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Immunocytochemical localisation of substance P-like nerves in the cardiac ganglia of the monkey (*Macaca fascicularis*)

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ABSTRACT

Substance P-like immunoreactive (SP-IR) nerves formed 2 types of relationships with nerve cells in the cardiac ganglia of the monkey (*Macaca fascicularis*). The first type consisted of varicose SP-IR nerve fibres that ramified throughout the cardiac ganglia, forming a loose network with several nerve cell bodies. The second type consisted of several nerve cell bodies enwrapped by a dense pericellular (basket-like) investment of varicose SP-IR nerve fibres. Numerous SP-IR nerve fibres formed perivascular networks around the walls of the blood vessels within the cardiac ganglia and the muscle. The cardiac muscle cells were also innervated either by an isolated single varicose SP-IR nerve fibre or by complex networks. At the ultrastructural level, the substance P reaction product appeared to be associated with the microtubules and the outer mitochondrial membranes of labelled axons. Substance P reaction product was also localised on the membranes of small agranular vesicles of the labelled axon terminals. The SP-IR axons and axon terminals were closely related to the nerve cell bodies and they occurred either singly or in small groups. Most of the axons were enwrapped by a sheath from the adjacent Schwann cells which often exhibited pseudopodia-like processes. None of the axon terminals was observed to make synaptic contact with the cell bodies. However, a few axoaxonal contacts involving SP-IR and non-SP-IR axon terminals were present, the significance of which is not understood. Several axon terminals lay close to blood vessels, and may modulate the activity of these vessels. It is hypothesised that the SP-IR nerves in the cardiac ganglia of the monkey may be involved in sensory innervation or in cardiovascular reflexes.

INTRODUCTION

Substance P-immunoreactive nerve fibres were first described in the mammalian heart by Reinecke et al (1980). Since then, the distribution of substance P-immunoreactive nerves in the heart of mammals has been reported by many workers (Weihe and Reinecke, 1981; Weihe et al. 1981, 1984; Wharton et al. 1981, 1988, 1990; Reinecke et al. 1982; Hougland and Hoover, 1983; Urban and Papka, 1985; Rechardt et al. 1986; Baluk & Gabella, 1989; Forsgren, 1989*a*; Forsgren et al. 1990). While most of these studies have been carried out in the heart of the rat and guinea-pig, human heart material (including cardiac ganglia) has also been investigated (Weihe et al. 1981; Wharton et al. 1988, 1990). Moreover, Weihe & Reinecke (1981) have further reported the presence of substance P-immunoreactive nerve fibres projecting towards the ganglion cells of the monkey heart. However, very little information is available at the ultrastructural level on the nature and distribution of substance P-containing nerves in the ganglia of the primate heart. The present study was therefore undertaken to immunolocalise the substance P-like nerves in the cardiac ganglia of a species of monkey (*Macaca fascicularis*). A preliminary report of the findings has been published (Tay & Wong, 1991).

MATERIALS AND METHODS

A total of 6 adult monkeys of both sexes (body weight 2–2.5 kg) was used for the present study. For perfusion, each animal was anaesthetised by an intraperitoneal injection of 0.5 ml of Sagatal (sodium pentobarbital, 60 mg/ml) per kg body weight. Artificial ventilation with air from a Harvard animal ventilator (model 683) through a tracheostomy was initiated before thoracotomy; 5 min before perfusion,

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1000 units of heparin and 2 ml of 1% sodium nitrite per kg body weight were given by intracardiac injection.

The animals were rapidly perfused through the left cardiac ventricle with 500 ml of Ringer's solution (pH 7.4) followed by 1000 ml of fixative (4% paraformaldehyde + 0.2% glutaraldehyde in 0.1 M phosphate buffer for electron microscopy, and 4% paraformaldehyde alone in 0.1 M phosphate buffer for light microscopy). The heart was then removed and postfixed in the same fixative for an additional 4 h.

