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Morphology of presumptive rapidly adapting receptors in the rat bronchus*

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INTRODUCTION

Electrophysiological studies of the cervical vagal nerve fibres show that there are four classes of sensory nerve endings in the lung (Coleridge & Coleridge, 1986). These are (i) pulmonary stretch receptors (PSR), (ii) irritant or rapidly adapting receptors (RAR), (iii) bronchial C fibres and (iv) J receptors. Of these, PSR and RAR are connected to myelinated fibres of the vagi while the latter two categories are associated with non-myelinated axons. PSR show sustained firing during inspiration, thus indicating a close structural association with the smooth muscle of the airways, and recent histological studies have provided evidence for such a proposition (Krauhs, 1984).

On the other hand, RAR are characterised by the rapid adaptation of their activity during sustained hyperinflation of the lung (Knowlton & Larrabee, 1946), their rate of firing falling off by about 30% after two seconds. Although they are principally found in the vicinity of the main bronchi their precise location in the bronchial wall is unclear (Sampson & Vidruk, 1975; Sant'Ambrogio, 1982). Most previous investigators have concentrated on the region of the bronchial epithelium (Das, Jeffery & Widdicombe, 1978, 1979; Hoyes & Barber, 1981; Jeffery & Reid, 1973; Lauweryns & Van Lommel, 1987) on the basis of evidence that RAR are stimulated by irritant vapours such as ammonia (Armstrong & Luck, 1974) and by light probing of the bronchial epithelial surface (Armstrong & Luck, 1974; Sampson & Vidruk, 1975).

However, electrophysiological studies in the anaesthetised dog have established that these receptors show a sustained increase in activity during both pulmonary venous congestion (Kappagoda, Man & Teo, 1987) and obstruction of the lymphatic drainage from the lung (Ravi, Teo & Kappagoda, 1988). In addition, they are sensitised by a reduction in the concentration of plasma proteins (Kappagoda & Ravi, 1988). These findings suggest that (i) the natural stimulus to the RAR is a function of the transfer of fluid from the intra- to the extravascular compartment of the bronchi and (ii) the RAR are located in the extravascular space in functional apposition to a component of the bronchial microvasculature. There are no descriptions in the literature of sensory nerve endings in apposition to bronchial venules or capillaries which could function in a manner which is consistent with the electrophysiological findings described above.

The present investigation was undertaken to determine whether sensory nerves exist in apposition to the bronchial microvessels. The rat was selected because the bronchial

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tissues could be fixed efficiently and conveniently for histological studies by perfusion techniques. The study was undertaken in four parts: (i) basic histology, (ii) demonstration of axonal transport of horseradish peroxidase, (iii) effects of cervical vagal sectioning and (iv) demonstration of the 'sensory transmitters', calcitonin gene related peptide (CGRP) and substance P in putative sensory endings. As a secondary aim, an attempt was made to confirm the existence of communications between the pulmonary veins and the bronchial venules. Such communications would provide an explanation for sensory endings located in the bronchial vasculature responding to changes in pressure within the pulmonary circulation.

MATERIALS AND METHODS

General method of preparation of bronchial tissue

Twenty two albino Wistar rats, each weighing approximately 250 g, were used for the study. Anaesthesia was induced with ether followed by an intraperitoneal injection of chloral hydrate (250 mg kg^{-1} body weight). The abdomen was opened and a cannula was inserted retrogradely into the descending aorta and advanced to a point just above the diaphragm. This route was adopted in order to perfuse the bronchial arteries, which originate from the thoracic aorta and from the intercostal and internal thoracic arteries.

Thoracotomy was performed following the cannulation. The animal was exsanguinated by making an incision in the right atrial appendage. Simultaneously, an infusion of approximately 50 cm^3 of Pipes buffered saline containing a final concentration of 10 mM Pipes buffer, 137 mM sodium chloride, 2·7 mM potassium chloride, 2·0 mM calcium chloride, 19·4 mM glucose, 0·0075 mM polyvinylpyrrolidone at a pH of 7·2 was initiated. The perfusion was maintained at a pressure of 100 mm Hg. The buffered saline was followed by 500 cm^3 of fixative, also maintained at 100 mm Hg. The fixative contained 2% glutaraldehyde, 2% formaldehyde, 2 mM calcium chloride in 0·1 M Pipes buffer at pH 7·2.

The lungs and attached airways were removed, the mediastinum was dissected under fixative and the primary and secondary bronchi were removed. These were cut into small pieces and further fixed by immersion in the same fixative for 4 hours. The pieces were rinsed in 0·1 M Pipes buffer at pH 7·2 made isotonic with sucrose and they were then separated into two batches. One batch was rinsed briefly in double distilled water and soaked in Jannssen's Intense II silver stain for 10 minutes to stain myelinated nerves for light microscopy. They were rinsed again in distilled water and returned to buffer. Both groups of tissue were then treated with 1% osmium tetroxide in 0·1 M Pipes buffer made isotonic with sucrose, dehydrated in graded solutions of ethanol and embedded in Taab embedding resin. Serial sections (1·0–2·0 μm) were prepared to identify nerve terminals by light microscopy (Krauhs, 1984). They were stained with methylene blue, viewed and photographed using a Zeiss Orthophot microscope. Thin sections (40–60 nm) were prepared and mounted on copper grids. These were double stained with uranyl acetate and lead citrate and viewed in a Philips EM300 transmission electron microscope.

