Cell Tissue Res (2006) 323: 197–209
DOI 10.1007/s00441-005-0074-3

REGULAR ARTICLE

Rodney L. Parsons · Sarah A. Locknar · Beth A. Young · Jennifer L. Hoard · Donald B. Hoover

Presence and co-localization of vasoactive intestinal polypeptide with neuronal nitric oxide synthase in cells and nerve fibers within guinea pig intrinsic cardiac ganglia and cardiac tissue

Received: 21 June 2005 / Accepted: 2 August 2005 / Published online: 12 October 2005

Abstract The presence of vasoactive intestinal polypeptide (VIP) has been analyzed in fibers and neurons within the guinea pig intrinsic cardiac ganglia and in fibers innervating cardiac tissues. In whole-mount preparations, VIP-immunoreactive (IR) fibers were present in about 70% of the cardiac ganglia. VIP was co-localized with neuronal nitric oxide synthase (nNOS) in fibers innervating the intrinsic ganglia but was not present in fibers immunoreactive for pituitary adenylate cyclase-activating polypeptide, choline acetyltransferase (ChAT), tyrosine hydroxylase, or substance P. A small number of the intrinsic ChAT-IR cardiac ganglia neurons (approximately 3%) exhibited VIP immunoreactivity. These few VIP-IR cardiac neurons also exhibited nNOS immunoreactivity. After explant culture for 72 h, the intraganglionic VIP-IR fibers degenerated, indicating that they were axons of neurons located outside the heart. In cardiac tissue sections, VIP-IR fibers were present primarily in the atria and in perivascular connective tissue, with the overall abundance being low. VIP-IR fibers were notably sparse in the sinus node and conducting system and generally absent in the ventricular myocardium. Virtually all VIP-IR fibers in tissue sections exhibited immunoreactivity to nNOS. A few VIP-IR fibers, primarily those located within the atrial myocardium, were immunoreactive for both nNOS and ChAT indicating they were derived from intrinsic cardiac neurons. We suggest that, in the guinea pig, the majority of intraganglionic and cardiac tissue VIP-IR fibers originate outside of the heart. These extrinsic VIP-IR fibers are also immunoreactive for nNOS and therefore most likely are a component of the afferent fibers derived from the vagal sensory ganglia.

Keywords Neuropeptide · Autonomic neuron · Intrinsic cardiac ganglia · Vagal afferent fibers · Guinea pig (Hartley)

This work was supported by NIH grant HL65481 (R.L.P.) and HL54633 (D.B.H.). Use of the DeltaVision Restoration microscope was provided through the Imaging/Physiology Core supported by NIH Grant P20 RR16435 from the COBRE program of the National Center for Research Resources.

R. L. Parsons (✉) · S. A. Locknar · B. A. Young
Department of Anatomy and Neurobiology,
College of Medicine, University of Vermont,
Burlington, VT 05405, USA
e-mail: Rodney.Parsons@uvm.edu
Tel.: +1-802-6562230
Fax: +1-802-6568704

J. L. Hoard · D. B. Hoover
Department of Pharmacology,
James H. Quillen College of Medicine,
East Tennessee State University,
Johnson City, TN 37614, USA

Introduction

Cardiac ganglia, once considered simple relay stations (Loffelholz and Pappano 1985), are now recognized as potential integrative centers innervated not only by preganglionic parasympathetic fibers, but also by sympathetic postganglionic and spinal and vagal afferent fibers (Randall and Wurster 1994; Ardell 2001; Parsons 2004). Each of these extrinsic fibers contains distinct neurotransmitter/neuromodulator combinations and the "chemical coding" of the various extrinsic inputs may be species-specific. Over the past few years, we have established the chemical coding of extrinsic inputs innervating the intrinsic cardiac ganglia in the guinea pig in order to improve the definition of the histochemical organization of this integrative neural network (Hardwick et al. 1995; Kennedy et al. 1998; Calupca et al. 2000a,b). We have also defined the actions of identified neurotransmitters on guinea pig cardiac neurons (Hardwick et al. 1995; Braas et al. 1998; Kennedy et al. 1998; Hardwick et al. 1997; Merriam et al. 2004).

A few years ago, we determined that the neuropeptide termed pituitary adenylate cyclase-activating polypeptide (PACAP) was present in fibers innervating the guinea pig intrinsic cardiac ganglia (Braas et al. 1998; Calupca et al. 2000a). In the guinea pig, PACAP, which is a member of the vasoactive intestinal polypeptide (VIP), secretin, and

glucagon family of peptides (Arimura 1998; Vaudry et al. 2000), has been extensively co-localized with the acetylcholine (ACh)-synthesizing enzyme, choline acetyltransferase (ChAT), in the parasympathetic preganglionic fibers innervating the intrinsic cardiac neurons (Calupca et al. 2000a). VIP-immunoreactive (IR) fibers and cells have also been identified within the intrinsic cardiac ganglia, and VIP-IR fibers have been shown to be distributed in the hearts of a number of mammals, including the guinea pig (Della et al. 1983; Weihe et al. 1984; Rechardt et al. 1986; Forsgren 1989; Seabrook et al. 1990; Anderson et al. 1993; Steele et al. 1994). However, whether the majority of the VIP-IR fibers represent an intrinsic or extrinsic input to the cardiac neurons and cardiac tissues is not known. In addition, whether the VIP-IR fibers are part of a specific input to cardiac tissues, such as parasympathetic preganglionic fibers, sympathetic postganglionic fibers, or spinal or vagal afferent fibers, has also not been established. In the central nervous system, VIP is rarely co-localized with the closely related peptide PACAP. In contrast, in the peripheral nervous system, these two neuropeptides are often found in the same fibers (Mawe and Ellis 2001; Fahrenkrug and Hannibal 2004). Nevertheless, whether VIP and PACAP are co-localized in fibers innervating the intrinsic cardiac ganglia and heart remains unknown. Consequently, we have undertaken an immunocytochemical analysis to determine (1) whether the majority of VIP-IR fibers within intrinsic cardiac ganglia and cardiac tissues originate from sources outside of the heart or are projections from cardiac neurons, (2) whether VIP is present in a select immunoidentified fiber projection, and (3) whether VIP and PACAP are co-localized in fibers innervating the intrinsic cardiac ganglia.

Materials and methods

General methods

Experiments were performed in vitro on atrial whole-mount preparations containing intrinsic cardiac ganglia or by using cardiac tissue sections from Hartley guinea pigs (mixed sex; 250–350 g). Guinea pigs were killed by halothane or isofluorane overdose followed by exsanguination or by deep anesthesia with sodium pentobarbital (75 mg/kg, i.p.) and removal of the heart. Animal protocols were approved by the Animal Care and Use Committees of the University of Vermont and East Tennessee State University and followed the methods described in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 85–23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

Cardiac ganglia whole-mount preparations

The heart was quickly removed and placed in cold standard Krebs solution (121 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 8 mM glucose). The pH was maintained at 7.4 by aeration with 95% O_2, 5% CO_2. The guinea pig possesses two areas of atrial tissue particularly rich in cardiac ganglia: an area around the superior vena cava and an area surrounding the coronary sinus, interatrial septum, and small cardiac vein (Mawe et al. 1996; Kennedy et al. 1998; Horackova et al. 1999; Leger et al. 1999; Calupca et al. 2000a,b). In the present study of the VIP innervation of the intrinsic cardiac ganglia, an atrial whole-mount preparation including the region around the superior vena cava, the coronary sinus, and small cardiac vein was prepared. The atrial muscle layers were dissected away to isolate clusters of cardiac neurons; the ventricular muscle, except for a small piece used to pin out the whole-mount preparation, was also removed.

For explant studies, the cardiac ganglia preparations were dissected under sterile conditions and were maintained at 37°C in culture media consisting of DMEM-F12 (1:1) containing 10% horse serum, gentamicin (10 μg/ml), amphotericin B (3.75 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml; Sigma, St. Louis, Mo., USA; Lynch et al. 1999). The preparations were placed on a slowly rotating shaker table in a 5% CO_2 incubator maintained at 37°C. The preparations were maintained for 72 h with the culture media being changed every 24 h.

Tissue sections

For these experiments, animals were pretreated with heparin (1,000 U/kg, i.p.) at least 30 min before anesthesia. Hearts were removed and perfused via the ascending aorta with 10 ml 0.01 M phosphate-buffered saline (PBS) containing heparin (10 U/ml), followed by 10 ml cold 4% paraformaldahyde in PBS containing 0.2% picric acid. Hearts were postfixed for 2 h at 4°C, transferred to 20% sucrose in PBS for 2 days at 4°C, and frozen on specimen plates by using TBS tissue freezing medium (Triangle Biomedical Sciences, Durham, N.C.) and powdered dry ice. Frozen tissue was stored at −80°C in 50 ml plastic tubes until sectioned. Short-axis sections of four hearts were cut at a thickness of 30 μm in a microtome cryostat at −20°C, beginning at the most superior aspect of the atria and ending in the ventricular myocardium at the superior aspect of the right and left bundle branches. Sections of heart were collected on slides coated with chrome alum and gelatin, dried, and stored at −20°C prior to staining.