The interatrial septum containing the cardiac ganglia was dissected out and placed in 0.1 M phosphate buffer. After 2 washes at 15 min each, the tissue was mounted on a metal chuck using Tissue-Tek (Miles Inc., USA) and liquid nitrogen. Frozen sections were cut at 40 μm thickness in a Histostat 1500 at −10 °C. Sections were mounted directly onto gelatinised glass slides and stored at −10 °C. These sections were then processed with a modification of the avidin–biotin–complex (ABC) technique (Tay et al. 1989). Briefly, the sections on slides were rinsed in phosphate-buffered saline (PBS) for 30 min and then incubated in 4% normal goat serum (NGS) for 2 h at room temperature (20 °C). Sections were washed in PBS for 1 h with several changes and subsequently incubated in anti-substance P (1:500) in 1% NGS for 20–48 h at 4 °C. Subsequent antibody detection was carried out by using the Vectastain ABC-kit (PK-4001, Vector Laboratories, Burlingame, California) against rabbit IgG with 3,3′ diaminobenzidine as a peroxidase substrate, and intensified with nickel ammonium sulphate. The buffer solutions for LM preparations contained 0.1% Triton-X 100, whereas buffer solutions for EM preparations contained no Triton-X 100. Sections for LM were dehydrated and coverslipped with Permount. For EM, the cardiac ganglia were identified and trimmed from the sections on slides and freely floated in vials containing 0.1% phosphate buffer. These freely floating sections were then osmicated and dehydrated in graded series of ethanol and embedded in Araldite. Ultrathin sections were cut on a Reichert E ultramicrotome, stained with lead citrate only and viewed in a JEOL 1200 CX electron microscope.

Rabbit antisubstance P (Incstar Corp., Minnesota) used at an antiserum dilution of 1:500 was satisfactory for both LM and EM preparations. Immunostaining was abolished by incubating the sections in 1% NGS minus the antiserum or by preincubation of the diluted antiserum with 50 μg/ml of synthetic substance P (Peninsula Laboratories Inc., California).

RESULTS

Light microscopy

Numerous varicose and nonvaricose nerve fibres in the cardiac ganglia and muscle of the monkey heart showed substance P-like immunoreactive (SP-IR) staining. Within the cardiac ganglia of the interatrial septum, the varicose SP-IR nerve fibres formed 2 types of relationships with the nerve cell bodies. The first consisted of varicose SP-IR nerve fibres that ramified throughout the cardiac ganglia, forming a loose network with several nerve cell bodies (Figs 1, 2). The second consisted of several nerve cell bodies receiving a dense pericellular (basket-like) investment of varicose SP-IR nerve fibres (Fig. 3). It was not possible to ascertain if the same nerve fibres contributed to the loose as well as the dense pericellular endings. SP-IR nerve cell bodies were not found in the cardiac ganglia of the monkey (Figs 1–3). It was also uncertain if the SP-IR nerve fibres made any synaptic contacts with themselves or with the cell bodies of the cardiac ganglia. Numerous varicose SP-IR nerve fibres also formed perivascular networks within the walls of the blood vessels (Fig. 4), both within the cardiac ganglia as well as the muscle in the interatrial septum. Nerve networks of varicose SP-IR fibres also ramified amongst the muscle fibres (Fig. 5). Some of these networks were simple, while others were comprised of numerous interlacing varicose SP-IR nerve fibres, weaving to form complex networks around groups of muscle fibres (Fig. 5). In the musculature adjacent to cardiac ganglia, an occasional isolated single varicose (Fig. 6) or nonvaricose SP-IR nerve fibre could be traced to course for a distance of several μm. The origins and terminations of these SP-IR axons in the cardiac ganglia, blood vessels and muscles have not been determined in the present study.

Electron microscopy

The substance P reaction product was characterised by electron-dense particles distributed in many axons and axon terminals, but not in the perikarya or dendrites of the cardiac ganglion cells. In the axons, the immunoreaction product appeared to be associated with the microtubules, the outer membranes of the mitochondria and the inner surface of the plasma membrane (Fig. 7). Very often several SP-IR axons coursed close to the non-SP-IR perikarya (Fig. 7). In other instances, a single axon terminal was found close to the perikarya of the neurons (Fig. 8).

Most of the axons and axon terminals were enwrapped by a sheath from the adjacent Schwann