Following the examination of the serial sections with the light microscope, selected samples containing identifiable terminals adjacent to venules were removed from siliconised glass slides using cyanoacrylate glue (UHU, Beecham) and resectioned for electron microscopy.

Labelling with WGA-HRP

Animals were anaesthetised as described previously. The right nodose ganglion was identified as a thickened region of the main vagal trunk adjacent to the base of the skull. Multiple injections of 10% wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) were made into the body of the ganglion using an extruded glass pipette with a tip diameter of approximately 100–200 μm. Two to six injections were routinely performed to a final volume of approximately 10 μl, in seven animals. The incisions were resutured and the animals were allowed to recover. They were left for 4–5 days to ensure transport of the WGA-HRP into both peripheral nerve terminals and cell bodies in the brain.

Preliminary studies were carried out to validate the accuracy of the injection site and the uptake of the label into the nerve cell bodies of the nodose ganglion. These studies were carried out using both diaminobenzidine (DAB) and tetramethylbenzidine (TMB) as substrates to visualise the distribution of the label. On the basis of this preliminary assessment, TMB was selected as the substrate of choice. The procedure is described in detail below.

After 4–6 days the animals were re-anaesthetised and the tissues fixed according to the procedure described above. In this instance the fixative was based on phosphate buffer and was composed of 0·1 M phosphate buffer containing 1% formaldehyde, 2% glutaraldehyde and 5% sucrose at pH 7·2. The animals were perfused with one litre of fixative followed immediately by one litre of 0·1 M phosphate buffer containing 5% sucrose at pH 7·2. The lungs and airways were removed and a block of tissue containing the right primary and secondary bronchi was removed from each animal. These blocks of tissue were cut on a Leitz freezing microtome into slices 50–70 μm in thickness. In order to check that transport of WGA-HRP had occurred (albeit in a centripetal direction, which is easier to identify than centrifugal transport) the hindbrain was also removed and transverse sections (50 μm) through the obex, at which level the vagal nuclei are readily identifiable, were prepared on a Vibratome (Oxford Instruments Ltd).

Tissue slices were processed for HRP activity essentially as described by Joosten, Gribnau & Dederen (1987). Sections were briefly washed in two changes of double distilled water and then incubated for twenty minutes in the following solution of TMB: 0·5 mg of TMB was dissolved in 2·5 cm^3 of Analar ethanol warmed to 40 °C and added to 97·5 cm^3 0·1 M phosphate buffer at pH 6·0 containing 250 mg ammonium heptamolybdate. After twenty minutes, 5·0 μl of 30% hydrogen peroxide was added for every 10·0 cm^3 of reaction medium. Additional aliquots were added every five minutes for twenty minutes. The reaction was then terminated by rinsing the sections four times in phosphate buffer at pH 5·0. They were then treated with 2% osmium tetroxide in 0·1 M phosphate buffer at pH 5·0 for four hours and embedded and sectioned as described previously.

Immunocytochemistry for calcitonin gene related peptide and substance P

A group of four animals was used for this procedure. They were anaesthetised as described above and fixed by perfusion in the same manner as for ultrastructural examination. The fixative contained 4% formaldehyde in 0·1 M Pipes buffer at pH 7·2. Glutaraldehyde was omitted to optimise the preservation of CGRP and substance P for immunogold labelling. Small pieces of bronchial wall were left unosmicated, partially dehydrated to 75% ethanol and embedded in LR White as described previously (Skepper & Navaratnam, 1988). Areas containing potential nerve terminals

were identified by light microscopy and thin sections from these regions were mounted on nickel grids. Confirmation that nerves examined by electron microscopy were, in fact, terminals was provided by subsequent serial sectioning at 1–2 μm. Any nerves that were not seen to terminate within 25 μm were excluded from examination with the transmission electron microscope.

Immunolabelling was carried out using polyclonal primary antisera to CGRP and substance P raised in rabbits (Cambridge Research Biochemicals) at dilutions of 1–100 to 1–500 in phosphate-buffered saline (PBS) containing 2% normal goat serum. After extensive washing in PBS the bound antibodies were visualised with goat anti-rabbit immunoglobulins bound to 10 nm colloidal gold particles. Immunocytochemical controls were carried out by omission of primary antisera, replacement of primary antisera with non-immune rabbit serum or pre-incubation of primary antisera with CGRP or substance P as appropriate. In all cases only negligible background labelling was apparent.

