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REGULAR ARTICLE

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Immunohistochemical analysis of intracardiac ganglia of the rat heart

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Abstract The neurochemistry of intracardiac neurons in whole-mount preparations of the intrinsic ganglia was investigated. This technique allowed the study of the morphology of the ganglionated nerve plexus found within the atria as well as of individual neurons. Intracardiac ganglia formed a ring-like plexus around the entry of the pulmonary veins and were interconnected by a series of fine nerve fibres. All intracardiac neurons contained immunoreactivity to PGP-9.5, choline acetyl transferase (ChAT) and neuropeptide Y (NPY). Two smaller subpopulations were immunoreactive to calbindin or nitric oxide synthase. Furthermore, a subpopulation (approximately 6%) of PGP-9.5/ChAT/NPY-immunoreactive cells lacking both calbindin and nitric oxide synthase (NOS) was surrounded by pericellular baskets immunoreactive to ChAT and calbindin. Vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activated peptide (PACAP), substance P and tyrosine hydroxylase (TH) immunoreactivity was observed in nerve fibres within the ganglion, but never in neuronal somata. Furthermore, immunoreactivity for NPY was not observed in pericellular baskets surrounding intracardiac neurons, despite being present in all intrinsic neuronal cell bodies. Taken together, the results of this study indicate a moderate level of chemical diversity within the intracardiac neurons of the rat. Such chemical diversity may reflect functional specialisation of neurons in the intracardiac ganglia.

Keywords Autonomic nervous system · Calcium-binding proteins · Chemical coding · Neuropeptides · ChAT · Rat (Sprague Dawley)

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Introduction

The parasympathetic postganglionic neurons found within the intracardiac ganglia have long been believed to faithfully relay activity from vagal preganglionic neurons in the brainstem. There is, however, a growing body of evidence suggesting that intracardiac ganglia, and the neurons within them, are arranged in a manner that might allow a degree of local, autonomous integration of neuronal activity (for review, see Randall et al. 1995). In the guinea pig, combined electrophysiological and cell-filling studies have identified different classes of neurons distinguishable by their electrophysiological characteristics, the synaptic inputs they receive and their morphology (Edwards et al. 1995). Reports of neuronal diversity have been made in other species, including pig (Smith 1999), dog (Xi et al. 1991a, 1991b) and rat (Selyanko 1992; Selyanko and Skok 1992). From these studies it has been concluded that intracardiac ganglia contain a range of different functional types of neurons, including interneurons (Edwards et al. 1995) and intrinsic sensory neurons (Gagliardi et al. 1988; Ardell et al. 1991; Edwards et al. 1995), which may form local reflex circuits.

Additionally, intracardiac neurons are believed to receive synaptic inputs from sympathetic and extrinsic sensory neurons (Saito et al. 1986; Slavikova et al. 1993, 2003; Onuoha et al. 1999; Calupca et al. 2000, 2002) in addition to vagal preganglionic axon inputs. Pharmacological studies suggest that transmitters such as noradrenaline, and peptides including substance P, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activated peptide (PACAP) and calcitonin-gene-related peptide (CGRP), alter the firing properties of intracardiac neurons, indicating that sensory and sympathetic neurons may also modify ganglionic transmission (Xu and Adams 1993; Cuevas and Adams 2000; Liu et al. 2000; Zhang et al. 2001).

A number of studies, particularly in the guinea pig, have looked at the chemical coding of intracardiac neurons in order to identify different functional subpop-

ulations (Steele et al. 1994, 1996a, 1996b). The term 'chemical coding' describes the finding that functionally unique subpopulations of neurons within autonomic ganglia share the same combinations of neuroactive substances. In other words, neurons that are functionally homologous form discrete subpopulations identifiable not only by their projection to different target tissue but also on the basis of their neurochemical content (Lundberg et al. 1982; Furness et al. 1989, 1992; Hökfelt 1991; Benarroch 1994; Grkovic and Anderson 1995, 1997).

In the rat, a number of studies have looked for the presence of different neuropeptides within the cardiac ganglia (Forsgren et al. 1990; Moravec et al. 1990; Klimaschewski et al. 1992; Sosunov et al. 1996, 1997; Onuoha et al. 1999). This is the first study to have investigated specific combinations of neuroactive substances in intracardiac neurons in the rat. In this study, we have identified three distinct neurochemical classes of intracardiac neurons. A fourth subpopulation of intracardiac neurons receiving chemically unique inputs was also identified. These distinct neurochemical classes may represent different functional groups of neurons.

Materials and methods

Animals

Sprague-Dawley rats of both sexes (21–28 days old) were used in the following studies. Animals were housed in a temperature-controlled room with a 12-h light:12-h dark cycle. Rats were fed ad libitum on a standard laboratory diet and water was freely available. The Animal Experimentation Ethics Committee of the University of Melbourne approved all experiments.

Preparation of tissue

For immunohistochemical studies, animals were anaesthetised with sodium pentobarbitone (Nembutal, 60 mg/kg i.p.) and when unresponsive to noxious stimuli, the chest was opened and the heart rapidly excised and placed in cold Krebs solution (concentration in mM: NaCl, 120; KCl, 5.9; CaCl, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; glucose, 8.0). Flat atrial preparations were prepared in cold Krebs solution in the following manner. The ventricles were removed by cutting along the atrioventricular groove and the aorta and pulmonary trunk were gently detached. The remaining tissue included the right and left atria, the left precaval vein, short lengths of the pulmonary veins and superior and inferior vena cava. The auricle of the right atrium was opened with a fine pair of scissors. The left auricle was removed near its attachment to the venous part of the atria. The superior and inferior vena cava, and left precaval vein were cut longitudinally and opened out. The preparation was stretched flat and pinned to a block of Sylgard and immersion fixed overnight in Zamboni's fixative (2% formaldehyde, 15% saturated picric acid in 0.1 M phosphate buffer; concentration in mM: Na_2PO_4, 7.5; $NaH_2PO_4 \cdot 2H_2O$, 2.5) at 4°C. Following fixation, specimens were washed in 0.01 M phosphate-buffered saline (PBS; concentration in mM: NaCl, 145; Na_2PO_4, 7.5; $NaH_2PO_4 \cdot 2H_2O$, 2.5) and divided into regions shown in Fig. 1. For whole-mount preparations, the epicardial connective tissue, which contains the cardiac ganglia, was peeled away from the myocardium and then processed immunohistochemically (see below). For sectioning, each region of atrium was cryoprotected in 20% sucrose in distilled water for a minimum period of 24 h at 4°C. Tissues were embedded in OCT tissue compound (Tissue Tek, Tokyo, Japan) and frozen in isopentane cooled with liquid nitrogen. Sections 10–14 μm thick were cut with a cryostat and were collected on gelatinised microscope slides.

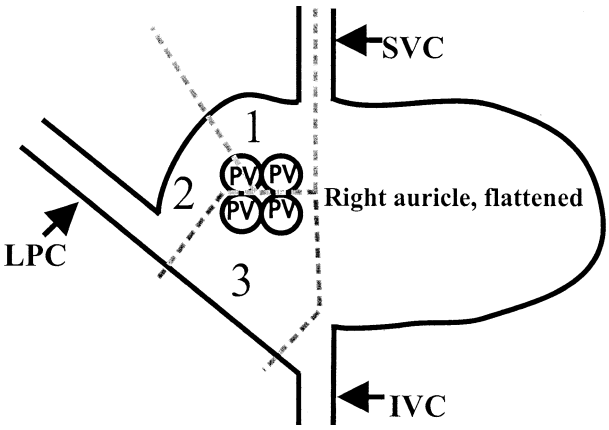


Fig. 1 Diagram of the flat atrial preparation used in this study. Cuts were made (*dashed lines*) to divide the atrial preparation into three regions (labelled as *1, 2, 3*). (*SVC* superior vena cava, *IVC* inferior vena cava, *LPCV* left pre-caval vein, *PV* pulmonary vein)

Immunohistochemistry on whole-mount preparations

All epicardial preparations were stored in 0.01 M PBS prior to immunohistochemical processing. Tissue was permeabilised in 0.5% Triton X-100 in PBS for 30 min and then for a minimum of 1 h in 10% normal horse serum in PBS at room temperature in a humid box to block non-specific antibody binding. Preparations were washed in 3×5 min changes in PBS and then incubated in species-specific double or triple combinations of primary antisera (see Table 1) for 48 h in a humid box at room temperature on a shaker table. Following incubation in primary antibodies, whole-mount preparations were washed 3 times for 30 min in 0.01 M PBS, and then incubated in the appropriate combinations of secondary antisera (see Table 1) for 24 h at room temperature on the shaker table. All antibodies were diluted in PBS containing 0.3% Triton X-100 and 0.1% sodium azide. The diluent for primary antibodies contained 5% normal horse serum to block non-specific antibody binding.