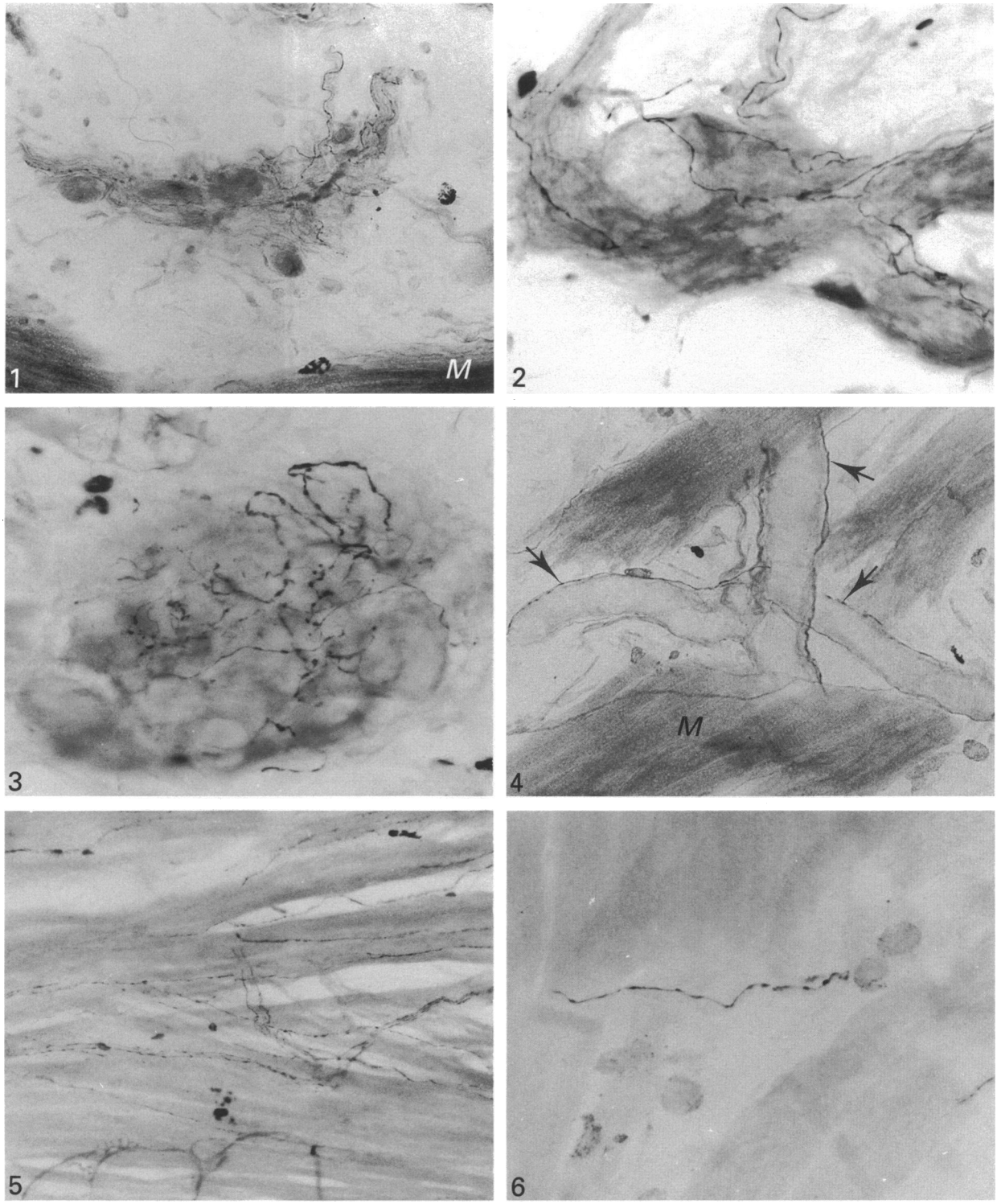


Fig. 1. Low magnification photomicrograph of an intrinsic cardiac ganglion. Several varicose SP-IR nerve fibres ramify throughout the ganglion, forming a loose network with several nerve cell bodies. *M*, cardiac muscle. ×270.

Fig. 2. High magnification of a cardiac ganglion showing several SP-IR nerve fibres forming a loose network around the nerve cell bodies. ×440.

Fig. 3. A cluster of several nerve cell bodies of an intrinsic cardiac ganglion is invested by a dense pericellular (basket-like) investment of varicose SP-IR nerve fibres. Note that all the ganglionic cell bodies are non-SP-IR. ×440.

Fig. 4. Photomicrograph showing some SP-IR nerve fibres forming perivascular networks (arrows) within the walls of the blood vessels. *M*, cardiac muscle. ×310.

Fig. 5. Numerous SP-IR nerve fibres ramifying amongst the cardiac muscle fibres. These SP-IR nerve fibres appear to form a nerve net around the fascicles of muscle fibres. ×310.

Fig. 6. Photomicrograph showing a solitary SP-IR nerve fibre traversing the cardiac muscle. Note the presence of varicosities along the length of the nerve fibre. ×440.

