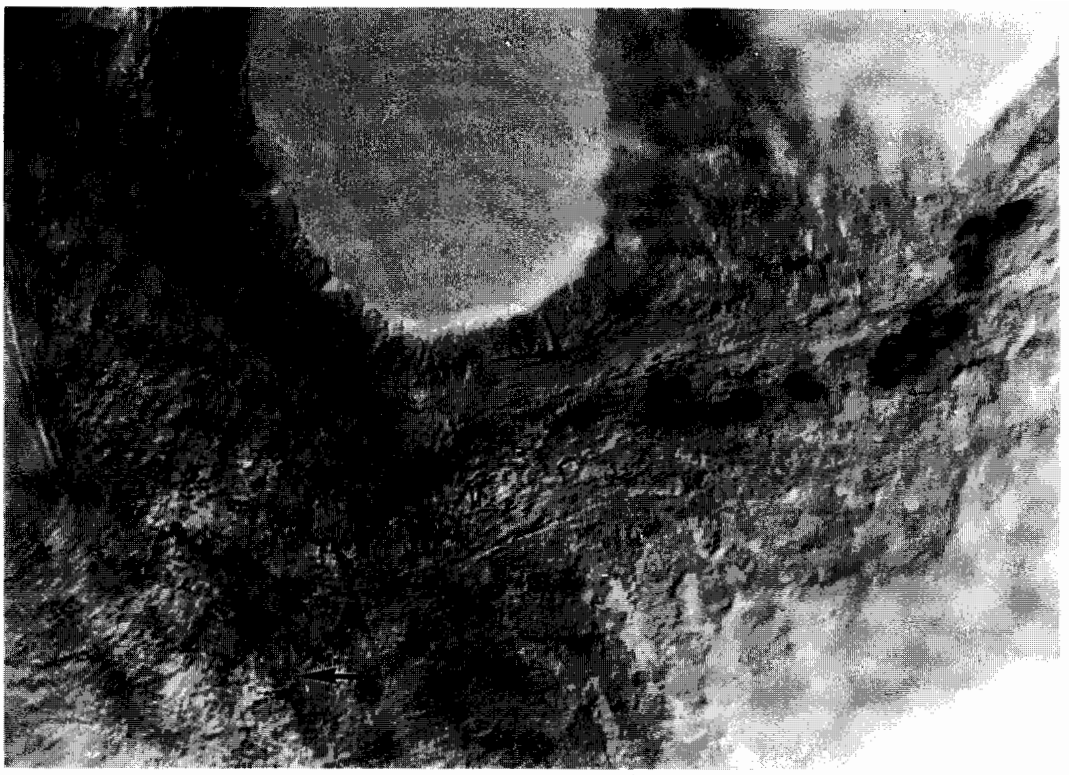

Fig. 6 - CGRP immunoreactive cells in an intrinsic ganglia next to a bronchial bifurcation located at the subepithelial level. It is possible to observe a CGRP immunoreactive nerve fibre in the pulmonary parenchyma (arrow). SBP method. OM x 250.

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