Immunohistochemistry

Cardiac ganglia whole-mount preparations Freshly dissected and explant-cultured preparations were fixed in 2% paraformaldehyde containing 0.2% picric acid for 2–5 h at 4°C. The preparations were labeled with antisera directed against specific neurotransmitters or transmitter synthetic enzymes (Table 1) following procedures described previously (Mawe et al. 1996; Braas et al. 1998; Kennedy et al. 1998; Lynch et al. 1999; Calupca et al. 2000a,b; Braas et al. 2004). The fixed whole-mount preparations were washed in

Table 1 Primary antisera utilized for immunohistochemistry (*VIP* vasoactive intestinal polypeptide, *nNOS* neuronal nitric oxide synthase, *ChAT* choline acetyltransferase, *PACAP* pituitary adenylate cyclase-activating polypeptide, *TH* tyrosine hydroxylase, *SP* substance P)

Antiserum	Species	Dilution	Supplier
VIP	Rabbit	1:100	CURE Research Center, UCLA, Los Angeles, Calif.
nNOS	Mouse	1:500	Sigma-Aldrich Corp., St. Louis, Mo. (Clone A-11)
nNOS	Sheep	1:400	Chemicon International, Temecula, Calif.
ChAT	Goat	1:100	Chemicon International
PACAP	Mouse	1:10	Jan Fahrenkrug, Copenhagen, Denmark (clone JH6F10)
TH	Mouse	1:400	Research Biochemical International, Natick, Mass. (clone TH-16)
SP	Rat	1:250	Fitzgerald Industries, Concord, Mass.

PBS, permeabilized with 0.5% Triton X-100, blocked with 4% horse serum, and incubated at 4°C overnight with combinations of antisera. The preparations were washed and incubated for 2 h at room temperature with appropriate secondary antisera conjugated to fluorescein isothiocyanate (FITC), indocarbocyanine (Cy3), or aminomethylcoumarin acetate (AMCA) (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA). Each cardiac ganglia preparation was washed, mounted with Citifluor (UKA Chemical Laboratory, Canterbury, England), and viewed with an Olympus AX70 fluorescence photomicroscope with filter sets appropriate for FITC, Cy3, and AMCA. The specificity of all the antisera has been characterized extensively (Calupca et al. 2000a,b; Mawe and Ellis 2001).

Slide-mounted sections Slide-mounted tissue sections were immunostained at room temperature as described previously (Hoover et al. 2004). Briefly, sections were washed in PBS, permeabilized with 0.4% Triton X-100 in PBS containing 0.5% bovine serum albumin (BSA), and blocked for 2 h in PBS containing 10% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, Pa.), 1% BSA, and 0.4% Triton X-100. Tissues were then triple-labeled for VIP, nNOS, and ChAT by incubation for 15–18 h with the same primary antisera as those used for the whole-mounts (Table 1). Tissues were then washed with PBS and incubated for 2 h with three species-specific donkey secondary antibodies conjugated to Alexa Fluor 488, 555, or 647 (Molecular Probes, Eugene, Ore.). Coverglasses were attached with Citifluor mounting medium and sealed with clear nail polish. Tissues were viewed and photographed by using an Olympus BX41 microscope equipped with an Optronics MagnaFire SP charge-coupled device camera.

Microscopic analysis of whole-mounts and tissue sections

Deconvolution microscopy of intrinsic cardiac ganglia The immunolabeled whole-mount preparations were imaged with a DeltaVision Restoration microscopy system (Applied Precision, Issaquah, Wash.). The system consisted of an Olympus IX-70 inverted microscope with a precision motorized stage and epifluorescence illumination. A 60× oil-immersion lens (NA=1.40) was used for all co-localization studies, and data were acquired as a z-series with a step-size of 0.2 μm. The following bandpass filters were used: AMCA, excitation 340–380 nm, emission 432–482 nm; FITC, excitation 480–500 nm, emission 509–547 nm; Cy3, excitation 541–569 nm, emission 580–654 nm. Post-processing of the z-stacks consisted of 15 iterations of deconvolution provided in the softWoRx package (Applied Precision). Projections were generated by using subsets of the z-stack, converted into TIFF images, and transferred into Adobe Photoshop (ver. 6.0) for minimal enhancement of brightness and contrast.

Confocal analysis of tissue sections Images were collected by using a Leica TCS SP2 laser scanning confocal microscope system. Specimens were scanned sequentially for each fluorophore to obtain separate images for each label and an overlay image of all three channels for each optical section. Maximum intensity projection images were obtained by using all optical sections in each series. Negative controls for each fluorochrome were also scanned by using the same parameter settings. Images were exported into Corel Graphics 11 and adjusted for brightness and contrast.

Statistical evaluation

Quantitative data are expressed as mean±SEM. Student's *t*-test was used to evaluate differences among groups, and differences were considered statistically significant if $P<0.05$.

Results

Whole-mount intrinsic cardiac ganglia preparations

VIP-IR fibers innervate the majority of the intrinsic cardiac ganglia and are present on atrial tissues

The pattern of VIP-IR fiber distribution was determined in 14 atrial whole-mount preparations containing the intrinsic cardiac ganglia. The number of discrete ganglia in each preparation ranged between 19 and 92 (42±5 ganglia/preparation). On average, VIP-IR fibers were present in 69±3% (range from 62% to 100%) of the intrinsic cardiac ganglia in these whole-mount preparations. The density of VIP-IR fibers present in individual ganglia varied widely. In some ganglia, only one or a few fibers were present, and these fibers formed pericellular arrangements around a limited number of cardiac neurons. In other ganglia, the VIP-IR

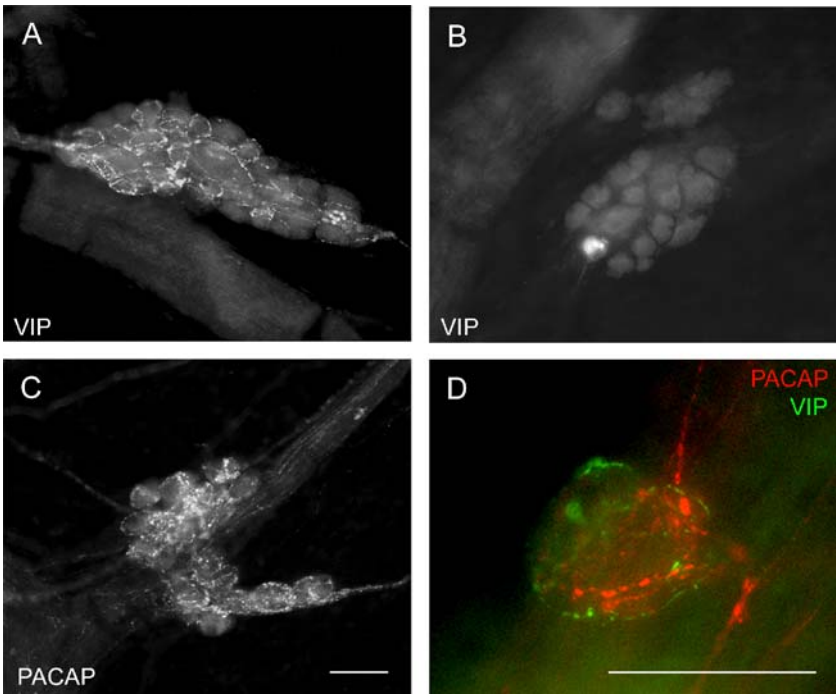

Fig. 1 Vasoactive intestinal polypeptide (VIP)-immunoreactive (-IR) fibers and pituitary adenylate cyclase-activating polypeptide (PACAP-IR) fibers form complexes within intrinsic cardiac ganglia. **a**, **c** VIP-IR and PACAP-IR fiber networks within different cardiac ganglia. **b** A ganglia with one VIP-IR neuron, but no VIP-IR fibers. **d** Double-labeled preparation showing that VIP and PACAP are present in separate fibers innervating a cardiac neuron. *Bars* 50 μm

fibers formed a complex network of fibers that surrounded many of the neurons within the ganglion (Fig. 1a).