After incubation in secondary antibodies, tissues were again washed in PBS and rinsed in 50% glycerol buffered with $NaHCO_3$ (pH 8.6) for 1 h. Whole-mounts were then arranged on slides, mounted in buffered glycerol and coverslipped.

Immunohistochemistry for sectioned tissue

Sections were air dried and immersed in 4% formaldehyde in 0.1 M phosphate buffer for 5 min, then washed in 3×5 min changes of 0.01 M PBS. Slides containing the sections were then rinsed in 0.5% Triton X-100 in 0.01 M PBS for 30 min and washed again in 0.01 M PBS (3×5 min). Sections were then incubated in species-specific double or triple combinations of primary antibodies for 24 h, washed and then incubated in the appropriate combinations of secondary antibodies for 2 h in a humid box at room temperature before washing in 0.01 M PBS. When required, streptavidin conjugates (see Table 1) were applied to sections after secondary antibody step, and incubated in a humid box for 90 min, before being rinsed in three changes of 0.01 M PBS. Sections were mounted in Dako fluorescence mounting medium (Dako Co., Carpinteria, CA) and coverslipped.

A number of control experiments were performed where the primary antibody was omitted from the diluent mixture, and

Table 1 Primary and secondary antisera used in this study

Antiserum	Lot	Species	Dilution	Source and characterisation
Primary				
Calbindin D28K	5.5	Rabbit	1:500	SWant Swiss Antibodies, Bellinzona, Switzerland
Calretinin	7696	Rabbit	1:3,000	SWant Swiss Antibodies, Bellinzona, Switzerland
Calretinin	20080804	Mouse	1:100	Chemicon International, Inc., Temecula, CA, USA
Calcitonin gene-related peptide	C1087	Rabbit	1:5,000	Genosys Biotechnol., Inc., Cambridge, England
Calcitonin gene-related peptide	B970925	Goat[a]	1:1,000	Biogenesis, Bournemouth, England
Choline acetyltransferase	AB144P	Goat[a]	1:50	Chemicon International, Inc., Temecula, CA, USA
Leu-enkephalin	550214	Rabbit	1:200	Incstar Corporation, Stillwater, MN, USA
Met-enkephalin-Arg-Gly-Leu	961400	Rabbit	1:500	Peninsula Laboratories, Inc., Belmont, CA, USA
Met-enkephalin	20060183	Mouse	1:100	Chemicon International, Inc., Temecula, CA, USA
Neuropeptide Y	-	Sheep	1:500	Gift from Drs. W. Blessing and J. Willoughby, Flinders University, Adelaide, Australia
Neuropeptide Y	550212	Rabbit	1:2,000	Incstar Corporation, Stillwater, MN, USA
Nitric oxide synthase	-	Sheep	1:5,000	Gift from Dr. P. Emson, University of Cambridge
PACAP	970737	Rabbit	1:500	Peninsula Laboratories, Inc., Belmont, CA, USA
Protein gene product 9.5 (PGP-9.5)	Lot 81475	Rabbit	1:2,000	Ultraclone Ltd., Isle of Wight, UK
Substance P	-	Rabbit	1:1,000	Gift from R. Murphy, Ludwig Institute for Cancer Research, Melbourne, Australia
Synaptophysin	087	Mouse	1:200	Dako Corporation, Carpenteria, CA, USA
Tyrosine hydroxylase	21030329	Rabbit	1:200	Eugene Tech International, Inc., Ridgefield Park, NJ, USA
Tyrosine hydroxylase	531220	Mouse	1:4,000	Incstar, Stillwater, MN, USA
Vasoactive intestinal peptide	910204C	Mouse	1:500	Biogenesis, Bournemouth, England
Vesicular acetylcholine transporter	19050693	Goat[a]	1:500	Chemicon International, Inc., Temecula, CA, USA
Secondary				
Mouse IgG, biotin-conjugated	F0718	Horse	1:100	Vector Laboratories, Inc., Burlingame, CA, USA
Mouse IgG, Texas red-conjugated	37997	Donkey	1:150	Jackson Immunoresearch, Westgrove, PA, USA
Rabbit IgG, Texas red-conjugated	47054	Donkey	1:150	Jackson Immunoresearch, Westgrove, PA, USA
Sheep IgG, FITC-conjugated	42784	Donkey	1:200	Jackson Immunoresearch, Westgrove, PA, USA
Streptavidin conjugates				
Streptavidin-Cy5	-	-	1:1,000	Amersham Pharmacia Biotech, Castle Hill, NSW, Australia
Streptavidin-fluorescein	-	-	1:50	Amersham Pharmacia Biotech, Castle Hill, NSW, Australia
Streptavidin-Texas red	-	-	1:50	Amersham Pharmacia Biotech, Castle Hill, NSW, Australia

secondary antibodies were applied as normal. This resulted in no staining of neuronal somata or nerve fibres. In one series of controls, where the primary antibody was omitted and the secondary step included donkey anti-mouse biotin, and the tertiary step with a streptavidin conjugate, there was staining of all SIF cells, but staining of principal neuronal somata or nerve fibres was not observed.

Preparation of brainstem, nodose and dorsal root ganglia

Brainstems and left and right nodose and dorsal root ganglia (T1–T6) were obtained from three animals that had been anaesthetised as described above and perfused through the ascending aorta via the left ventricle with 0.9% saline containing heparin (1,000 IU/l) followed by Zamboni's fixative. Tissues were removed and postfixed for 2 h in the same fixative at 4°C and were washed (3×10 min) and stored in 0.01 M PBS. Brainstems, nodose and dorsal root ganglia were cryoprotected, sectioned and processed immunohistochemically following the same protocol for sectioned intracardiac ganglia.

Microscopy

All whole-mounts and sections were examined using a Zeiss Axioskop fluorescence microscope fitted with filters for separate visualisation of Fast Blue/AMCA, Texas Red and FITC. For fluorescence microscopy, images were taken using an ImagePoint cooled charged coupled device camera (Photometrics, Tucson, AZ) and V for Windows imaging software (Digital Optics, Auckland, New Zealand). Digital manipulation was kept to a minimum; images were sharpened and brightness and contrast were adjusted if required. A Biorad 1024 confocal microscope fitted to a Zeiss Axioskop was utilised for analysis of triple immunolabelling and whole-mount specimens. All colocalisation studies were also carried out on the confocal microscope. All images taken in the confocal microscope were analysed using Confocal Assistant (version 4.02, Todd Clarke Brelje). All plates were prepared using CorelDraw10 software (version 10.427, Corel Co., Dublin, Ireland 2000).