Sectioning the cervical vagus

One group of six animals was anaesthetised and the right main vagal trunk was exposed and sectioned immediately below the nodose ganglion. The incision was sutured and the animals allowed to recover for 2–4 days. At the end of this period they were re-anaesthetised, fixed and bronchial tissue prepared for electron microscopy as described above. Evidence of degeneration was sought in the putative nerve endings.

Demonstration of communications between the pulmonary veins and the bronchial venules

Two rats were anaesthetised as described above. A nylon cannula was inserted into the left atrium. Two other nylon cannulae (internal diameter 0·8 mm) were inserted into the left and right ventricles. The latter two cannulae were equipped with small latex balloons which could be distended with saline (0·5 ml). When these balloons were distended, they filled the cavities of both ventricles. When all the cannulae were in place, the tissues were fixed as described previously.

After fixation, Batson's casting resin (Polysciences Inc) containing 0·2% particulate red pigment was injected through the cannula into the left atrium at a pressure of approximately 100 mm Hg. To ensure complete filling of the bronchial circulation, the viscosity of the resin was reduced by replacing 30% of the base solution with methyl methacrylate monomer. The total volume of resin injected was approximately 0·75 cm^3. After completion of the injection, the carcass of the animal was left overnight for the resin to set. The bronchial tree was excised, embedded and sections were prepared for light microscopy.

RESULTS

General distribution of the bronchial venules

The histological studies were limited to the primary and secondary bronchial tissue. Typically, the bronchi were lined by columnar epithelium with a ciliated border. The submucosa consisted of loose connective tissue containing an abundance of blood vessels. There was a well-developed layer of smooth muscle outside the connective tissue. The venules, which were identified as thin walled vessels with no internal elastic lamina and a thin muscle layer of 1–2 cells in thickness with a single layer of endothelium, were disposed in two plexi on either side of the layer of muscle (Fig. 1). The injections of resin confirmed the existence of communications between the pulmonary veins and the bronchial venules. For instance, Figure 2 shows bronchial

venules filled with resin in which particles of pigment are apparent, following injection of the pigmented resin into the pulmonary veins.

Nerves containing both myelinated and non-myelinated elements were frequently seen adjacent to the extramuscular venules (Fig. 1) and they were followed through successive serial sections. In some instances the myelinated elements disappeared, leaving an encapsulated terminal which contained only non-myelinated elements (Fig. 3). These encapsulated terminals were consistently observed close to the extramuscular vascular plexus (often within 2 μm of individual vessels) when the bronchi were sectioned in a transverse orientation. None was identified in the epithelial layer, in the submucous coat or amongst the smooth muscle cells. Serial sections established that such terminals were independent of ganglion cells, many examples of which were found elsewhere in the bronchial walls.

Electron microscopy of nerve terminals

In order to establish criteria for identifying these nerve endings by electron microscopy, nerves were followed through serial sections until demyelination began to occur. At this stage, sections were removed from slides and re-sectioned for examination with the electron microscope. For example, Figure 3 is a light micrograph showing a nerve trunk in apposition to a venule. Successive sections of this trunk showed loss of myelination and the appearance of an encapsulated swelling of the distal end of the nerve. When examined by electron microscopy, the terminal was found to be composed of non-myelinated, convoluted axons which were still partially ensheathed in Schwann cell cytoplasm. These axons were enclosed in a loose monolayer of attenuated cells, the tapering extremities of which partly overlapped each other. The intercellular space between the capsular cells measured approximately 20 nm and occasional desmosomes were identified; no tight junctions were found. Within the capacious capsule, there were numerous collagen fibres loosely arranged around the nerve terminals.

Similar encapsulated structures were identified, adjacent to venules in non-silver stained materials prepared solely for ultrastructural study. They were followed through subsequent serial thick sections to confirm their identity as terminals. In such preparations, the features of the endings were defined in greater detail (Fig. 4).

Encapsulated nerve terminals were not found in the bronchial epithelium, submucosa or muscularis. However, free unmyelinated axon profiles were found in the muscular coat and, more rarely, in the submucosa.

WGA-HRP transport

Uptake into the nodose ganglion cells

The accuracy of the initial injection site of WGA-HRP was monitored in three animals by excision of the nodose ganglion and adjacent portions of the vagus 2–4 hours after the injection. These tissues were sectioned and incubated for HRP activity as described above. In all cases reaction product was found in the extracellular spaces surrounding the neuronal cell bodies and within membrane-bound structures in their cytoplasm (Fig. 5).

Transport to ipsilateral vagal nuclei

Four to six days after injection of WGA-HRP, labelled nerve fibres were found ramifying in the nucleus of the tractus solitarius (Fig. 6), and labelled cell bodies were demonstrated in the dorsal motor nucleus of the vagus and in the nucleus ambiguus of the right side. No labelled cell bodies or nerve fibres were seen on the left side.