VIP-IR fibers innervating cardiac tissues have been suggested to originate from the cells in the intrinsic cardiac ganglia (Weihe et al. 1984; Henning and Sawmiller 2001). However, we have found few VIP-IR cells in freshly fixed whole-mount preparations of guinea pig intrinsic cardiac ganglia (Fig. 1b). All guinea pig cardiac neurons exhibit immunoreactivity for ChAT (Mawe et al. 1996). Therefore, the presence of VIP-IR cells relative to the total number of cardiac neurons was determined from the ratio: [number of VIP-IR cells/number of ChAT-IR cells]×100 (Lynch et al. 1999; Braas et al. 2004). In five preparations double-labeled with antiserum directed against ChAT and antiserum against VIP, the percentage of cardiac neurons immunoreactive for VIP was 2.7±1.5%. Furthermore, the presence of VIP-IR fibers was not correlated with the presence of VIP-IR cells. Some ganglia contained both VIP-IR cells and fibers, whereas other ganglia exhibited VIP-IR cells with no evidence of an intraganglionic VIP-IR fiber network (Fig. 1b). Furthermore, in most cases, VIP-IR fibers were evident in ganglia that did not contain VIP-IR cells (Fig. 1a).

VIP-IR fibers were also evident outside the cardiac ganglia within the small interganglionic connectives running between clusters of cardiac neurons. Commonly, VIP immunoreactivity was more robust within the ganglia and decreased progressively with distance from the ganglion in the interganglionic bundles. In some whole-mount preparations, VIP-IR fibers were also evident in large nerve bundles and coursing across the epicardial tissue. Occasionally, VIP-IR fibers appeared to be closely associated with blood vessels. The extraganglionic VIP-IR fibers were most evident in preparations in which dissection had been less extensive, and in which more cardiac tissue layers remained. The distribution of the extraganglionic VIP-IR fibers was examined thoroughly in tissue sections (see below).

VIP-IR fibers innervating the intrinsic cardiac ganglia originate from sources outside the heart

Previously, we and other investigators have reported that nerve fibers that originate outside of the heart degenerate when whole-mount preparations of cardiac ganglia are maintained in explant culture for a few days (Steele et al.

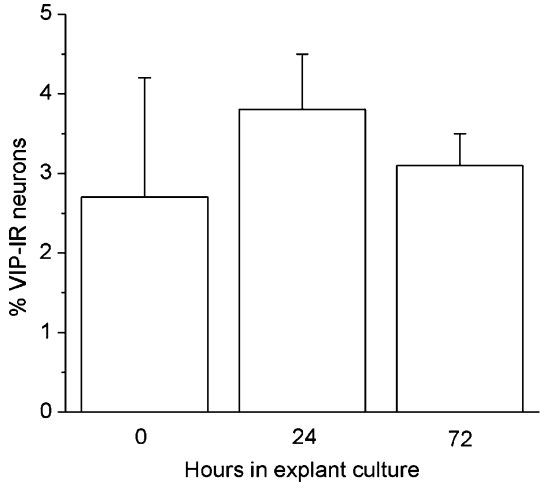

Fig. 2 The presence of VIP-IR neurons does not increase in explant-cultured cardiac ganglia whole-mount preparations. The percentage of VIP-IR neurons was determined from the ratio: [number of VIP-IR neurons/number of ChAT-IR neurons]×100 determined in freshly dissected and explant-cultured cardiac ganglia whole-mount preparations

1994; Calupca et al. 2000a,b). To test whether the VIP-IR fibers in the cardiac ganglia originated outside the heart, whole-mount cardiac ganglia preparations were placed in explant culture for 72 h and were then immunostained for the presence of VIP-IR fibers. After 72 h in explant culture, no intrinsic cardiac ganglia were innervated by VIP-IR fiber networks, even though a few VIP-IR neurons were still present. In many cases when individual neurons were present in a ganglion, the processes extending from these neurons could be followed for some distance. These intrinsic VIP-IR fibers did not form pericellular complexes around neighboring cells; rather, they left the ganglion and entered nearby interganglionic fiber bundles (not shown).

Previously, peptide expression by guinea pig cardiac neurons has often been shown to increase following explant culture (Parsons 2004). Therefore, we determined whether the number of cells expressing VIP increased in explant-cultured whole-mount preparations. We found that the percentage of VIP-IR cells did not significantly increase for up to 72 h in explant culture (Fig. 2).

VIP and PACAP are not co-localized in freshly dissected cardiac ganglia whole-mount preparations

Calupca et al. (2000a) demonstrated that, in the guinea pig, PACAP-IR fibers innervated every cardiac ganglion. We confirmed that all intrinsic cardiac ganglia were innervated by PACAP-IR fibers, and baskets of PACAP-IR fibers surrounded virtually every neuron within each ganglion (Fig. 1c). In additional experiments, we tested whether VIP and PACAP were co-localized in fibers surrounding the

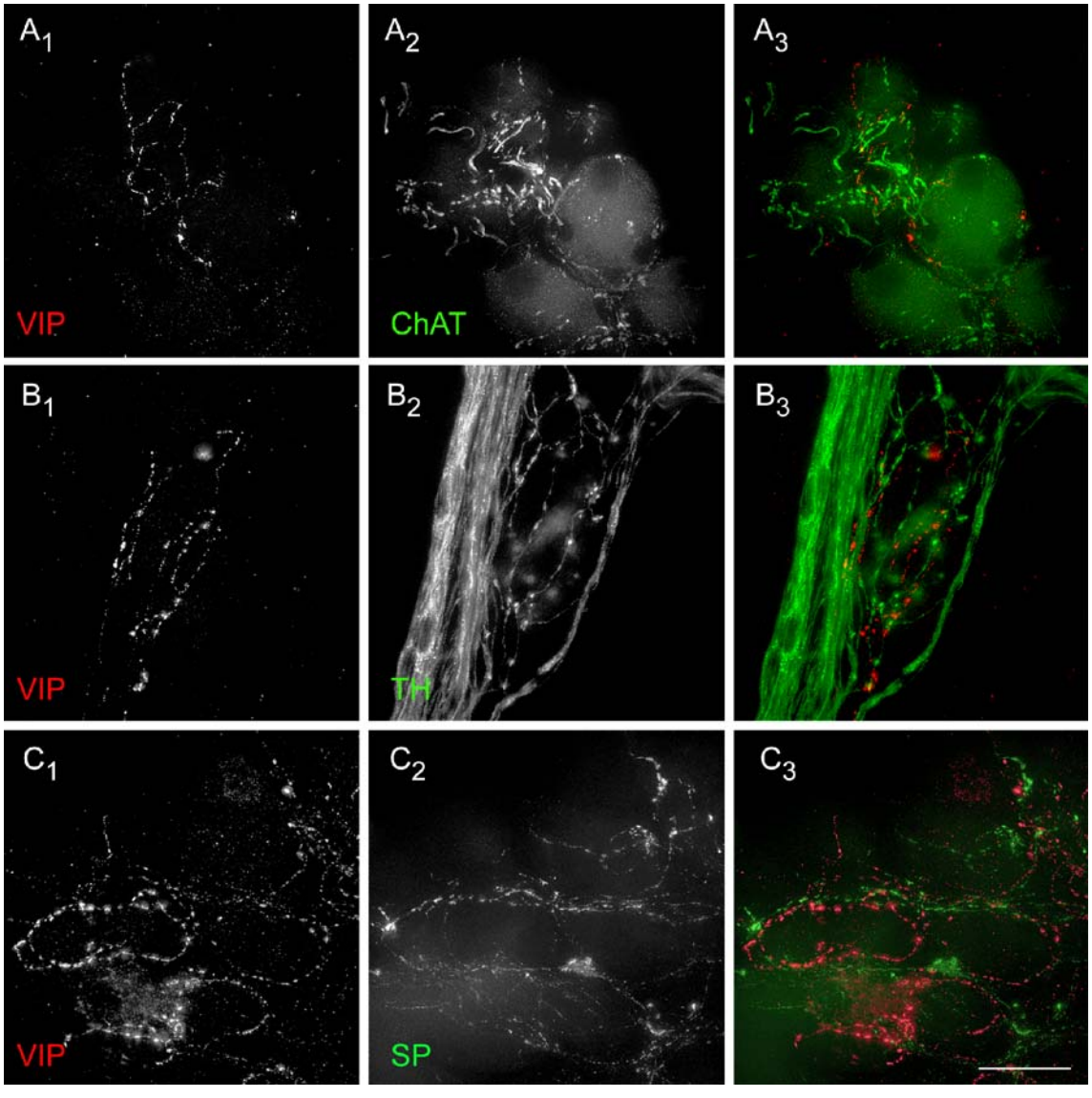

Fig. 3 Fluorescent images from immunolabeled guinea-pig whole-mounts analyzed with the DeltaVision restoration microscopy system (for an explanation of abbreviations, see Table 1). $\mathbf{a}_1$ Cy3-VIP. $\mathbf{a}_2$ FITC-ChAT. $\mathbf{a}_3$ Merged image. Projection of 30 slices (thickness=6 μm). $\mathbf{b}_1$ Cy3-VIP. $\mathbf{b}_2$ FITC-TH. $\mathbf{b}_3$ Merged image. Projection of 20 slices (thickness=4 μm). $\mathbf{c}_1$ Cy-3 VIP. $\mathbf{c}_2$ FITC-SP. $\mathbf{c}_3$ Merged image. Projection of 40 slices (thickness=8 μm). *Bar* 30 μm

cardiac neurons within ganglia. Whole-mount ganglia preparations were labeled with an antiserum directed against VIP and an antiserum directed against PACAP. PACAP-IR fibers were present in all ganglia; some of the same ganglia were also innervated by VIP fibers. Inspection of ganglia containing both VIP-IR fibers and PACAP-IR fibers revealed that these two neuropeptides were not co-localized in the fibers that formed pericellular complexes around the cardiac neurons (Fig. 1d).