Results

Topographical anatomy of intracardiac ganglia in the rat

The topography of the intrinsic ganglia and neuronal connectives was visualised in a single whole-mount preparation of subepicardial connective tissue surrounding the entry of the pulmonary veins stained for choline acetyl transferase (ChAT) immunoreactivity. Great care was taken to ensure that the correct orientation of the preparation was maintained during the dissection and immunohistochemical processing. Examination of this preparation (Fig. 2A, B, D) showed three or four large ganglia, each consisting of up to several hundred neurons, interconnected by a continuous plexus of fine nerve trunks which formed a ring that completely encircled the

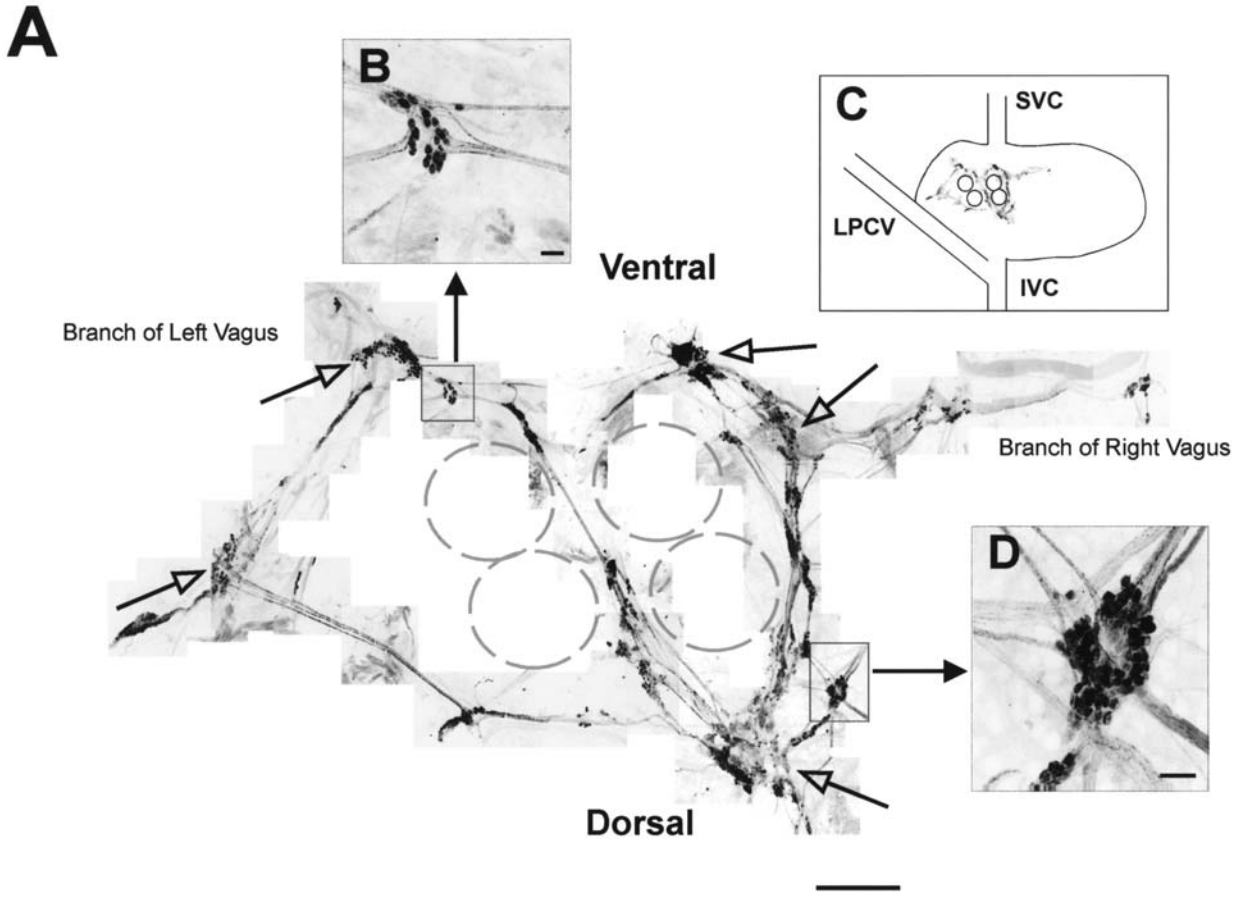


Fig. 2 Inverted fluorescence confocal photomontage of the plexus of intracardiac ganglia **(A)**. The atrial epicardium was peeled in one piece from the region of atria depicted in the schema **(C)**, dissected and stained as a whole-mount preparation for ChAT immunoreactivity. Individual large (*open arrows*) and small ganglia (see **B** and **D** *insets*) were interconnected by a series of fine nerve trunks, and formed a ring-like plexus around the entry of the pulmonary veins (*dashed circles in* **A**). Note that all images are single optical sections and not z-series stacks; thus total neuronal numbers are underrepresented in these images. *Scale bars* 1 mm **(A)**, 100 μm **(B, D)**

entry of the pulmonary veins. Small, satellite ganglia containing around 50 or fewer neurons were found throughout this network (Fig. 2B, D). Both neurons and bundles of axons in the interconnecting nerves were easily visualised with ChAT immunohistochemistry.

For most studies, for practical reasons, this atrial preparation was divided into three regions of tissue as determined by pre-specified anatomical landmarks (Fig. 1). The three regions generated contained at least one, and sometimes two, large ganglia. The manner in which these three pieces of tissue were generated enabled comparisons to be made between three distinct sets of ganglia defined by their location. The use of whole-mounts enabled us to investigate the morphology of individual neurons. Sectioned tissue was used for quantitative studies, as the thickness of the ganglion made it difficult to quantify the numbers of neurons present.

Neurochemical coding of intracardiac neuronal somata

Throughout the study, tests were carried out to determine whether neurochemical subpopulations were more common in any particular region of the flat atrial preparation. No differences were observed in relation to the neurochemical types of neurons observed and their frequency in any one region. Hence, the following descriptions represent the entire population of intracardiac neurons studied. Table 2 summarises the results of the following analysis.

Choline acetyltransferase and neuropeptide Y immunoreactivity was present in all intracardiac principal neuronal somata

The general neuronal marker PGP-9.5 was used to identify all intracardiac neurons. ChAT immunoreactivity was seen in all principal neuronal somata (as indicated by PGP-9.5 immunoreactivity) in the intrinsic cardiac gan-

Table 2 Neurochemical subpopulations of intracardiac neurons in the rat

Neurochemical subpopulation	% (number of profiles, number of animals)	SEM
ChAT/NPY	82.8%[a]	NA
ChAT/NPY, with calbindin input	6.6% (135/2,046, 5)	11.7
ChAT/NPY/Calb	6.6% (180/2,720, 6)	6.6
ChAT/NPY/NOS	4%[b]	-

[a] Calculated from proportion of neurons remaining that are not immunoreactive to NOS or calbindin
[b] Klimaschewski (1992)

glia, indicating that these neurons use acetylcholine as their primary neurotransmitter (Fig. 3A, A'). Immunoreactivity appeared as an even cytoplasmic stain, with the nucleus left unstained. There was considerable variability in the intensity of the stain in individual neurons. Clusters of small cells (5–10 μm) within the ganglia were not stained for ChAT and it was later established that these were TH-immunoreactive presumably small intensely fluorescent (SIF) cells.

Neuropeptide Y (NPY) immunoreactivity was seen in all principal neuronal somata in the intrinsic cardiac ganglia (Fig. 3B, B'). Immunoreactivity appeared as granular, perinuclear labelling. The co-localisation of ChAT and NPY was 100%; hence, all intracardiac neurons in the rat express NPY. This was confirmed using two different antibodies raised against NPY. A small number of SIF cells also showed immunoreactivity to NPY.

Calbindin-D28K immunoreactivity is present in principal neuronal somata and is colocalised with ChAT and NPY immunoreactivity

Immunoreactivity for calbindin-D28K (calbindin) was observed in a subpopulation of neuronal somata, and in many pre-terminal and terminal axons in the ganglia.