Transport to putative RAR in the bronchial wall

Approximately fifty nerve endings in the bronchial wall, with the characteristics described above, were examined with the electron microscope and a substantial proportion of the demyelinated axons (typically 2–4 for each terminal profile examined) were found to contain electron-dense deposits of TMB reaction product, thus confirming HRP transport (Fig. 7). These profiles were subsequently followed through serial thick sections to confirm their identity as terminals. In all sections of bronchi from animals injected with HRP, nerves in close apposition to the airway epithelium were examined for the presence of TMB reaction product but none was found.

Effects of cervical vagotomy

The tissues were examined for evidence of degeneration 2–4 days after sectioning the right vagus. Some encapsulated nerve terminals were found to have degenerating axons characterised by increased electron density of the axoplasm and deterioration of the myelin sheath (Fig. 8).

Immunocytochemistry for CGRP and substance P

Immunogold labelling demonstrated that both CGRP and substance P could be localised within electron-dense vesicles in the unmyelinated axons of the encapsulated terminals (Figs. 9, 10). Immunolabelled, free nerve varicosities were also found amongst the smooth muscle cells after using primary antisera to substance P (Fig. 11). Free nerve varicosities which were immunoreactive for CGRP were also occasionally found in submucosal locations between the muscularis and the bronchial epithelium (Fig. 12). No encapsulated terminals similar to those found close to vessels of the extramuscular vascular plexus were observed within either of the latter locations.

DISCUSSION

Rapidly adapting receptors were first defined by Knowlton & Larrabee (1946) in terms of their electrophysiological responses to sustained inflation of the lungs. These receptors show a rapid increase in activity when the lungs are inflated to three times the tidal volume. However, when the inflation is sustained, the discharge shows a characteristic adaptation, with the activity declining by more than 30% within two seconds. This feature is in sharp contrast to the response of PSR, which demonstrate sustained activity to the same stimulus. Both RAR and PSR transmit action potentials in myelinated fibres of the cervical vagi.

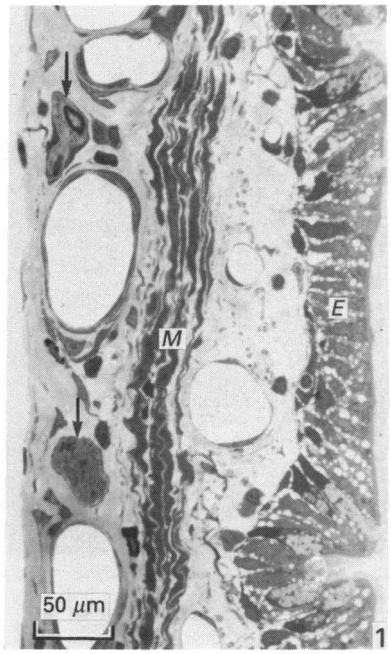
Fig. 1. Light micrograph of a section through a secondary bronchus showing its epithelial lining (*E*) and muscle coat (*M*). Venules in the bronchial wall are disposed in two layers, viz. submucous and extramuscular. Note the small nerve bundles (arrows) near the extramuscular venules.

Fig. 2. Light micrograph of a section through a bronchus showing pigmented particles, which had been introduced through the left atrium, embedded in the resin within the lumen of an extramuscular venule (*V*) confirming the existence of communications between this vessel and pulmonary veins. *E*, epithelial lining; *M*, bronchial muscle.

Fig. 3. Light micrograph of a semithin section treated with Jannssen's Intense II silver stain. It shows a myelinated nerve apposed to a venule. One end of the nerve has shed its myelin sheath and is expanding to form a nerve terminal (*N*).

Fig. 4. Electron micrograph of an encapsulated nerve terminal near a bronchial venule. The terminal comprises coiled unmyelinated axons loosely surrounded by a thin capsule of overlapping fibrocytes. The axons are generally ensheathed by Schwann cells but the axolemma is denuded in small areas.