VIP is co-localized in extrinsic NOS-IR fibers and in some of the intrinsic NOS-IR cardiac neurons

PACAP is co-localized in ChAT-IR preganglionic parasympathetic fibers innervating the cardiac neurons (Calupca et al. 2000a). We considered that the extrinsic VIP-IR fibers might be a separate population of preganglionic parasympathetic fibers. Therefore, we tested for the co-localization of VIP and ChAT. From inspection of preparations double-labeled with an antiserum against ChAT and an antiserum against VIP, we saw no evidence that VIP was co-localized in ChAT-positive fibers (Fig. 3a).

Other fiber types, such as sympathetic postganglionic fibers and spinal sensory and vagal sensory fibers, are well established as innervating the intrinsic cardiac ganglia (Parsons 2004). In the guinea pig, these different extrinsic fibers are chemically coded, each containing specific neurotransmitters/neuromodulators (Parsons 2004). We took advantage of this chemical coding to determine whether the VIP immunoreactivity was primarily present in a specific type of extrinsic fiber. Different whole-mount cardiac ganglia preparations were tested for the co-localization of VIP with tyrosine hydroxylase (TH), a marker for sympathetic postganglionic fibers, with substance P (SP), a marker for spinal afferent fibers, and with nNOS, a marker of vagal afferent fibers (Parsons 2004). The VIP immunoreactivity appeared not to be present in fibers that were immunoreactive for either TH (Fig. 3b), or SP (Fig. 3c). Moreover, numerous cells were smaller than the cardiac neurons and were TH-IR. None of these small intensely fluorescent cells appeared to exhibit VIP immunoreactivity (data not shown). In contrast, essentially all of the VIP-IR fibers exhibited immunoreactivity for nNOS (Fig. 4). Triple-labeled preparations with antisera against VIP, nNOS, and PACAP revealed numerous VIP and nNOS fibers surrounding a cluster of cardiac neurons. PACAP-IR fibers also formed pericellular complexes on these same cells. When Fig. 4a–c were merged in Fig. 4d, it was apparent that VIP-IR and nNOS-IR were co-localized, whereas PACAP was not.

We also tested whether nNOS and VIP were co-localized in intrinsic cardiac neurons. All of the VIP-IR intrinsic cardiac neurons exhibited nNOS immunoreactivity, but many more neurons exhibited nNOS immunoreactivity alone. Previously, Calupca et al. (2000b) had shown that the number of nNOS-IR cardiac neurons, like that reported here for VIP-IR neurons, did not increase following explant culture. As all extrinsic nNOS-IR and VIP-IR fibers de-

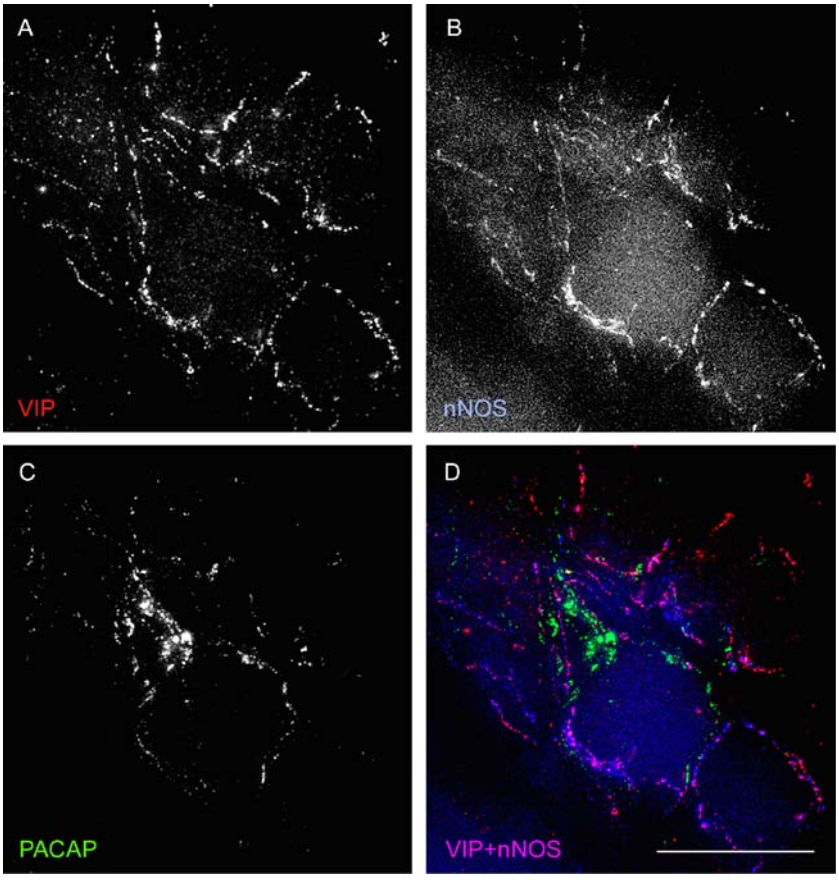

Fig. 4 Images from a triple-labeled guinea-pig atrial whole-mount obtained with the DeltaVision restoration microscopy system. **a** Cy3-VIP. **b** AMCA-nNOS. **c** FITC-PACAP. **d** Merged image. Projection of 30 slices (thickness=6 μm). *Bar* 30 μm

generate over time in explant culture, it is easier to count immunoreactive neurons in 72-h explant-cultured preparations. Therefore, we quantified the co-localization of VIP and nNOS in the intrinsic cardiac neurons in three 72-h explant-cultured preparations. VIP-IR neurons were present in each preparation with the number ranging from 17 to 74 (52±18 VIP-IR neurons). The number of nNOS-IR neurons was greater in these same preparations (124±72 NOS-IR neurons, range of 61 to 200 per preparation). All of the VIP-IR neurons (Fig. 5a$_1$) exhibited immunoreactivity for nNOS (Fig. 5a$_2$). In whole-mount preparations that had been triple-labeled with antisera against VIP, nNOS, and ChAT, the VIP-IR/nNOS-IR cells also exhibited immunoreactivity for ChAT (Fig. 5a$_3$). In the example shown in Fig. 5a, fibers originating from these three cardiac neurons left the ganglion to enter a nearby fiber bundle without making intraganglionic connections and did not exit the nerve bundle to innervate cardiac neurons in nearby ganglia. In many explant-cultured whole-mount ganglia, intrinsic neurons that were only nNOS-IR were present with some that exhibited immunoreactivity for both VIP and nNOS. (Fig. 5b$_{1,2}$).

Although the percentage of VIP-IR neurons did not increase over time in explant culture, axonal growth did occur. This was evident at the ends of fiber bundles severed during the dissection. VIP-IR and nNOS-IR fibers could be followed as they exited the cut ends of small nerve trunks. Some of these fibers exhibited immunoreactivity for both VIP and nNOS (Fig. 5c$_{1,2}$), whereas many others were immunoreactive for only nNOS (data not shown).

Sectioned tissue

Localization of VIP immunoreactivity

VIP-IR nerve fibers in tissue sections were localized primarily to the atria, perivascular connective tissue, and intrinsic cardiac ganglia. The most prominent cluster of myocardial VIP-IR fibers occurred in the posterior wall of the right atrium, beginning at a level slightly inferior to the sinus node and continuing in the inferiomedial direction to the interatrial septum and the junction with the inferior vena cava. The superior aspect of this cluster had the highest density of VIP-IR nerve fibers (Fig. 6a,b). VIP-IR fibers occurred sporadically in other regions of the right atrium and appendage (Fig. 6c), but the overall abundance was low. Several VIP-IR nerve fibers occurred within discrete regions of the posterior interatrial septum, but a significant portion of these were located in connective tissue (Fig. 6d).