In the intrinsic somata, double-label immunohistochemistry showed that calbindin immunoreactivity, which appeared as a diffuse, even cytoplasmic stain that often included the nucleus, was always colocalised with ChAT or NPY immunoreactivity (Fig. 3C, C', E, E'). In whole-mount preparations, calbindin neurons had diverse morphologies, with both unipolar and multipolar somata present within the intrinsic ganglia. It was difficult to analyse the morphology of calbindin-immunoreactive neurons due the abundance of calbindin-immunoreactive preterminal nerve fibres in the ganglion.

Sections of cardiac ganglia were used to quantify this novel population of cardiac neurons. Every fourth section was analysed (every 40–60 μm) by counting profiles of cell bodies immunoreactive for calbindin as a proportion of all cell body profiles in the section, as indicated by ChAT immunoreactivity. Calbindin immunoreactivity accounted for 6.6% of all neuronal profiles counted (SEM=6.6, n=2,720 nucleated cell profiles from six animals). There were no detectable differences in the numbers of calbindin-immunoreactive neuronal profiles in ganglia located in different regions of the ganglionated plexus.

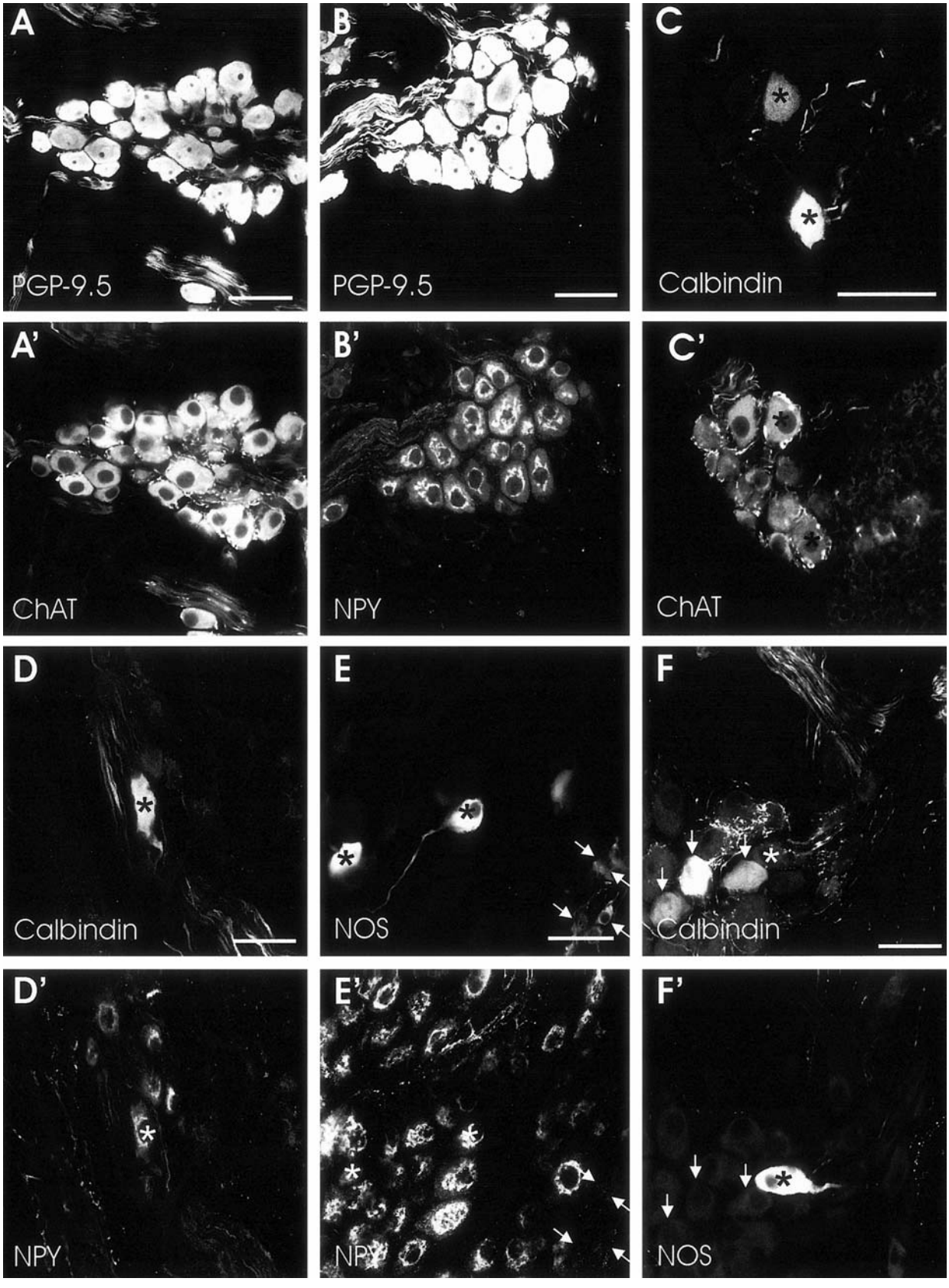
Nitric oxide synthase immunoreactivity is present in principal neuronal somata and is colocalised with ChAT and NPY immunoreactivity

Immunoreactivity for nitric oxide synthase (NOS) was observed in neuronal somata, dendrites and preterminal axons coursing through nerve trunks. NOS immunoreactivity was always colocalised with ChAT and NPY immunoreactivity in neuronal somata, but never in calbindin-immunoreactive neurons (Fig. 3E, E', F, F'). NOS immunoreactivity was not observed in specific pericellular baskets surrounding intracardiac neurons. Previous studies have demonstrated that NOS-immunoreactive neurons account for 4% of intracardiac neurons in the rat (Klimaschewski et al. 1992), so no attempt was made to quantify the size of the subpopulation in this study. NOS immunoreactivity was observed in the cytoplasm and in neural processes at great distances from the cell body in whole-mount preparations. Thus it was possible to analyse NOS-immunoreactive cell bodies morphologically using immunohistochemistry and confocal microscopy alone.

NOS-immunoreactive neurons exhibited great morphological diversity consisting of unipolar neurons with smooth somata, or with small spines in their somata or multipolar neurons with short or long processes (Fig. 4A–E). Unipolar neurons generally projected their axons into interganglionic nerve bundles and, while it was possible to follow their axons for some distance, it was not possible to identify the terminal endings of these axons, as there were often a number of immunoreactive fibres in the same nerve twisting around each other. The processes of the multipolar neurons on the other hand were generally confined to the same ganglion in which the immunoreactive cell body was found, and axons from these neurons were rarely observed entering nerve trunks. The processes of multipolar NOS-immunoreactive neurons did not make obvious specialised terminal endings on any structure within the ganglion.

Other neuropeptides examined in this study were not expressed in the intracardiac neuronal somata