Fig. 5. Electron micrograph showing HRP reaction product within the nodose ganglion. Labelling is found outside the neuronal perikarya as well as in lysosomes within the neuronal cytoplasm.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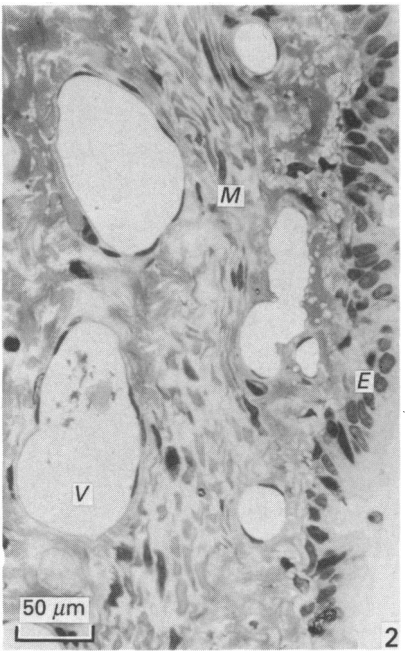
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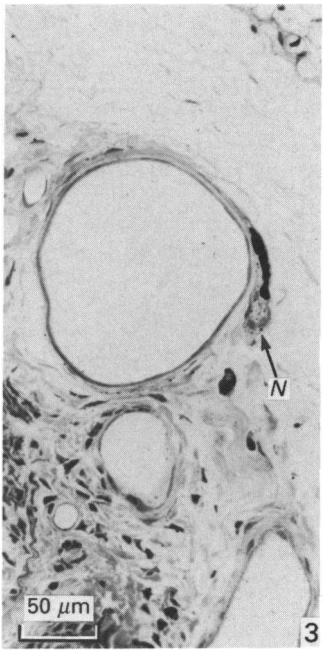
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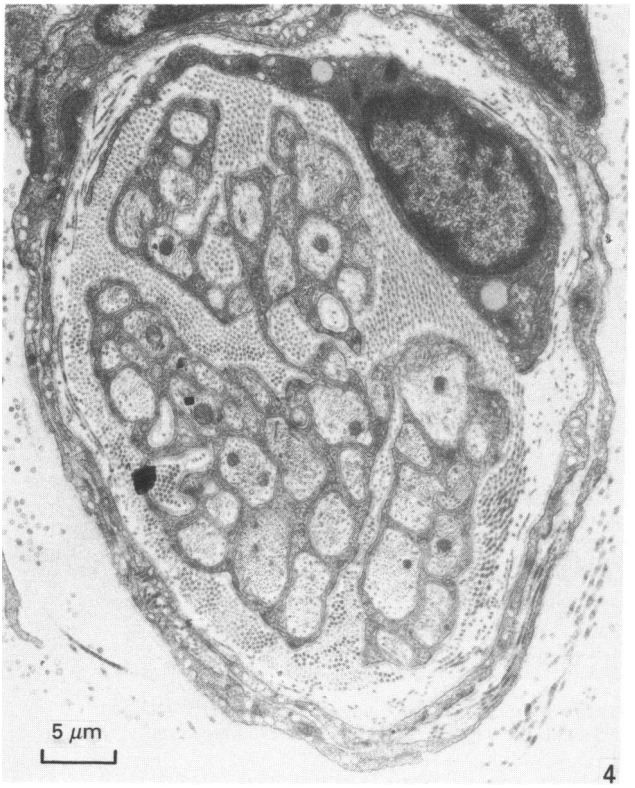
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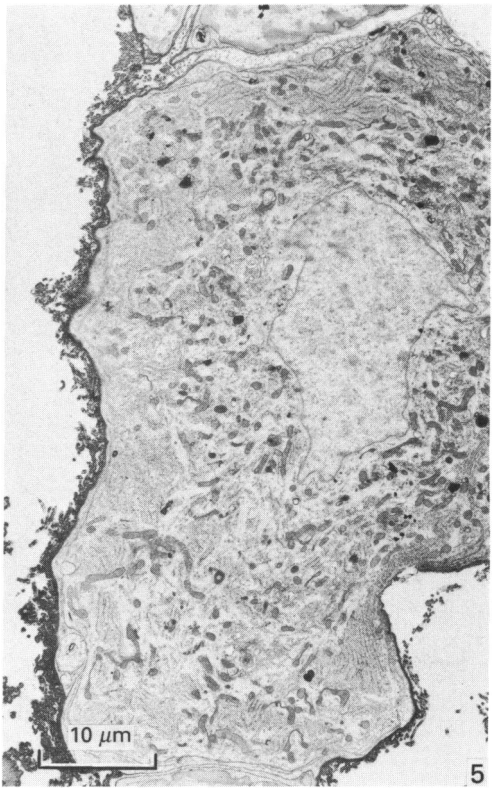
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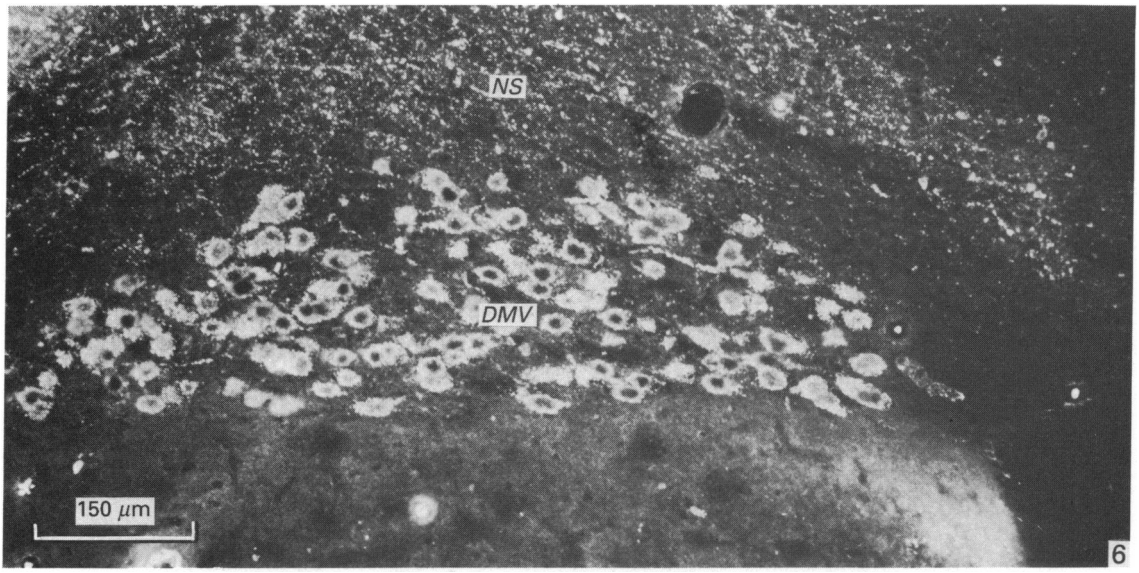