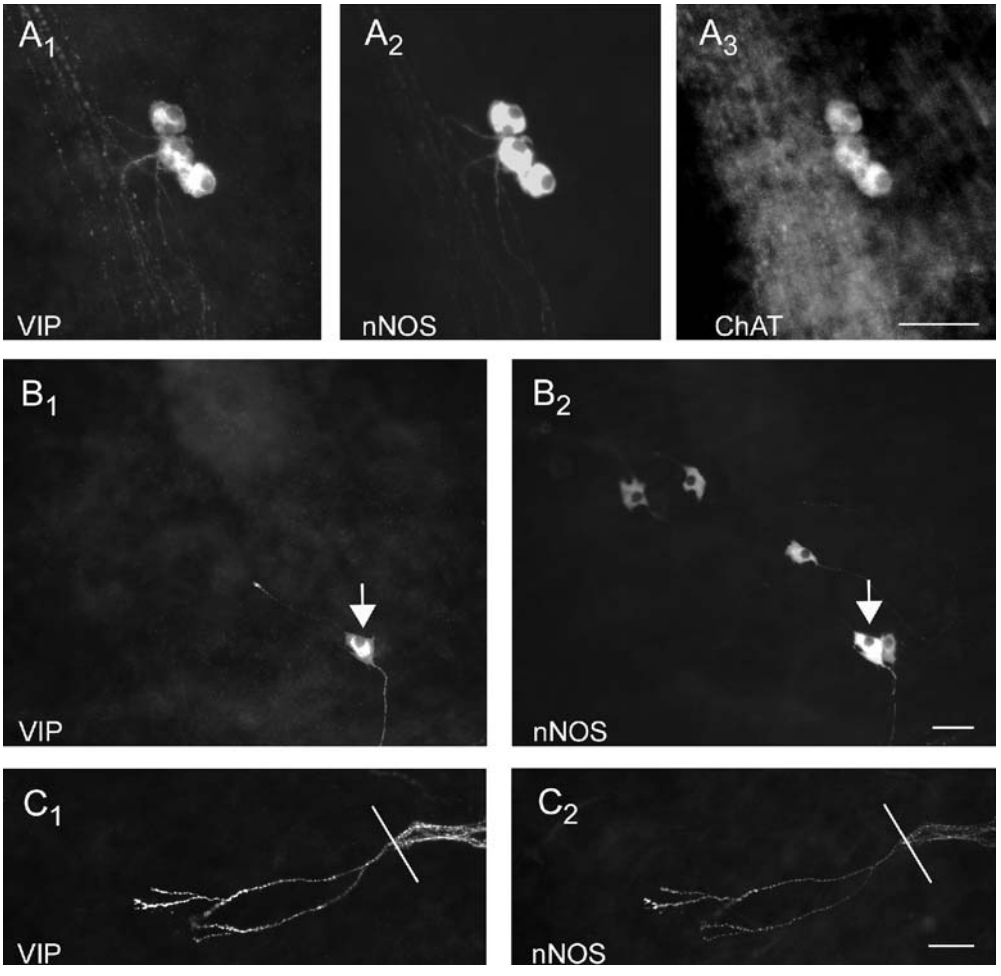

Fig. 5 VIP-IR cells and VIP-IR fibers present in 72-h explant preparations. **a**$_{1,2,3}$ Co-localization of VIP (**a**$_1$) with nNOS (**a**$_2$) and ChAT (**a**$_3$) in the same intrinsic cardiac neurons. **b**$_{1,2}$ Note the presence of more nNOS-IR (**b**$_2$) neurons than VIP-IR (**b**$_1$) neurons in the 72-h explant-cultured cardiac ganglia whole-mounts (*arrow* an intrinsic neuron immunoreactive for both VIP and nNOS). **c**$_{1,2}$ Fibers derived from intrinsic neurons and immunoreactive for VIP (**c**$_1$) and nNOS (**c**$_2$). These fibers extend out of the cut ends of nerve trunks after 72 h in explant culture (*line* original cut end of the nerve trunk). *Bars* 50 μm

Only occasional VIP-IR fibers traveled among myocytes of the atrial septum. VIP immunoreactivity was observed neither in the left atrial appendage nor in most of the left atrium (Fig. 6e). However, the left atrial endocardium contained a dense plexus of VIP-IR fibers at the septal and posterior walls of the main chamber (Fig. 6e). Endocardial VIP-IR fibers were also evident near the entry of the pulmonary veins. Although most left atrial muscle lacked VIP innervation, a few labeled fibers coursed through the thin anterior wall and adjacent connective tissue of the aorta as the annulus of the mitral valve was approached. A similar pattern of VIP-IR nerves was associated with the right atrial approach to the tricuspid valve. Occasional VIP-IR fibers were seen within the atrioventricular (AV) valves. The ventricular myocardium generally lacked VIP-IR nerves except for rare fibers in the interventricular septum and right ventricular wall near the AV junction. Only occasional VIP-IR nerve fibers were encountered in the sinus and AV nodes, the AV bundle, or the bundle branches.

VIP-IR nerve fibers appeared in the adventitia of some large and intermediate coronary arteries located near the base of the heart (Fig. 7a) and around a few smaller vessels in the right atrium. Many VIP-IR fibers were associated with the inferior vena cava (Fig. 6f) and the pulmonary trunk (Fig. 7b).

The highest density of VIP-IR nerve fibers appeared within the intrinsic cardiac ganglia. Many of these ganglia occurred in connective tissue at the posterior atrial surface and would probably have been included in whole-mount preparations (Fig. 6e). However, VIP-IR nerve fibers also innervated additional ganglia located between folds of the posterior inferior interatrial septum (Fig. 6f) or within the myocardium of the interatrial septum or AV bundle (Fig. 7c,d). As observed with whole-mounts, not all ganglia received VIP input. In particular, VIP-IR fibers were rare in ganglia located near the sinus node. VIP-IR cell bodies were uncommon in tissue sections and usually appeared in ganglia located at inferior levels. Some occurred in small ganglia located within the interatrial septum or in the AV bundle.

Colocalization of VIP with nNOS and ChAT

Triple-labeled sections were evaluated by laser scanning confocal microscopy to determine the extent that VIP immunoreactivity was localized to ChAT-IR and nNOS-IR nerve fibers. More extensive screening for double labeling was carried out by conventional fluorescence microscopy. These experiments demonstrated that most VIP-IR nerve

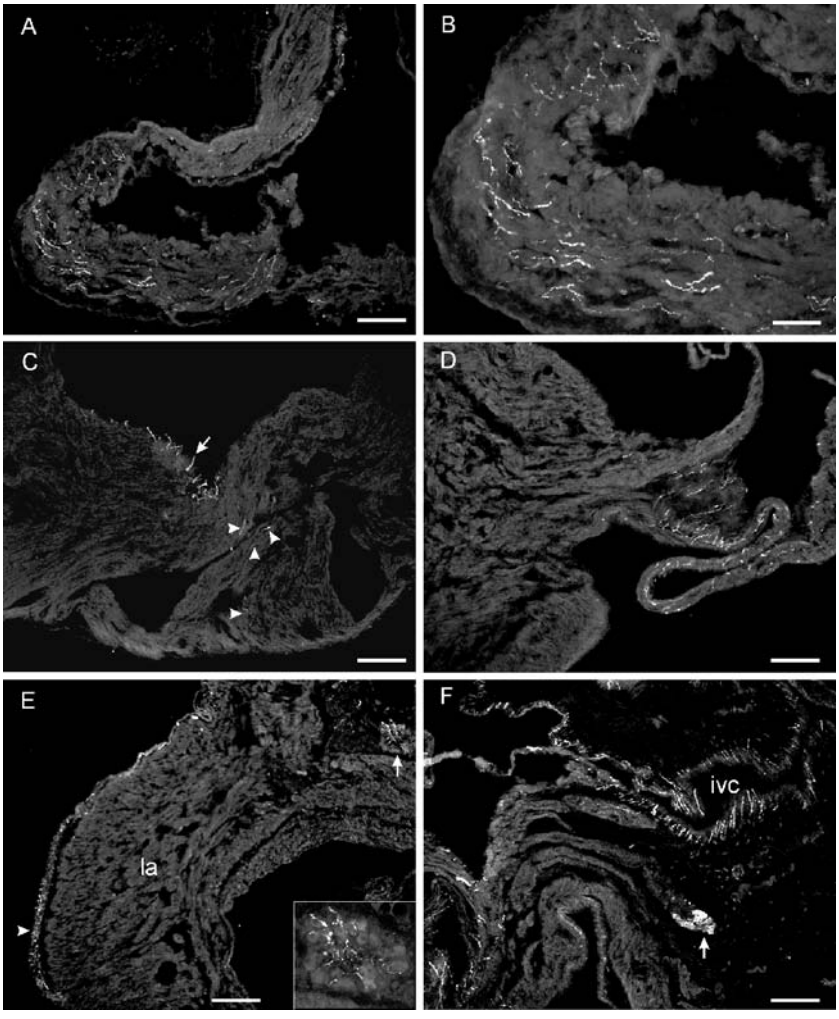

Fig. 6 Fluorescence micrographs showing principal sites of VIP-IR nerve fibers in short-axis sections through atria and surrounding tissue. **a**, **b** Area of posterior right atrium with the highest density of myocardial VIP-IR nerves fibers (**b** is an expansion of **a**). **c** Area of right atrium with a cluster of VIP-IR fibers located at the endocardium (*arrow*) and occasional labeled fibers found within the myocardium (*arrowheads*). **d** VIP-IR nerve fibers located primarily in non-myocyte regions of posterior interatrial septum (anterior is to the *left*; right and left atrial chambers are to the *top* and *bottom*, respectively). **e** Section through left atrium (*la*) showing VIP-IR fibers localized to the endocardium (*arrowhead*) and a ganglion (*arrow*) located at the posterior edge. *Insert* Ganglion at higher magnification. VIP-IR nerve fibers are not present in the left atrial myocardium. **f** Section through a more inferior level of the atria where the inferior vena cava (*ivc*) attaches to the right atrium. Orientation is the same as shown in **d**. VIP-IR nerve fibers are present in the wall of the vena cava, the posterior interatrial septum (*lower left*), and a ganglion (*arrow*). *Bar* 100 μm (**b**), 200 μm (**a**, **c**–**f**)