Through the process of screening different antibodies it was noted that there were no neuronal somata immunoreactive to PACAP, VIP, substance P, CGRP, leu-enkephalin, met-enkephalin or met-enkephalin-arg-glu-leu. These observations were made in a minimum of three animals.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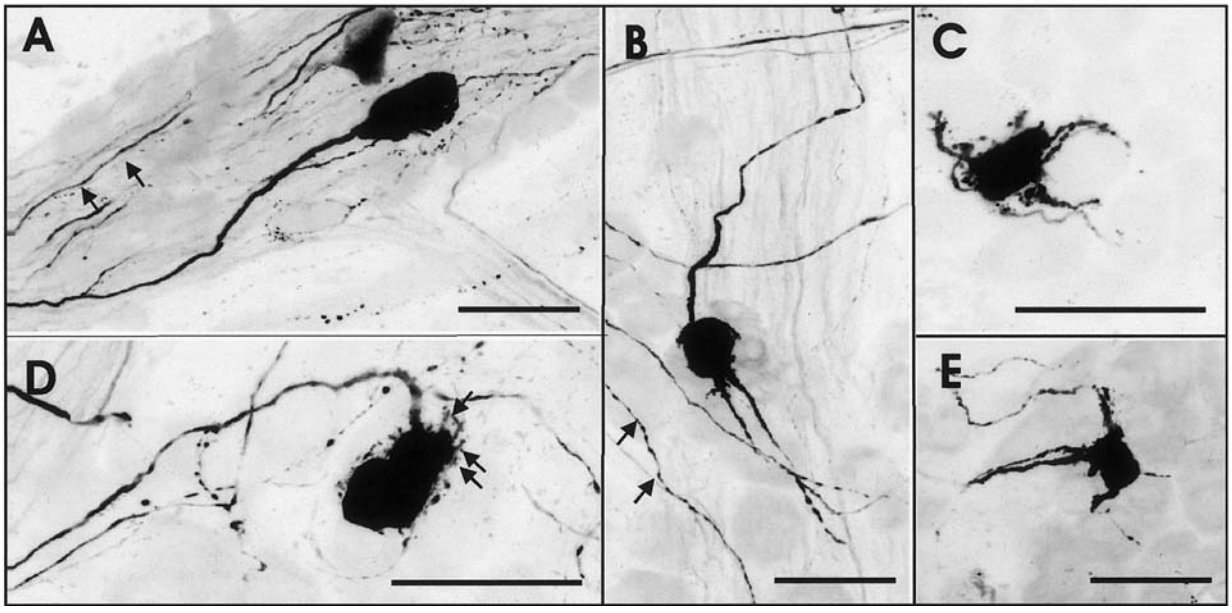


Fig. 4 Inverted fluorescence projected z-series of NOS-immunoreactive intracardiac neurons **(A–E)** depicting the morphological diversity of this subpopulation. Unipolar neurons had either smooth cell bodies **(A)** or short 'spiky' processes (**D**, *arrows*) on their somata. Multipolar neurons had very long processes **(B)**, very short processes **(C)** or a combination of both **(E)**. Note also the presence of NOS-immunoreactive nerve fibres passing thorough the ganglia that do not make pericellular baskets (**A, B,** *arrows*). *Scale bars* 50 μm

Neurochemical coding of nerve fibres and terminals in the intracardiac ganglia

Synaptophysin immunoreactivity was used to identify nerve terminals

Synaptophysin immunoreactivity was observed in boutons surrounding many intracardiac neurons (Figs. 5A', 6B'). There were some examples of intracardiac neuronal somata that were not surrounded by synaptophysin-immunoreactive terminals. The somata of SIF cells were also immunoreactive for synaptophysin. The intensity of staining in SIF cells made it impossible to visualise any synaptophysin-immunoreactive terminals surrounding these cells.

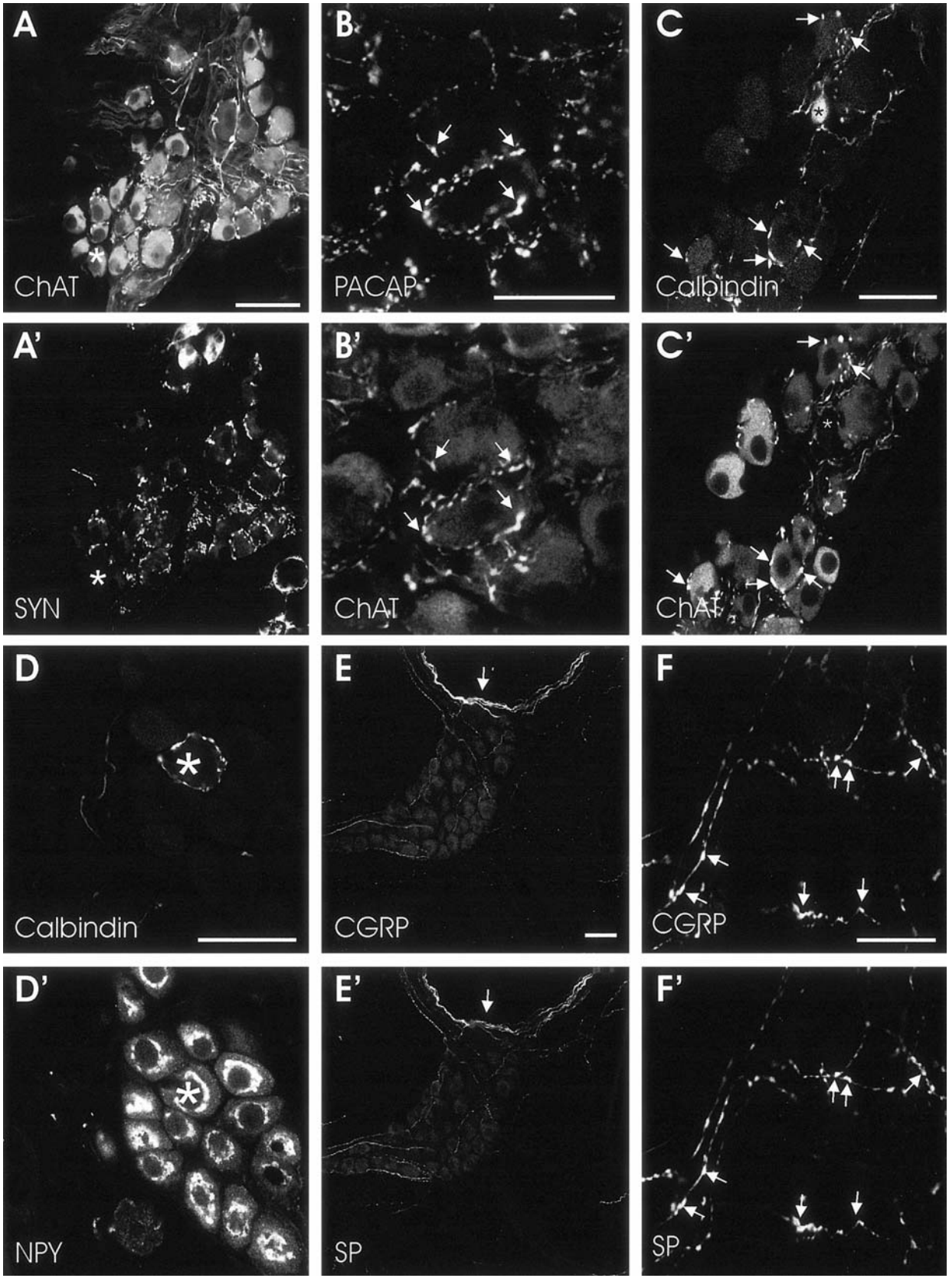
ChAT and PACAP immunoreactivity is colocalised in boutons surrounding most intracardiac neurons

ChAT immunoreactivity was also observed in pericellular boutons surrounding the cell bodies of most principal neurons and was generally more intense than that seen in the cell body of the neurons (Fig. 5A, A'). ChAT immunoreactivity was also weakly expressed in bundles of axons within the interganglionic connections (Fig. 2A, B, D). Synaptophysin immunoreactivity was colocalised with ChAT in many, but not all, boutons. Some ChAT-immunoreactive boutons lacked synaptophysin immunoreactivity and vice versa. PACAP immunoreactivity was also colocalised with ChAT immunoreactivity. Almost all ChAT-immunoreactive boutons appeared to be also immunoreactive to PACAP (Fig. 5B, B').