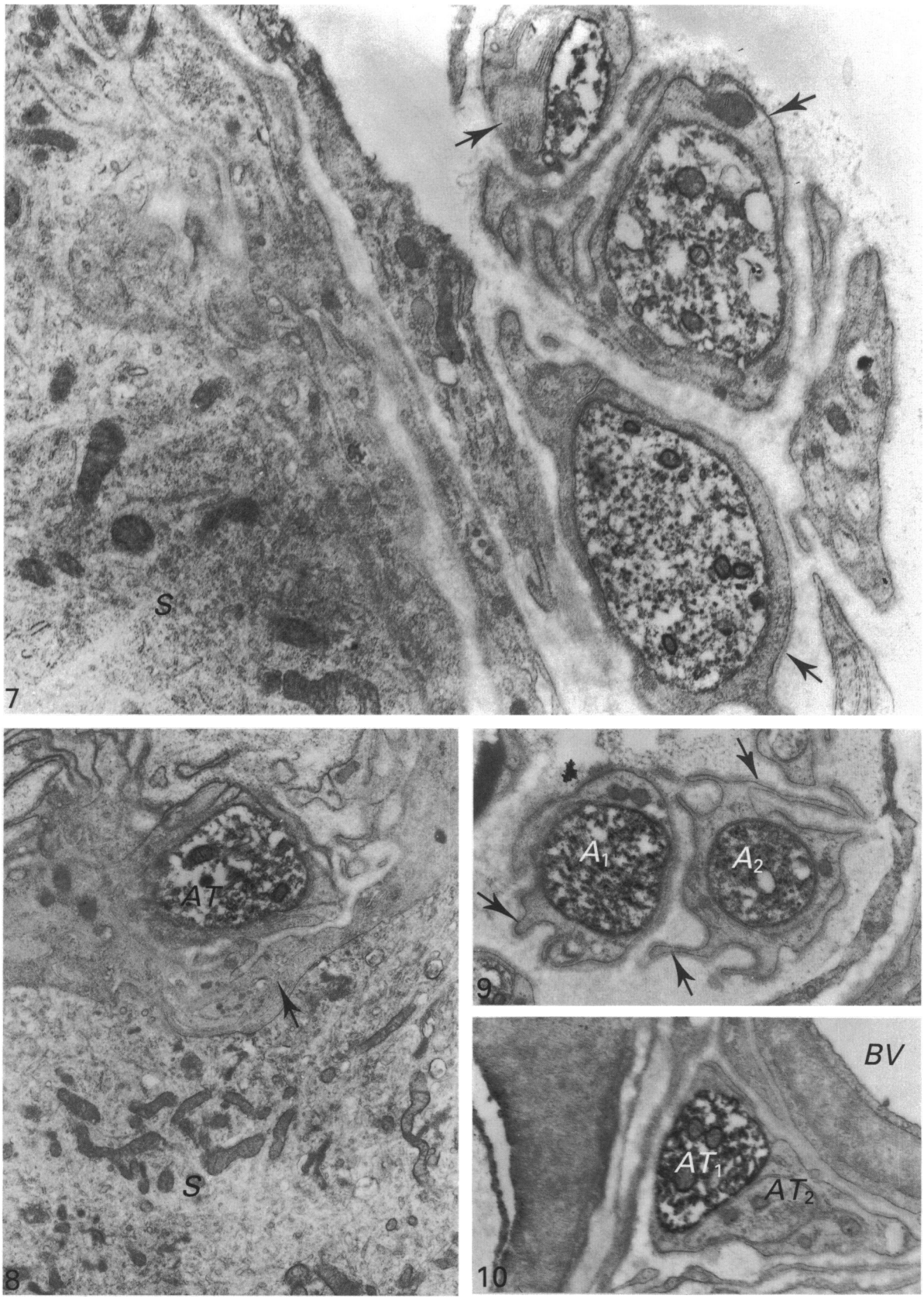


Fig. 7. Electron micrograph showing 3 SP-IR axons within a cardiac ganglion. Note the close proximity of these 3 SP-IR axons to the non-SP-IR nerve cell (*S*). These 3 SP-IR axons are all ensheathed by their own Schwann sheaths (arrows). Labelling of the substance P-reaction product is found on the inner plasma membrane, outer mitochondrial membranes and microtubules. ×21000.

Fig. 8. A solitary SP-IR axon terminal (*AT*) lying adjacent to a non-SP-IR nerve cell (*S*). Note the capsule around the surface of the cell soma (arrow) and the Schwann sheath around the axon terminal. ×13500.

cells (Figs 7, 9, 10, 11). Several of these Schwann cells surrounding the axons exhibited pseudopodia-like projections (Figs 9, 11). In the interstitial spaces, some of the axons and axon terminals were associated with blood vessels (Figs 10, 11). None of these SP-IR axon terminals was observed to make close contact with the endothelial cells of the blood vessels. In many instances, SP-IR axon terminals appeared to be in close contact with non-SP-IR axon terminals (Figs 12, 13). In these SP-IR axon terminals, the immunoreaction product appeared to be closely related to the outer membranes of mitochondria, inner surface of the plasma membrane and membrane of the small agranular vesicles (Fig. 13). So far, SP-IR axon terminals have not been observed to make close contacts with other SP-IR axon terminals in the cardiac ganglia of the monkey.