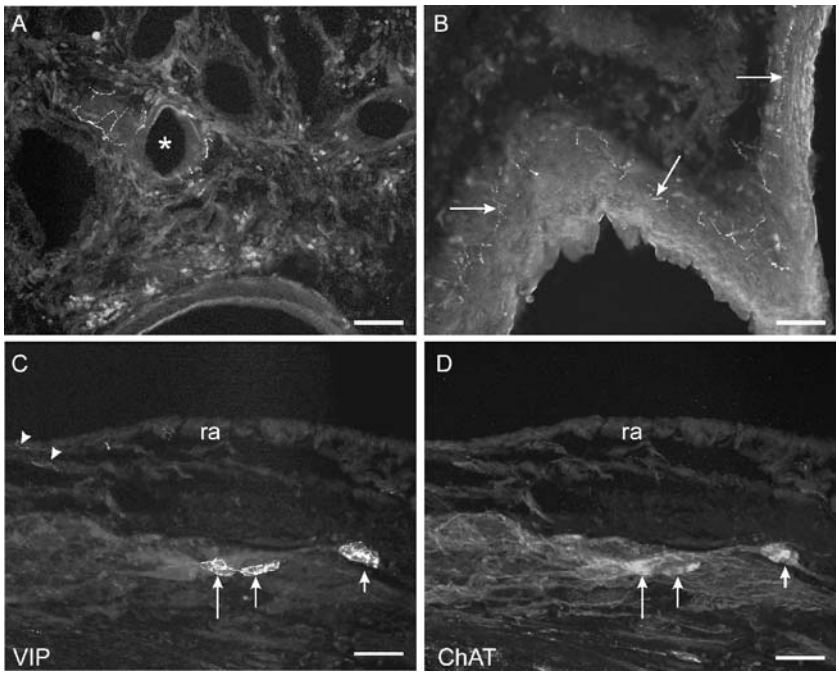

Fig. 7 Fluorescence micrographs showing examples of perivascular VIP-IR fibers (**a**, **b**) and VIP-IR fibers innervating small ganglia located in the atrioventricular (AV) bundle (**c**). **a** Several blood vessels present in connective tissue at the base of the aorta (*asterisk* the only vessel with VIP-IR fibers). **b** Numerous VIP-IR fibers are associated with the pulmonary trunk (*arrows*). **c**, **d** Section through the AV bundle showing double-labeling for VIP and ChAT immunoreactivity (right atrial chamber is at the *top*; anterior is to the *left*). **d** The horizontal band of ChAT-IR nerve fibers marks the AV bundle (*arrows* small ganglia with ChAT-IR neurons). **c** VIP-IR fibers surround neurons in each of the ganglia but are not present within the AV bundle. Note the VIP-IR fibers (*arrowheads*) in the adjacent right atrium (*ra*). *Bars* 100 μm

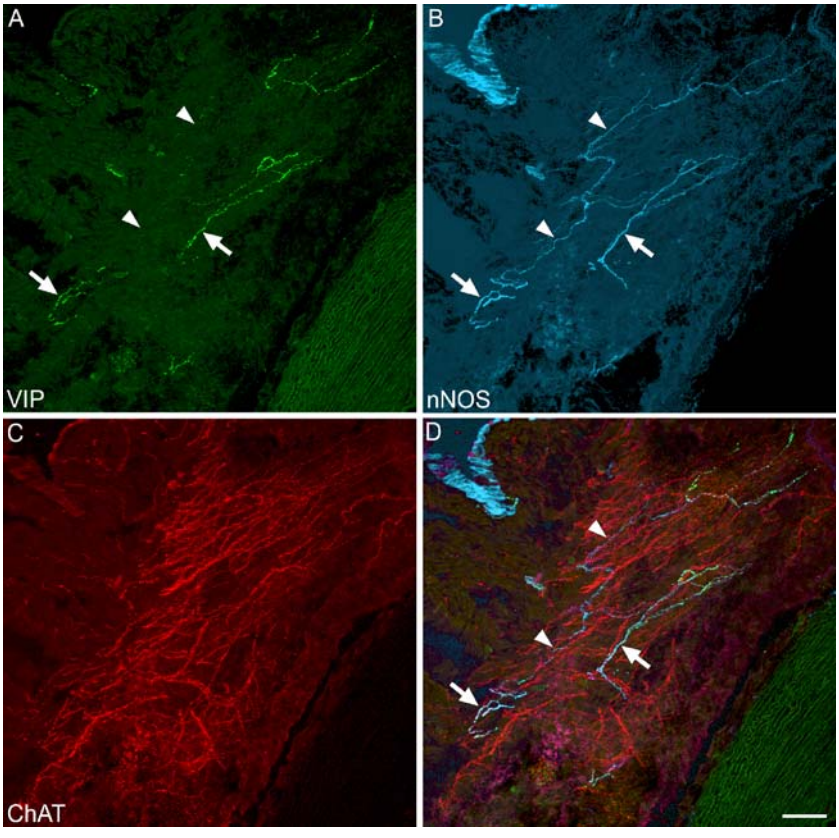

Fig. 8 Sequentially scanned confocal images of nerve fiber staining in the sinus node. Whereas the vast majority of nerve fibers exhibited only ChAT immunoreactivity (**c**, **d**), a few were also immunoreactive for both VIP (**a**, **d, arrows**) and nNOS (**b**, **d**, *arrows*). **d** Overlay of all three immunostainings. Single optical slice approximately 0.4 μm thick. *Bar* 80 μm

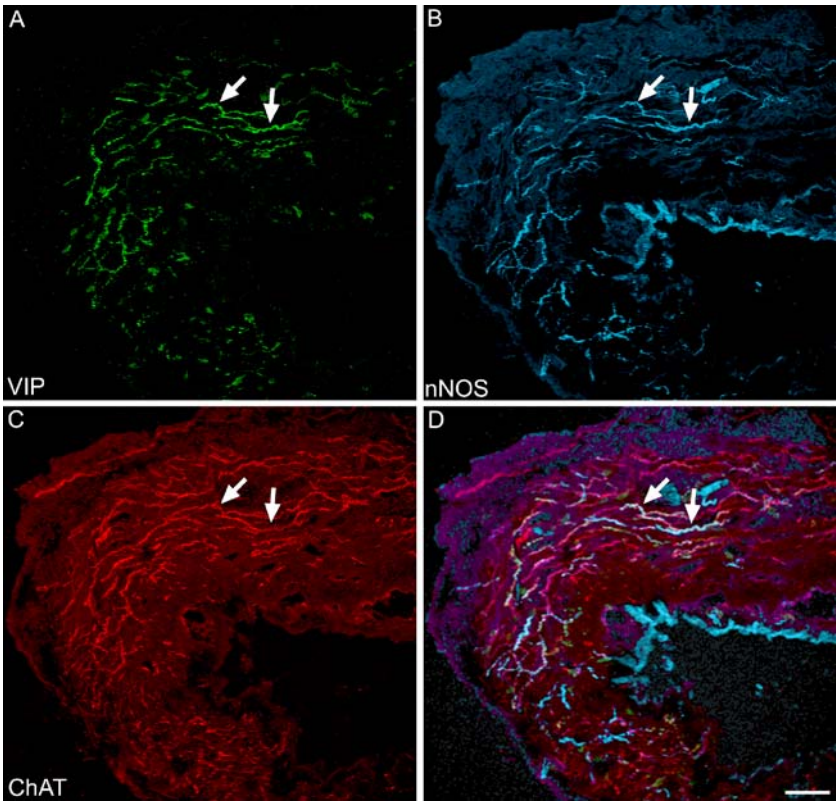

Fig. 9 Sequentially scanned confocal images of nerve fiber staining in the posterior right atrium. Together with abundant staining of fibers for ChAT (**c**) and nNOS (**b**), a considerable number of VIP-IR fibers were also present at this level (**a**). **d** Overlay of immunostainings (*arrows* a few examples of triple-labeled fibers). Maximum projection images were obtained from 14 optical sections spanning a tissue thickness of 5.2 μm. *Bar* 80 μm

fibers exhibited nNOS immunoreactivity (Figs. 8, 9, 10). A majority of VIP/nNOS-IR fibers located in the myocardium at the sinus node (Fig. 8) and posterior right atrium (Fig. 9) also contained ChAT immunoreactivity, but nerve fibers labeled for ChAT alone were far more abundant at these sites. Occasional nerve fibers at one or both of these right atrial locations stained for ChAT and either nNOS or VIP. A few fibers were observed that stained for nNOS alone,