Calbindin immunoreactivity is expressed in terminals forming pericellular baskets surrounding a subpopulation of intracardiac neurons

Pericellular baskets of calbindin-immunoreactive terminals were observed surrounding 6.6% of principal neuron cell bodies (SEM=11.7, n=2,046 nucleated cell profiles from five animals, Fig. 5C, C', D, D'). Calbindin-immunoreactive terminals in pericellular baskets were always immunoreactive for ChAT also and did not surround many principal neurons that contained calbindin or NOS immunoreactivity. Calbindin-immunoreactive baskets were only observed around principal neurons that were immunoreactive to calbindin in two cases or NOS in

Fig. 3A–F Paired confocal micrographs stained with different combinations of primary antibodies and visualised with Texas Red and FITC-conjugated secondary antibodies. All intracardiac neurons are immunoreactive to the general neuronal marker PGP-9.5 **(A, B)**. Additionally, all intracardiac neurons are immunoreactive to choline acetyl transferase **(A, A')** and neuropeptide Y **(B, B')**. Calbindin-immunoreactive somata were always immunoreactive to ChAT (**C, C',** *asterisk*) and NPY (**D, D',** *asterisk*). NOS immunoreactivity was present in a subpopulation of neurons that coexpressed ChAT (not shown) and NPY immunoreactivity **(E, E')**. Calbindin (*arrows*) and NOS (*asterisk*) immunoreactivity was never coexpressed in neuronal somata or in pericellular baskets surrounding principal neuronal somata **(F, F')**. *Scale bars* 50 μm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Calbindin
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CGRP
D'
NPY
E'
SP
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SP

one case. Thicker, non-varicose calbindin-immunoreactive terminals were observed surrounding some clusters of SIF cells, which were never NOS- or calbindin-immunoreactive.

In whole-mount preparations, there were numerous calbindin-immunoreactive axons in the interganglionic nerves. Numerous calbindin-immunoreactive fibres traversed the ganglion in large bundles close to the surface of the ganglion, possibly within the connective tissue surrounding the ganglion.

Absence of NPY immunoreactivity in pericellular baskets surrounding intracardiac neurons

NPY immunoreactivity was not observed in nerve fibres forming pericellular baskets around any neuronal somata in the ganglion. NPY-immunoreactive terminals were present in the atrial myocardium and in the walls of blood vessels. In whole-mount preparations, NPY-immunoreactive axons were observed in non-varicose preterminal axons in nerves connecting the ganglia (Figs. 3B', D', E', 5D').

Substance P and CGRP immunoreactivity in nerve terminals in the intracardiac ganglia

Substance P and CGRP immunoreactivity was observed in numerous nerve fibres but never in the somata of neurons or SIF cells. Double-labelling studies showed that substance P and CGRP were always colocalised in the same nerve fibres within the ganglia (Fig. 5E, E', F, F'). Within each ganglion, substance P/CGRP-immunoreactive fibres were varicose and appeared to pass closely to numerous intracardiac neurons, but did not appear to make specialised nerve endings in the ganglion or make specific pericellular baskets around intracardiac neurons.

Fig. 5 Paired confocal micrographs stained with different combinations of primary antibodies and visualised with Texas Red and FITC-conjugated secondary antibodies show the coexpression of neuroactive substances in nerve terminals within the intracardiac ganglia. Nerve terminals that were immunoreactive to ChAT **(A)** also contain synaptophysin immunoreactivity **(A')**, surrounded many intracardiac neurons. There were intracardiac neurons present that were not surrounded by synaptophysin-immunoreactive boutons (**A, B,** *asterisk*). PACAP (**B,** *arrows*) and ChAT immunoreactivity (**B',** *arrows*) was colocalised in pericellular baskets surrounding many intracardiac neurons. Calbindin immunoreactivity was also observed in pericellular baskets (**C,** *arrows,* **D,** *asterisk*) that surrounded a subpopulation of intracardiac neurons that were immunoreactive to ChAT **(C, C')** and NPY immunoreactivity (**D, D',** *asterisk*). Calbindin-immunoreactive baskets contained ChAT immunoreactivity (**C',** *arrows*) but lacked NPY **(D')**. Note the SIF cell (**C, C',** *asterisk*), indicated by its size and lack of ChAT immunoreactivity, which also contains calbindin immunoreactivity. Colocalised immunoreactivity for CGRP (**E, F,** *arrows*) and substance P (**E', F',** *arrows*) was present in numerous nerve terminals in the cardiac ganglia. These terminals did not form specific pericellular baskets around any intracardiac neurons. *Scale bars* 30 μm **(B, F)**, 50 μm (all others)

In whole-mount specimens, a dense network of varicose substance P/CGRP-immunoreactive fibres appeared to be present on the surface of the ganglion, possibly within the connective tissue surrounding the ganglion (Fig. 5E, E').

Tyrosine hydroxylase immunoreactivity is present in SIF cells and nerve terminals in the intracardiac ganglia

Tyrosine hydroxylase (TH) immunoreactivity was observed in a population of small cells (5–10 μm diameter) that formed tight clusters near the outer surface of most ganglia. On the basis of their intense TH immunoreactivity and small size, they were identified as SIF cells (Heym et al. 1994). Principal neurons were never immunoreactive for TH (Fig. 6A, A').

TH immunoreactivity was also observed in numerous terminal and pre-terminal axons throughout the atrial myocardium, the walls of blood vessels and in large nerve trunks. In the ganglia, varicose TH-immunoreactive terminals were observed (Fig. 6A, A') and appeared to contact some intracardiac neuronal somata. These terminals, however, did not form distinct pericellular baskets around any intracardiac neurons. Only rarely did TH-immunoreactive varicosities contain colocalised synaptophysin immunoreactivity (Fig. 6B, B'). In contrast, nerve fibres in the myocardium and blood vessel walls always contained colocalised TH and synaptophysin immunoreactivity (Fig. 6C, C').

Neurochemistry of neurons in the brainstem, nodose and dorsal root ganglia

In the rat, preganglionic parasympathetic neurons that innervate the heart have been described previously as occupying the dorsal motor nucleus of vagus, nucleus ambiguus and the intermediate zone between these nuclei in the medulla (Cheng et al. 1999; Cheng and Powley 2000). With the aid of a rat brain atlas, these nuclei were identified in the present study as clusters of neurons immunoreactive for ChAT, confirming their cholinergic nature. Further analysis for the presence or absence of neuropeptides was carried out in order to determine whether some of the terminals observed within the intracardiac ganglia could be derived from preganglionic neurons.

Calbindin and ChAT immunoreactivity are not colocalised in neurons of the brainstem, nodose or dorsal root ganglia

Colocalised calbindin and ChAT immunoreactivity, which was localised in discrete pericellular baskets surrounding a small population of intracardiac neurons, was never observed in any neurons within the vagal preganglionic nuclei. Calbindin immunoreactivity was