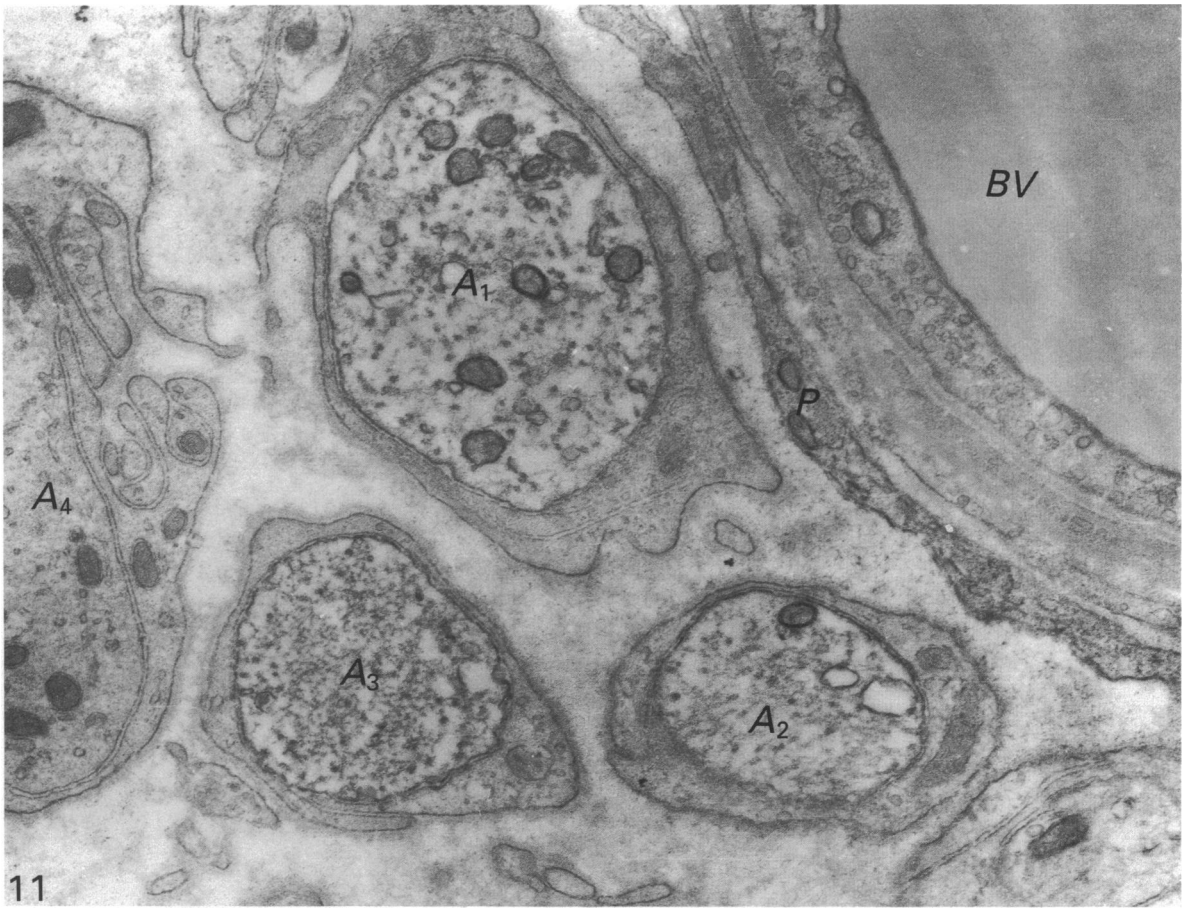
DISCUSSION

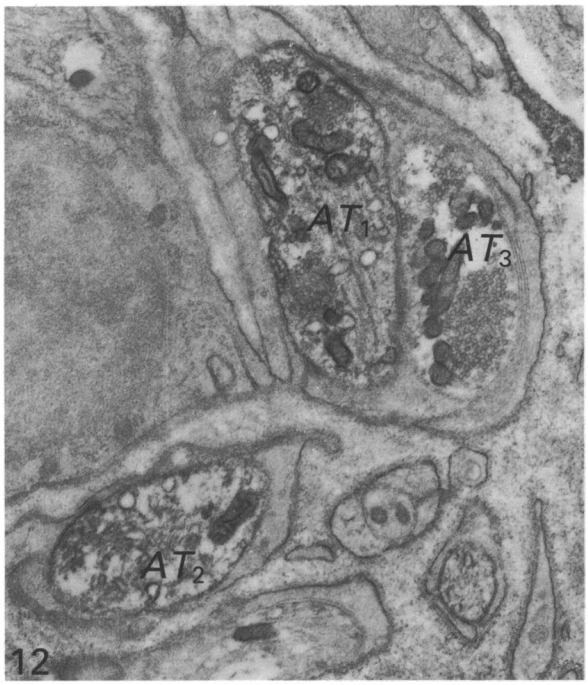
Although it has been well documented that some of the ganglion cells in the mammalian heart display neuropeptide Y- and vasointestinal polypeptide-like immunoreactivities (Weihe & Reinecke, 1981; Hassall & Burnstock, 1984, 1987; Reinecke & Forssmann, 1984; Weihe et al. 1984; Dalsgaard et al. 1986; Forsgren, 1989*b*, *c*), so far most workers have reported that none of the ganglion cells are SP-IR in the mammals examined (Weihe & Reinecke, 1981; Wharton et al. 1981; Hougland & Hoover, 1983; Weihe et al. 1984; Urban & Papka, 1985; Rechardt et al. 1986; Forsgren et al. 1990). Baluk and Gabella (1989) found that a very small population of intrinsic ganglion cell bodies were SP-IR. They further observed that the SP-IR nerve cell bodies were also surrounded by baskets of SP-IR nerve fibres. However, they could not distinguish which SP-IR fibres were of intrinsic and which of extrinsic origin. The results of the present study show that none of the cardiac ganglion cells of the monkey (*Macaca fascicularis*) is SP-IR. Although no SP-IR intrinsic ganglion cell bodies have been encountered in the present study, it cannot be excluded that a small population of SP-IR cell bodies may also be present in the monkey heart. The absence of SP-IR cell bodies in the monkey heart may be attributed to the limited sampling employed in this study. It is interesting to note that Baluk and Gabella (1989) employed colchicine treatment and whole-mount preparations, which possibly account for the localisation of SP-IR intrinsic ganglion cell bodies in the guinea-pig heart.

In the cardiac ganglia, numerous varicose and nonvaricose SP-IR nerve fibres are present, some of them being closely associated with the ganglion cells. Similar results have been reported by other workers in the mammalian heart (Reinecke & Forssmann, 1984; Dalsgaard et al. 1986; Papka & Urban, 1987; Forsgren, 1989*c*). The close relationships of the SP-IR nerve fibres to nerve cell bodies in the cardiac ganglia suggest that these nerve fibres may be modulating the ganglionic activities. Rechardt et al. (1986) reported that SP-IR nerves often formed large glomerular-like nerve ending