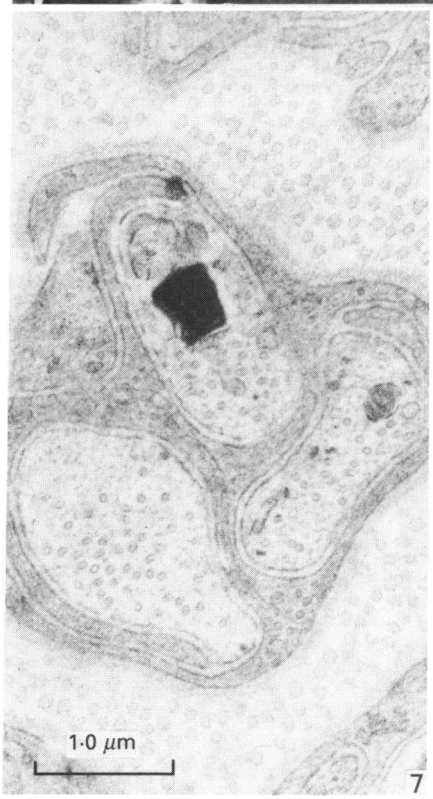

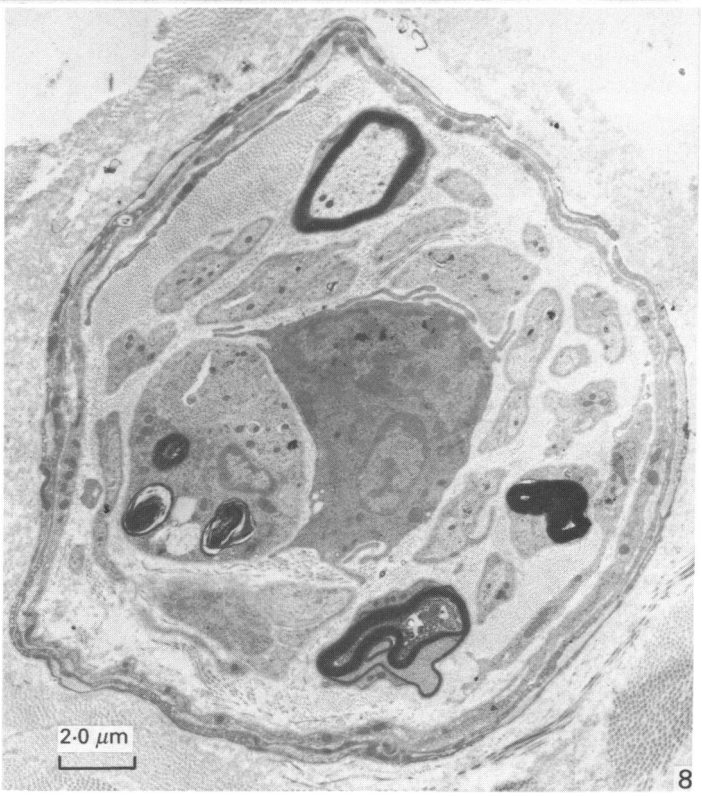

Fig. 6. A section through the hindbrain viewed with dark field illumination showing HRP reaction product within cells of the dorsal motor nucleus of the vagus (*DMV*) and in nerve fibres ramifying in the nucleus solitarius (*NS*).

Fig. 7. Electron micrograph of an encapsulated nerve terminal near a bronchial venule showing HRP reaction product inside an unmyelinated axon.

Fig. 8. Electron micrograph of an encapsulated nerve terminal near a bronchial venule showing degenerating axon profiles (myelinated and unmyelinated) 3 days after cervical vagotomy.

It is generally accepted that the RAR are most profuse in the trachea and main bronchi (see Sant'Ambrogio, 1982 for review), but there is some evidence to suggest that they may also extend to the distal portions of the airways (Sampson & Vidruk, 1975). Although the RAR are activated by a variety of chemical agents (e.g. vapours of ether, alcohol and ammonia), both the physiological stimulus to the RAR and their fine structure remain unresolved (Coleridge & Coleridge, 1986). The distribution of the RAR within the depth of the bronchial wall from the epithelium to the extramuscular regions is incompletely defined.