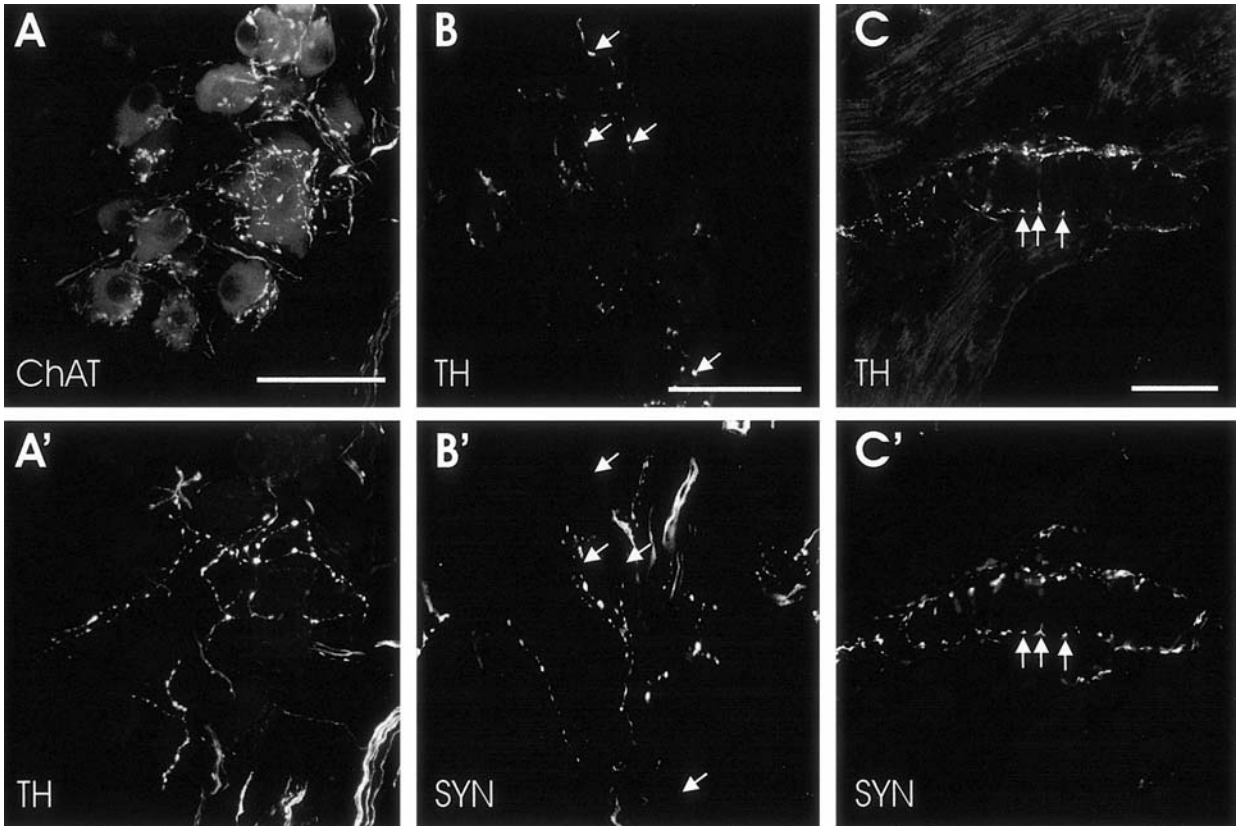

Fig. 6 Paired confocal micrographs stained with different combinations of primary antibodies and visualised with Texas Red and FITC-conjugated secondary antibodies show the coexpression of neuroactive substances in nerve terminals within the intracardiac ganglia. TH-immunoreactive terminals within intrinsic ganglia **(A', B)** were not ChAT-immunoreactive **(A)**. No intracardiac neurons were immunoreactive to TH (**A, A',** z-series stacks). Note that these terminals did not make specific baskets around any intracardiac neurons **(A')**. In the ganglia, nerve terminals immunoreactive to TH rarely contained colocalised synaptophysin immunoreactivity (**B, B',** *arrows*). In contrast TH-immunoreactive terminals surrounding blood vessels **(C, C')** and in the myocardium (not shown) often contained colocalised synaptophysin immunoreactivity (*arrows*). *Scale bars* 50 μm

only observed in a population of small cells within the dorsal motor nucleus that lacked ChAT immunoreactivity. These calbindin-immunoreactive cells were round and much smaller than the neurons expressing ChAT immunoreactivity.

In the nodose and dorsal root ganglia (T1–T6), many calbindin-immunoreactive somata were present. ChAT, however, was not expressed in any of the calbindin-immunoreactive somata in the dorsal root and nodose ganglia.

Discussion

Location and organisation of cardiac ganglia

The intracardiac ganglia are located on the base of the heart around the entry of major blood vessels. Previous reports have described the location of intracardiac ganglia in both sectioned (Pardini et al. 1989) and whole-mount (Cheng et al. 1997, 1999) rat heart preparations. In the present study, it was observed that the nerve plexus surrounding the pulmonary veins was made up of three or four large ganglia as well as a number of smaller ganglia, all interconnected by a plexus of nerve trunks. While the locations of the ganglia correspond to those described in a previous study (Cheng et al. 1999), this study demonstrates that the ganglia are part of a single plexus rather than being discrete entities. Our finding that, at the neurochemical level, the ganglia contain the same types of neurons supports this. Therefore, at the anatomical level, it appears that rat intracardiac ganglia are part of a single plexus and interganglionic communication may be permitted through the network of nerve fibres linking the ganglia.

Rat intracardiac ganglia are quite large, generally comprising up to several hundred neurons. Previous studies in the rat have estimated the total number of intracardiac neurons above the atrioventricular groove at 4,000, organised into four major groups (Pardini et al. 1987). These observations contrast greatly with findings in other small mammals such as guinea pigs, which have an estimated 1,500 neurons, the majority of which are organised into ganglia containing fewer than 20 cells (Leger et al. 1999). Thus even within small mammals, there is a great deal of diversity in the gross anatomy of

the ganglia and indeed the plexus of which they are part of.

Identification of neurochemically distinct subpopulations of intracardiac neurons

In the present study, three chemically distinct classes of intrinsic cardiac neurons were described. All principal neurons in the cardiac ganglion are immunoreactive to ChAT, suggesting that they release acetylcholine, and all neurons contained immunoreactivity to NPY. Two small populations of neurons were identified by their immunoreactivity to NOS or calbindin in addition to ChAT and NPY. Thus the three neurochemical classes of principal neurons that are described in this study are ChAT/NPY, ChAT/NPY/calbindin and ChAT/NPY/NOS.

A striking feature of rat intrinsic cardiac neurons is the lack of neuropeptide diversity when compared to the guinea pig (Steele et al. 1994; Horackova et al. 1999). There were no neuronal somata immunoreactive to VIP, substance P, CGRP, leu-enkephalin, met-enkephalin or met-enkephalin-arg-glu-leu, results that are consistent with previous immunohistochemical studies in rats (Forsgren et al. 1990; Moravec et al. 1990). Furthermore, we could not find any somata of principal neurons that were immunoreactive to TH, which is also consistent with other studies in the rat (Moravec et al. 1990; Slavikova et al. 2003).

This is the first study to report intrinsic cardiac neurons with calbindin immunoreactivity in the heart of any species. Calbindin immunoreactivity was present in approximately 7% of neuronal profiles, in pericellular baskets and in numerous pre-terminal fibres in large nerve trunks and in the conrective tissue surrounding the individual cardiac ganglia. Previous studies in other autonomic ganglia have shown that calbindin immunoreactivity identifies functional subpopulations of neurons (Kuramoto et al. 1990; Messenger and Furness 1991; Mann et al. 1995; Grkovic and Anderson 1997). Therefore, the intracardiac neuron somata that express calbindin immunoreactivity identified in this study could represent a functionally homologous subpopulation of neurons. As yet, the target of the calbindin-immunoreactive neurons has not been identified.

NOS-immunoreactive neurons exhibit diverse morphology

NOS-immunoreactive neurons account for 4% of the total intracardiac neuron population in the rat (Klimaschewski et al. 1992), which is consistent with our observations. This study showed that NOS immunoreactivity is always colocalised with ChAT and NPY immunoreactivity in cell bodies, but never with calbindin.

The morphological differences observed in the NOS-immunoreactive neurons may further divide this neurochemical subpopulation into different morphological types. Previously, it has been demonstrated that rat intracardiac neurons are diverse morphologically, with unipolar and multipolar neurons being described (Pauza et al. 1997). In the present study, it was noted that some NOS-immunoreactive neurons projected axons into nerve trunks, while others had their processes contained within the same ganglion. On this anatomical evidence alone, the two types of NOS-immunoreactive neurons may serve different physiological functions. NOS-immunoreactive fibres were observed winding through the cardiac ganglia and in connective tissue surrounding the ganglion. There were, however, no specific pericellular baskets made by NOS-immunoreactive fibres around any principal neuron somata. These findings indicate that intrinsic neurons that are NOS-immunoreactive do not form obvious axon-somatic contacts with other intracardiac neurons. We cannot, however, rule out the possibility of axodendritic and axon-axonal contacts being made.