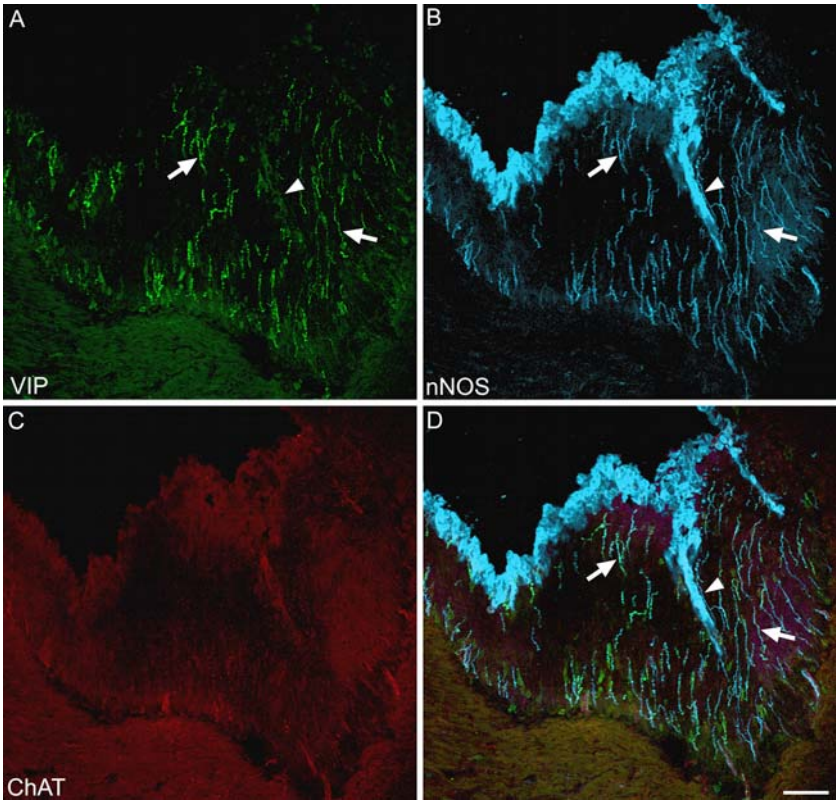

Fig. 10 Sequentially scanned confocal images of nerve fiber staining in the endocardium of the left atrium. A dense plexus of VIP-IR nerve fibers was evident in the endocardium (**a**), and the vast majority of these fibers was also nNOS-immunoreactive (**b**, **d**, *arrows*). A few fibers (*arrowheads*) were labeled for nNOS only (**b**, **d**). No ChAT-IR nerve fibers were observed in the endocardium (**c**). Maximum projection images were obtained from 16 optical sections spanning a tissue thickness of 6 μm. **d** Overlay of immunostainings. *Bar* 80 μm

but fibers exhibiting VIP immunoreactivity alone were rare. Most perivascular and endocardial VIP-IR nerve fibers were also immunoreactive for nNOS but did not exhibit ChAT immunoreactivity (Fig. 10). Several fibers around the pulmonary trunk and within the left atrial endocardium (Fig. 10) only displayed immunoreactivity for nNOS; relatively few were VIP-IR only.

Discussion

We have analyzed the distribution of VIP-IR fibers in the intrinsic cardiac ganglia and cardiac tissue sections of the guinea pig. VIP-IR fibers are present in approximately 70% of the intracardiac ganglia, although not all cells within each innervated ganglion receive pericellular complexes. In contrast, essentially every intracardiac neuron in each ganglion is surrounded by a complex of PACAP-IR fibers. Based on the dramatic loss of intraganglionic VIP-IR fibers in 72-h explant-cultured whole-mount preparations, we suggest that most of the VIP-IR fibers within cardiac ganglia, like the PACAP-IR fibers (Calupca et al. 2000a), are derived from sources extrinsic to the heart. Furthermore, from the inspection of double-labeled preparations, immunoreactivity for VIP and PACAP appears to be present in different fibers forming pericellular complexes around individual neurons within the cardiac ganglia. VIP is co-localized with nNOS immunoreactivity. We have previously postulated that most of the extrinsic nNOS-IR fibers within the ganglia are processes of vagal afferent fibers (Calupca et al. 2000b). Therefore, we now propose that the extrinsic intraganglionic VIP-IR fibers are nNOS-IR vagal afferent fibers. However, given that VIP and nNOS also co-localize in the cell bodies of a few intrinsic cardiac neurons, a minor component of the interganglionic fibers immunoreactive for both VIP and nNOS might represent axons derived from neurons within the whole-mount ganglia preparation. No difference has been seen in the percentage of cells exhibiting VIP immunoreactivity between control and explanted cardiac ganglia preparations. Although the number of cells expressing VIP does not increase in intrinsic cardiac neurons following explant culture, the numbers of cardiac neurons expressing other peptides, such as neuropeptide Y and PACAP, have been shown to increase in these preparations (Parsons 2004). Moreover, in prior studies of explant-cultured rat sympathetic neurons, the numbers of VIP-IR cells and PACAP-IR cells have been demonstrated to increase dramatically (Hyatt-Sachs et al. 1993; Moller et al. 1997). These results suggest that neuropeptide expression following explantation is peptide- and neuron-specific.

In the 72-h explant preparations, VIP-IR cells give rise to axons that can be followed long distances. These VIP-IR fibers do not form complexes with adjacent cells in the ganglion of origin or within other ganglia; rather they exit the ganglion and enter nearby nerve bundles. The axonal extension of the VIP-IR cells is reminiscent of the intrinsic nNOS-IR fibers in explant-cultured preparations (Calupca et al. 2000b). We suggest that this observation provides further support for our hypothesis that the VIP/nNOS fibers making connections within intracardiac ganglia are essentially all extrinsic fibers derived from sources outside the heart.

Our experiments with sections of guinea pig heart provide additional evidence that many epicardial ganglia receive dense innervation by VIP-IR fibers and further establish that VIP-IR processes extend to some ganglia located within the myocardium near the AV node and AV bundle. However, VIP-IR fibers are generally sparse in nodal, conductile, and working myocardium. The posterior wall of the right atrium is the only exception, since a moderate density of VIP-IR fibers travel through the myocardium at discrete levels of this region. The majority of the remaining VIP-IR fibers occurs in non-myocyte regions, such as the atrial endocardium and the adventitia of the pulmonary trunk and inferior vena cava. These VIP-IR fibers most probably have an extrinsic source, since VIP-IR neurons are sparse in tissue sections. Furthermore, most VIP-IR fibers in tissue sections are also immunolabeled for nNOS, but not for ChAT, suggesting that they could have a vagal afferent origin.

Early studies of VIP innervation of the heart identified VIP-IR cell bodies in intrinsic cardiac ganglia of several species (Weihe et al. 1984), and subsequent physiological studies of the dog have provided support for the concept that VIP functions as a co-transmitter with ACh in cholinergic nerves that innervate the sinus node and other regions of the myocardium (for a review, see Henning and Sawmiller 2001). The present findings provide some support for this view, since some of the VIP-IR fibers observed at the sinus node also exhibit immunoreactivity for ChAT. VIP and ChAT also colocalize in most of the VIP-IR fibers innervating regions of the posterior right atrium, where they are most abundant. However, the vast majority of the cholinergic neurons and nerve fibers in the guinea pig heart do not exhibit any VIP immunoreactivity. These anatomical observations suggest that direct physiological actions of VIP on the guinea pig heart would occur primarily in discrete regions of the posterior right atrium, and that endogenous VIP would exert little or no influence on nodal function, AV conduction, or myocardial contractility in this species.