Many of the investigations designed to elucidate their structure have been influenced by the claim that the RAR are located within the airway epithelium (Das *et al.* 1978, 1979). The electrophysiological studies reported by Armstrong & Luck (1974) and Sampson & Vidruk (1975) were instrumental in defining the gross anatomical location of these receptors. Armstrong & Luck probed the epithelial surface in anaesthetised cats using glass rods, while Sampson & Vidruk used a cotton-tipped probe for the same purpose in anaesthetised dogs. Both studies confirmed that the receptor activity of RAR originated from the main airways. In neither instance did the authors claim that their stimulation was restricted to epithelium. In fact, transduction of such a mechanical stimulus to receptors in the subepithelial layers is clearly possible. Even though an epithelial location for the RAR may be convenient to explain some of the 'irritant' properties of these receptors, other observations remain unexplained. For instance, when local anaesthetics were instilled in the form of aerosols, the RAR, in contrast to low threshold PSR, were not consistently blocked (Fahim & Jain, 1979). Further, after superficial destruction of the tracheobronchial mucosa, it was found that mechanosensitivity (to local probing) of RAR was abolished while the responses to inflation and deflation were retained (Mortola, Saint'Ambrogio & Clement, 1975). These findings suggest that, in addition to a possible location within the epithelium, the RAR may be distributed at several distinct anatomical locations within the bronchial wall.

Recent investigations have suggested that the RAR are activated by forces influencing the movement of fluid from microvessels to the extravascular compartment, viz. pulmonary venous congestion (Kappagoda *et al.* 1987), lymphatic obstruction (Ravi *et al.* 1988) and reduction in the concentration of plasma proteins (Kappagoda & Ravi, 1988). The investigation reported in this paper has revealed the existence of nerve endings in the vicinity of the bronchial venules. These endings appear to be derived from myelinated nerves which lose their myelin sheath distally. Serial sections show that the terminals are not related to ganglion cells, thus ruling out the possibility that they are preganglionic fibres, a distinction which is not always easy to make (see El-Bermani & Chang, 1979).

WGA-HRP transport and vagal nerve section studies have provided strong circumstantial evidence in support of the proposition that these endings are sensory in nature. In addition, immunocytochemical studies have established that these structures are immunoreactive for CGRP and substance P. CGRP innervation in the lung is thought to play a predominantly sensory role (Cadieux *et al.* 1986), as is substance P innervation (Terenghi *et al.* 1983). However, it is recognised that the evidence presented in this paper does not confirm that the receptor activity, which characterises RAR, originates from these structures. It is emphasised that nerve endings having these basic characteristics were not encountered in apposition to the smooth muscle of the airways.

Further, the retrograde injection of pigmented resins into the pulmonary veins has confirmed previous observations that significant communications exist between the