Neurochemistry of nerve terminals in the intracardiac ganglia

PACAP/ChAT-immunoreactive terminals in the intracardiac ganglia

In this study, it was found that PACAP immunoreactivity was colocalised with ChAT immunoreactivity in terminals surrounding intracardiac neuron somata, but was never observed in the cell body of intracardiac neurons. Given the density of the ChAT/PACAP terminals within the intracardiac ganglia, it is likely that these terminals are those of preganglionic parasympathetic neurons. In the brainstem, PACAP immunoreactivity was not observed in the neurons of the dorsal motor nucleus of the vagus or in the nucleus ambiguus. PACAP-immunoreactive neurons were observed in the dorsal root ganglia and very rarely in the nodose ganglia. However, these somata always lacked ChAT immunoreactivity. It is possible that PACAP, a neuropeptide, is not found in high concentration within the cell body, and therefore cannot be detected with immunohistochemistry. Therefore, it is most likely that PACAP is present in preganglionic parasympathetic terminals. The receptors for PACAP have also been identified in the neonatal rat (DeHaven and Cuevas 2002) and the guinea pig (Braas et al. 1998) where PACAP immunoreactivity is also present in nerve fibres and a subpopulation of intrinsic neurons (Braas et al. 1998). Like VIP, PACAP has been demonstrated to change the firing properties of intracardiac neurons (Braas et al. 1998). Therefore, it is likely that PACAP released from vagal preganglionic terminals would modify cholinergic neurotransmission.

Calbindin-immunoreactive terminals form selective baskets around a subpopulation of ChAT/NPY-immunoreactive neurons in the intracardiac ganglia

A major finding of this investigation was the identification of calbindin in a number of fibres throughout the cardiac ganglia. Given that the density of calbindin-immunoreactive fibres is high, and calbindin-immunoreactive intracardiac neurons only account for approximately 7% of neurons present, it is likely that calbindin-immunoreactive fibres are derived, at least in part, from extrinsic sources. Previous studies from our laboratory (Anderson 1998; Richardson et al., unpublished observations) have identified a subpopulation of sympathetic neurons in the stellate ganglion that contain calbindin-immunoreactive that can be labelled from the heart using retrograde neuronal tracing. However, given that the calbindin-immunoreactive terminals around intrinsic neurons observed in this study were immunoreactive to ChAT and not TH, it is highly unlikely that these baskets are derived from sympathetic postganglionic neurons. Sensory neurons from the rat nodose (Ichikawa and Helke 1996, 1997) and dorsal root (Honda 1995) ganglia have been shown to contain calbindin immunoreactivity. Afferent neurons from the nodose ganglion have been reported to innervate SIF cell clusters and not principal neuron somata (Cheng et al. 1997). Therefore, it is likely that calbindin-immunoreactive fibres surrounding SIF cells are derived from the nodose ganglion. The calbindin-immunoreactive baskets surrounding principal neurons, however, cannot be derived from neurons from the nodose ganglion as it has been shown that axons of afferent neurons in the nodose ganglia (Cheng et al. 1997) do not project to principal ganglion cells. Furthermore, in the present study, ChAT immunoreactivity was not present in cell bodies of neurons in the dorsal root or nodose ganglia; hence terminals that contain ChAT and calbindin immunoreactivity cannot be derived from either source of extrinsic sensory neurons.

Parasympathetic preganglionic neurons in the brainstem lack calbindin immunoreactivity and, therefore, it is concluded that pericellular baskets surrounding intracardiac neurons that contain colocalised calbindin and ChAT immunoreactivity are not derived from preganglionic neurons. For now, a source for these pericellular baskets is unclear and is an avenue for further investigation.

The lack of NPY-immunoreactive terminals in the intracardiac ganglia

The lack of NPY immunoreactivity in varicosities forming pericellular baskets in the ganglion, despite every neuron in the intracardiac ganglia expressing NPY immunoreactivity in their cell body, suggests that, in the rat, intracardiac neurons do not make projections to other intracardiac neurons, and therefore do not form intrinsic nerve circuits contained within the heart. Such a situation would differ from other small species such as the guinea pig where intracardiac neurons that receive only local synaptic input have been described (Edwards et al. 1995).

NPY immunoreactivity is present in all intracardiac neurons but not in terminals within the intracardiac ganglia. Therefore, if intracardiac neurons were to form local circuits NPY must be excluded from their nerve terminals. While neuropeptides are commonly reported to be present in nerve terminals and difficult to detect in the cell body, the reverse situation whereby a neuropeptide is present in the somata and not in terminals has yet to be reported.

Tyrosine hydroxylase-immunoreactive terminals generally lack synaptophysin immunoreactivity

We have confirmed previous observations of TH-immunoreactive terminals in rat intrinsic cardiac ganglia (Forsgren et al. 1990; Moravec et al. 1990). TH-immunoreactive terminals did not appear to form pericellular baskets around principal neurons, and they generally lacked synaptophysin immunoreactivity, in contrast to the terminals present in the myocardium or around blood vessels. Intraganglionic sympathetic terminals provide inputs to intrinsic cardiac neurons in the pig (Smith 1999) and dog (Gagliardi et al. 1988), while in the guinea pig, distinct pericellular baskets of TH-immunoreactive terminals, probably of sympathetic origin, surround some intracardiac neurons (Steele et al. 1994). While isolated rat intracardiac neurons are responsive to noradrenaline (Xu and Adams 1993; Ishibashi et al. 2003), the absence of an intimate relationship between TH-immunoreactive terminals and intracardiac somata, coupled with the absence of synaptophysin, a marker of synaptic vesicles (reviewed in Winkler 1997), suggests that any sympathetic transmission in the rat cardiac ganglia must have some unusual features.

Substance P- and CGRP-immunoreactive nerve fibres

The presence of substance P- and CGRP-immunoreactive fibres in rat cardiac ganglia has been previously reported (Forsgren et al. 1990; Moravec et al. 1990; Onuoha et al. 1999). Double-label immunohistochemistry showed that substance P and CGRP were always colocalised in nerve fibres in the cardiac ganglia. Like the TH-immunoreactive fibres, substance P- and CGRP-immunoreactive fibres were varicose, but never made specific pericellular baskets around intracardiac neurons. Furthermore, whole-mount specimens showed an extensive network of substance P- and CGRP-immunoreactive nerve fibres in the connective tissue surrounding the ganglion. The origin of these fibres is unlikely to be the brainstem (vagal preganglionic) as studies identifying CGRP-immunoreactive neuronal somata in the nucleus ambiguus, along with other brainstem nuclei of the cat and the rat, have demonstrated that these neurons innervate striated muscle in the larynx and pharynx, and the tongue, respectively

(Takami et al. 1985; McWilliam et al. 1989). Given that substance P is not found in vagal nuclei, or in sympathetic postganglionic neurons, it is likely that substance P- and CGRP-immunoreactive axons are derived from sensory neurons from the dorsal root ganglia (Wiesenfeld-Hallin et al. 1984).

Physiological studies have demonstrated the effects on firing properties that tachykinins such as substance P have on intracardiac neurons, suggesting that afferent neurons also play a modulatory role within the cardiac ganglia (Hardwick et al. 1995; Thompson et al. 1998; Zhang et al. 2001). In the rat, experiments in isolated cultured intracardiac neurons demonstrate that substance P can inhibit ACh-induced currents through nicotinic receptors (Cuevas and Adams 2000). The findings of the current study demonstrate an anatomical substrate that may permit such interactions between sensory and intracardiac neurons to occur in rats.

Concluding remarks

In the present study, it was demonstrated that rat intracardiac neurons display a degree of neurochemical heterogeneity, with four distinct subpopulations of intrinsic neurons identified. These neurochemical subpopulations may represent different functional groups of neurons. When compared to the guinea pig, it appears that the rat intracardiac nervous system is much simpler from a neurochemical point of view. This study, however, did not employ colchicine or organ culture methods that have been shown to cause increased expression of many neuropeptides (Ekblad et al. 1996; Lynch et al. 1999). Alternatively, the lack of diversity may be due simply to species differences.

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