VIP and PACAP exert their influence through three G-protein-coupled receptors, the PAC_1 receptor and two VPAC receptors (Vaudry et al. 2000). The PAC_1 receptor is selective for PACAP, with VIP being approximately 1,000-fold less effective. In contrast, the VPAC receptors are equally sensitive to VIP and PACAP. The cardiac neurons of adult guinea pig express the PAC_1 receptor, and activation of these receptors with PACAP increases neuronal excitability and evokes action potentials from some cells (Braas et al. 1998). VIP has little or no effect on the excitability of guinea pig intracardiac neurons (Braas et al. 1998), as expected based on its low potency at PAC_1 receptors, but does cause dose-dependent tachycardia in isolated perfused guinea pig hearts (Hoover 1989). Positive chronotropic responses can be evoked by VIP in many species (Henning and Sawmiller 2001) and most likely occur through the

activation of VPAC receptors on sinus nodal cells. The present findings suggest that there is a mismatch of VPAC receptors and VIP nerves at the sinus node of guinea pig. Mismatch of VIP receptors and nerves might also occur in humans, since VIP-IR nerve fibers are sparse in human sinus node (Crick et al. 1994), although exogenous VIP causes tachycardia by a non-reflex mechanism (Unwin et al. 1987). PACAP can have dual chronotropic effects in isolated cardiac preparations from guinea pigs, causing bradycardia through the stimulation of PAC_1 receptors on cholinergic neurons (Seebeck et al. 1996; Chang et al. 2005) and tachycardia that is probably mediated by VPAC receptors on nodal cells, which are not innervated by PACAP-IR fibers (Chang et al. 2005). The function of VIP-IR fibers in guinea pig cardiac ganglia is unknown, but VIP might modulate ganglionic transmission by augmenting the response of cardiac neurons to ACh, as demonstrated for rat intracardiac neurons (Liu et al. 2000) or by acting presynaptically to influence the release of ACh.

Acknowledgements We thank Drs. Jan Fahrenkrug and Jens Hannibal for the generous gift of PACAP antiserum.

References

Anderson FL, Hanson GR, Reid B, Thorpe M, Kralios AC (1993) VIP and NPY in canine hearts. Distribution and effect of total and selective parasympathetic denervation. Am J Physiol 265: H91–H95

Ardell JL (2001) Neurohumoral control of cardiac function. In: Sperelakis N (ed) Heart physiology and pathophysiology. Academic, San Diego, pp 45–59

Arimura A (1998) Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol 48:301–331

Braas KM, May V, Harakall SA, Hardwick JC, Parsons RL (1998) Pituitary adenylate cyclase-activating polypeptide expression and modulation of neuronal excitability in guinea pig cardiac ganglia. J Neurosci 18:9766–9779

Braas KM, Rossignol TM, Girard BM, May V, Parsons RL (2004) Pituitary adenylate cyclase activating polypeptide (PACAP) decreases neuronal somatostatin immunoreactivity in cultured guinea-pig parasympathetic ganglia. Neuroscience 126:335–346

Calupca MA, Vizzard MA, Parsons RL (2000a) Origin of pituitary adenylate cyclase-activating polypeptide (PACAP)-immunoreactive fibers innervating guinea pig parasympathetic cardiac ganglia. J Comp Neurol 423:26–39

Calupca MA, Vizzard MA, Parsons RL (2000b) Origin of neuronal nitric oxide synthase (NOS)-immunoreactive fibers in guinea pig parasympathetic cardiac ganglia. J Comp Neurol 426:493–504

Chang Y, Lawson LJ, Hancock JC, Hoover DB (2005) Pituitary adenylate cyclase-activating polypeptide: localization and differential influence on isolated hearts from rats and guinea pigs. Regul Pept 129:139–146

Crick SJ, Wharton J, Sheppard MN, Royston D, Yacoub MH, Anderson RH, Polak JM (1994) Innervation of the human cardiac conduction system. A quantitative immunohistochemical and histochemical study. Circulation 89:1697–1708

Della NG, Papka RE, Furness JB, Costa M (1983) Vasoactive intestinal peptide-like immunoreactivity in nerves associated with the cardiovascular system of guinea-pigs. Neuroscience 9: 605–619

Fahrenkrug J, Hannibal J (2004) Neurotransmitters co-existing with VIP or PACAP. Peptides 25:393–401

Forsgren S (1989) Vasoactive intestinal polypeptide-like immunoreactivity in the bovine heart: high degree of coexistence with neuropeptide Y-like immunoreactivity. Cell Tissue Res 256: 125–135

Hardwick JC, Mawe GM, Parsons RL (1995) Evidence for afferent fiber innervation of parasympathetic neurons of the guinea pig cardiac ganglion. J Auton Nerv Syst 53:166–174

Hardwick JC, Mawe GM, Parsons RL (1997) Tachykinin-induced activation of non-specific cation conductance via NK_3 neurokinin receptors in guinea-pig intracardiac neurons. J Physiol (Lond) 504:65–74

Henning RJ, Sawmiller DR (2001) Vasoactive intestinal peptide: cardiovascular effects. Cardiovasc Res 49:27–37

Hoover DB (1989) Effects of guinea pig vasoactive intestinal peptide on the isolated perfused guinea pig heart. Peptides 10: 343–347

Hoover DB, Ganote CE, Ferguson SM, Blakely RD, Parsons RL (2004) Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc Res 62:112–121

Horackova M, Armour JA, Byczko Z (1999) Distribution of intrinsic cardiac neurons in whole-mount guinea pig atria identified by multiple neurochemical coding. A confocal microscope study. Cell Tissue Res 297:409–421

Hyatt-Sachs H, Schreiber RC, Bennett TA, Zigmond RE (1993) Phenotypic plasticity in adult sympathetic ganglia in vivo: effects of deafferentation and axotomy on the expression of vasoactive intestinal peptide. J Neurosci 13:1642–1653

Kennedy AL, Harakall SA, Lynch SW, Braas KM, Hardwick JC, Mawe GM, Parsons RL (1998) Expression and physiological actions of neuropeptide Y in guinea pig parasympathetic cardiac ganglia. J Auton Nerv Syst 71:190–195

Leger J, Croll RP, Smith FM (1999) Regional distribution and extrinsic innervation of intrinsic cardiac neurons in the guinea pig. J Comp Neurol 407:303–317

Liu D-M, Cuevas J, Adams DJ (2000) VIP and PACAP potentiation of nicotinic ACh-evoked currents in rat parasympathetic neurons is mediated by G-protein activation. Eur J Neurosci 12: 2243–2251

Loffelholz K, Pappano AJ (1985) The parasympathetic neuroeffector junction of the heart. Pharmacol Rev 37:1–24

Lynch SW, Braas KM, Harakall SA, Kennedy AL, Mawe GM, Parsons RL (1999) Neuropeptide Y (NPY) expression is increased in explanted guinea pig parasympathetic cardiac ganglia neurons. Brain Res 827:70–78

Mawe GM, Ellis LM (2001) Chemical coding of intrinsic and extrinsic nerves in the guinea pig gallbladder: distributions of PACAP and orphanin FQ. Anat Rec 262:101–109

Mawe GM, Talmage EK, Lee KP, Parsons RL (1996) Expression of choline acetyltransferase immunoreactivity in guinea pig cardiac ganglia. Cell Tissue Res 285:281–286

Merriam LA, Barstow KL, Parsons RL (2004) Pituitary adenylate cyclase-activating polypeptide enhances the hyperpolarization-activated nonselective cationic conductance, I_h, in dissociated guinea pig intracardiac neurons. Regul Pept 123:123–133

Moller K, Reimer M, Ekblad E, Hannibal J, Fahrenkrug J, Kanje M, Sundler F (1997) The effects of axotomy and preganglionic denervation on the expression of pituitary adenylate cyclase activating peptide (PACAP), galanin and PACAP type 1 receptors in the rat superior cervical ganglion. Brain Res 775: 166–182

Parsons RL (2004) Mammalian cardiac ganglia as local integration centers: histochemical and electrophysiological evidence. In: Dun N, Machado B, Pilowsky P (eds) Mechanisms of cardiovascular regulation. Kluwer Academic Publishers, Norwell, pp 335–356

Randall WC, Wurster RD (1994) Peripheral innervation of the heart. In: Levy MN, Schwartz PJ (eds) Vagal control of the heart: experimental basis and clinical implications. Futura, Armonk, pp 21–32

Rechardt L, Aalto-Setala K, Purjeranta M, Pelto-Huikko M, Kyosola K (1986) Peptidegic innervation of human atrial myocardium: an electron microscopical and immunocytochemical study. J Auton Nerv Syst 17:21–32

Seabrook GR, Fieber LA, Adams DJ (1990) Neurotransmission in neonatal rat cardiac ganglion in situ. Am J Physiol 259:H997–H1005

Seebeck J, Schmidt WE, Kilbinger H, Neumann J, Zimmermann N, Herzig S (1996) PACAP induces bradycardia in guinea-pig heart by stimulation of atrial cholinergic neurones. Naunyn-Schmiedeberg's Arch Pharmacol 354:424–430

Steele PA, Gibbins IL, Morris JL, Mayer B (1994) Multiple populations of neuropeptide-containing intrinsic neurons in the guinea-pig heart. Neuroscience 62:241–250

Unwin RJ, Reed T, Thom S, Calam J, Peart WS (1987) Effects of indomethacin and (±)-propranolol on the cardiovascular and rennin responses to vasoactive intestinal polypeptide (VIP) infusion in man. Br J Clin Pharmacol 23:523–528

Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H (2000) Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol Rev 52: 269–324

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 Tissue Res 236:527–540