loops between the right auricular muscle cells in the human heart. They further found substance P-like immunofluorescent nerves in the vicinity of blood vessels. Similar findings have been observed in the present study. Based on the close relationship between blood vessels and the SP-IR axons, it is hypothesised that these axons possibly modulate the activities of the blood vessels, and thus the coronary blood flow, including blood vessels to the cardiac ganglia. The exact mechanisms of the modulatory processes are still not known; however, it is possible that the substance P is released into the interstitial microenvironment, thereby influencing the regulation of the contractility of the blood vessels. Because none of the cell bodies in the cardiac ganglia was SP-IR, it is therefore hypothesised that the SP-IR nerves in the cardiac ganglia of the monkey may have been derived from the nodose ganglia as postulated earlier by Lundberg et al. (1978) and Rechardt et al. (1986). Some of these SP-IR nerve fibres could also have their cell bodies in the dorsal root ganglia of the spinal nerves. The possibility that a few of these SP-IR nerve fibres may be of intrinsic origin should not be excluded. In the present study, some of the SP-IR axon terminals approaching the ganglion cells share a common Schwann sheath. At the regions where the SP-IR axon terminals make close contacts with other non-SP-IR axonal profiles, they appear to lose most of the Schwann sheath. It is thus possible that these SP-IR axon terminals also modulate the release of neurotransmitters from other axon terminals. The axoaxonal close contacts resemble the puncta adherentia shown by Peters et al. (1976). Similar close contacts have been observed in the CNS (Sotelo & Palay, 1970) and these appear to be compatible with a sensory innervation of the cardiac ganglia. More-

Fig. 9. Two ensheathed SP-IR axons (A_1, A_2) traversing the interstitial space of an intrinsic cardiac ganglion. Note the pseudopodia-like projections of the Schwann cells (arrows). × 16500.

Fig. 10. A SP-IR axon terminal (AT_1) lies adjacent to a blood vessel (*BV*). Note that the SP-IR axon appears to make axoaxonal close contact with another non-SP-IR axon terminal (AT_2). × 16500.