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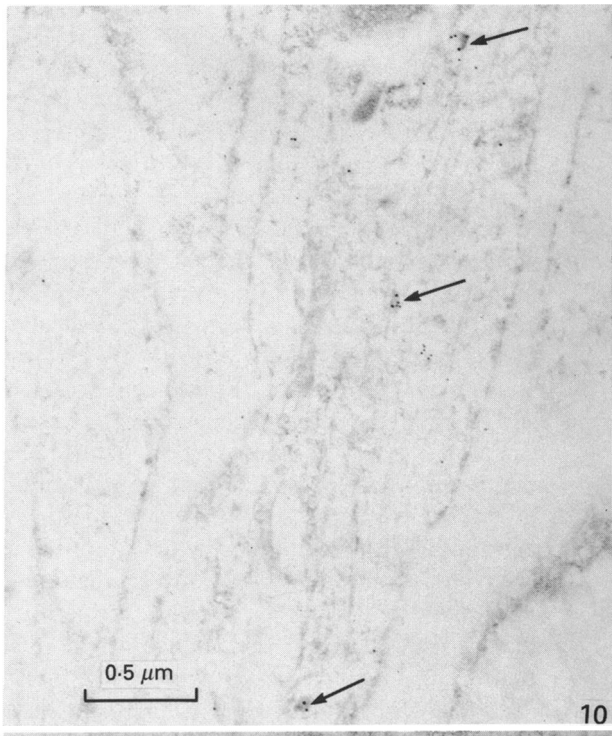
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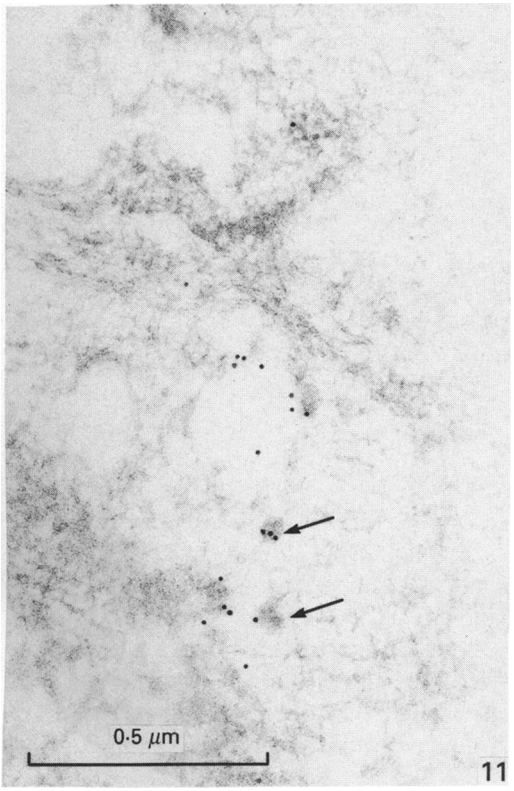
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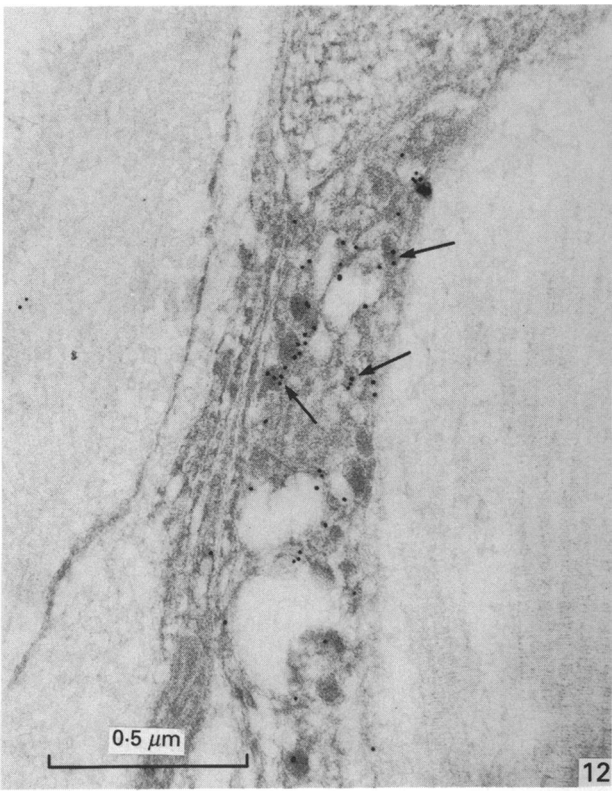
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bronchial veins and the pulmonary vasculature in several species including the dog (de Burgh Daly & Hebb, 1956). The existence of such communications provides an anatomical basis for sensory endings located in areas supplied by the bronchial circulation responding to changes in pressure in the pulmonary circulation. These findings, taken collectively, suggest that the structures described here could be the RAR. Although the location of the nerve endings is consistent with the physiological observations relating to the RAR, the nature of the transduction occurring at the terminal remains to be resolved. Indeed, the precise site of the trigger zone of the putative sensory axon is not clear. In all the encapsulated structures observed in this study, the unmyelinated axons remained partially ensheathed by Schwann cell cytoplasm.

SUMMARY

The present investigation was undertaken in rats to determine whether sensory nerves exist in apposition to the bronchial microvessels which may function as rapidly adapting receptors (RAR). The primary and secondary bronchi on both sides were removed and processed for light and electron microscopy. Nerves were frequently found in relation to venules external to the muscle coat of bronchi. They comprised myelinated axons which ended individually as non-myelinated convoluted terminals enclosed within a loose capsule of attenuated cells. Serial sections showed that these terminals were not related to ganglion cells. Cervical vagal section and injection of HRP-WGA into the nodose ganglion provided corroborative evidence of the sensory nature of these terminals. Vagal section caused degenerative changes in the encapsulated nerve terminals in the bronchial walls and horseradish peroxidase labelling was demonstrable in such terminals. Moreover, immunocytochemical studies demonstrated the presence of calcitonin gene regulated peptide and substance P in these structures. It is suggested that they comprise the RAR. Encapsulated nerve terminals were not found in the epithelial layer, in the submucous coat or in the muscularis of bronchi.

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Fig. 9. Thin section of an encapsulated nerve terminal after immunogold labelling for CGRP. Immunogold decoration is particularly profuse over a large dense-core vesicle (arrow).

Fig. 10. Thin section of an encapsulated nerve terminal after immunogold labelling for substance P. Immunogold decoration is concentrated over dense-core vesicles (arrows).

Fig. 11. Thin section of an unencapsulated nerve varicosity within the muscularis of the bronchial wall after immunogold labelling for CGRP. Immunogold decoration is concentrated over large dense-core vesicles (arrows).

Fig. 12. Thin section of an unencapsulated nerve varicosity within the submucosa, between the muscularis and the bronchial epithelium, after immunogold labelling for CGRP. Immunogold decoration is concentrated over large dense-core vesicles (arrows).

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