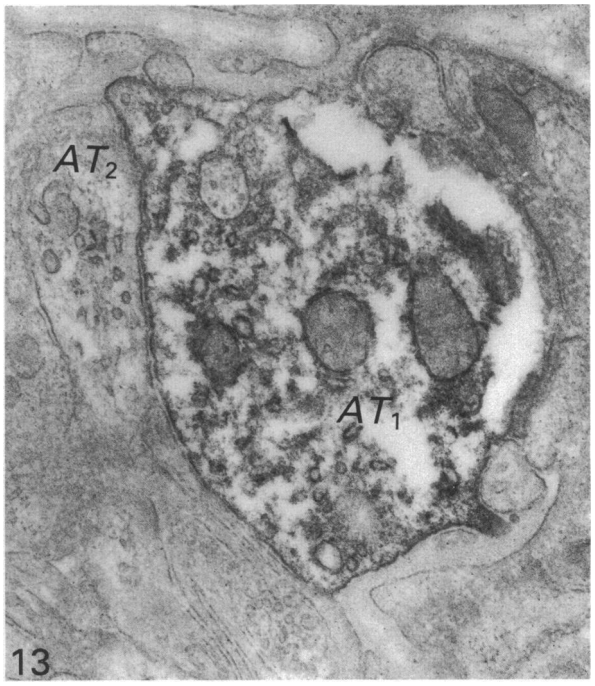


Fig. 11. Three ensheathed SP-IR axons (A_1, A_2, A_3) lie close to a blood vessel (BV). Note the presence of a non-SP-IR axon (A_4) on the left and a fibroblast process (P) intervening between the 3 axons and the blood vessel. ×26250.

Fig. 12. Two labelled SP-IR axon terminals (AT_1, AT_2) lying in the interstitial space of a cardiac ganglion. An axon terminal (AT_1) is in close contact with another non-SP-IR axon terminal (AT_3). ×15000.

Fig. 13. High magnification of an SP-IR axon terminal (AT_1) making close contact with another non-SP-IR axon terminal (AT_2). Note that the contact area resembles a punctum adherens. Observe that most of the substance P-reaction product is localised on the inner plasma membrane, outer mitochondrial membranes and membranes of the small agranular vesicles. ×35000.

over, the large pericellular (basket-like) networks around the ganglion cells of the monkey heart may well represent the large sensory or baroreceptor type of endings. Papka et al. (1981) have also postulated that substance P-containing nerves in the guinea pig heart are possibly sensory in function since the substance P-like immunoreactivity is depleted by capsaicin. In the monkey heart, there appears to be a paucity of neuromuscular contacts involving SP-IR axon terminals. Such contacts may be present, but owing to sampling problems have not been observed in the present study. Further work will be undertaken to determine the origins of these SP-IR nerves to the cardiac ganglia employing sympathectomy and vagotomy.

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REFERENCES

BALUK P, GABELLA G (1989) Some intrinsic neurons of the guinea-pig heart contain substance P. *Neuroscience Letters* **104**, 269–273.

DALSGAARD CJ, FRANCO-CERECEDA A, SARIA A, LUNDBERG JM, THEODORSSON-NORHEIM E, HOKFELT T (1986) Distribution and origin of substance P- and neuropeptide Y-immunoreactive nerves in the guinea-pig heart. *Cell and Tissue Research* **243**, 477–485

FORSGREN S (1989*a*) The distribution of nerve fibers showing substance P-like immunoreactivity in the conduction system of the bovine heart: dense innervation in the atrioventricular bundle. *Anatomy and Embryology* **179**, 485–490.

FORSGREN S (1989*b*) Neuropeptide Y-like immunoreactivity in relation to the distribution of sympathetic fibres in the heart conduction system. *Journal of Molecular and Cellular Cardiology* **21**, 279–290.

FORSGREN S (1989*c*) Vasoactive intestinal polypeptide-like immunoreactivity in the bovine heart: high degree of co-existence with neuropeptide Y-like immunoreactivity. *Cell and Tissue Research* **256**, 125–135.

FORSGREN S, MORAVEC M, MORAVEC J (1990) Catecholamine-synthesizing enzymes and neuropeptides in rat heart epicardial ganglia; an immunohistochemical study. *Histochemical Journal* **22**, 667–676.

HASSALL CJS, BURNSTOCK G (1984) Neuropeptide Y-like immunoreactivity in cultured intrinsic neurones of the heart. *Neuroscience Letters* **52**, 111–115.

HASSALL CJS, BURNSTOCK G (1987) Immunocytochemical localization of neuropeptide Y and 5-hydroxytryptamine in a subpopulation of amine-handling intracardiac neurones that do not contain dopamine β-hydroxylase in tissue culture. *Brain Research* **422**, 74–82.

HOUGLAND MW, HOOVER DB (1983) Detection of substance P-like immunoreactivity in nerve fibres in the heart of the guinea-pigs but not rats. *Journal of the Autonomic Nervous System* **8**, 295–301.

LUNDBERG JM, HÖKFELT T, NILSSON G, TERENIUS L, REHFELD J, ELDE R, SAID S (1978) Peptide neurons in the vagus, splanchnic and sciatic nerves. *Acta Physiologica Scandinavica* **104**, 499–501.

PAPKA RE, FURNESS JB, DELLA NG, COSTA M (1981) Depletion by capsaicin of substance P-immunoreactivity and acetylcholinesterase activity from nerve fibres in the guinea-pig heart. *Neuroscience Letters* **27**, 47–53.

PAPKA RE, URBAN L (1987) Distribution, origin and sensitivity to capsaicin of primary afferent substance P-immunoreactive nerves in the heart. *Acta Physiologica Hungarica* **69**, 459–468.

PETERS A, PALAY SL, WEBSTER H DE F (1976) *The Fine Structure of the Nervous System: The Neurons and Supporting Cells*. Philadelphia: W. B. Saunders.

RECHARDT L, AALTO-SETALA K, PURJERANTA M, PELTO-HUIKKO M, KYOSOLA K (1986) Peptidergic innervation of human atrial myocardium: an electron microscopical and immunocytochemical study. *Journal of the Autonomic Nervous System* **17**, 21–32.

REINECKE M, FORSSMANN WG (1984) Regulatory peptides (SP, NT, VIP, PHI, ENK) of autonomic nerves in the guinea-pig heart. *Clinical and Experimental Hypertension* **6**, 1867–1871.

REINECKE M, WEIHE E, CARRAWAY RE, LEEMAN SE, FORSSMANN WG (1982) Localization of neurotensin immunoreactive nerve fibres in the guinea-pig heart: evidence derived by immunohistochemistry, radioimmunoassay and chromatography. *Neuroscience* **7**, 1785–1795.

REINECKE M, WEIHE E, FORSSMANN WG (1980) Substance P-immunoreactive nerve fibres in the heart. *Neuroscience Letters* **20**, 265–269.

SOTELO C, PALAY SL (1970) The fine structure of the lateral vestibular nucleus in the rat. II. Synaptic organization. *Brain Research* **18**, 93–115.

TAY SSW, WILLIAMS TH, JEW JY (1989) Neurotensin immunoreactivity in the central nucleus of the rat amygdala: an ultrastructural approach. *Peptides* **10**, 113–120.

TAY SSW, WONG WC (1991) Substance P-like immunoreactivity in the ganglia and muscle of the heart of the monkey (*Macaca fascicularis*). *Anatomical Record* **229**, 88A.

URBAN L, PAPKA RE (1985) Origin of small primary afferent substance P-immunoreactive nerve fibers in the guinea-pig heart. *Journal of the Autonomic Nervous System* **12**, 321–331.

WEIHE E, REINECKE M (1981) Peptidergic innervation of the mammalian sinus node: vasoactive intestinal polypeptide, neurotensin, substance P. *Neuroscience Letters* **26**, 283–288.

WEIHE E, REINECKE M, FORSSMANN WG (1984) Distribution of vasoactive intestinal polypeptide-like immunoreactivity in the mammalian heart. Interrelation with neurotensin and substance P-like immunoreactive nerves. *Cell and Tissue Research* **236**, 527–540.

WEIHE E, REINECKE M, OPHERK D, FORSSMANN WG (1981) Peptidergic innervation (substance P) in the human heart. *Journal of Molecular and Cellular Cardiology* **13**, 331–333.

WHARTON J, GULBENKIAN S, MERIGHI A, KUHN DM, JAHN R, TAYLOR KM, POLAK JM (1988) Immunohistochemical and ultrastructural localization of peptide-containing nerves and myocardial cells in the human atrial appendage. *Cell and Tissue Research* **254**, 155–166.

WHARTON J, POLAK JM, GORDON L, BANNER NR, SPRINGALL DR, ROSE M, KHAGANI A, WALLWORK J, YACOUB MH (1990) Immunohistochemical demonstration of human cardiac innervation before and after transplantation. *Circulation Research* **66**, 900–912.

WHARTON J, POLAK JM, MCGREGOR GP, BISHOP AE, BLOOM SR (1981) The distribution of substance P-like immunoreactive nerves in the guinea-pig heart. *Neuroscience* **6**, 2193–2204.