Organization of a Vertebrate Cardiac Ganglion: A Correlated Biochemical and Histochemical Study

R. L. Parsons, D. S. Neel, T. W. McKeon,¹ and R. E. Carraway²

Departments of Anatomy and Neurobiology and ¹Physiology and Biophysics, University of Vermont, Burlington, Vermont 05405, and ²Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

A correlated biochemical and histochemical study was undertaken to identify and quantify the presence of different biogenic amines and a substance P-like peptide within the parasympathetic cardiac ganglion of the mudpuppy (*Necturus maculosus*). Tissue extracts of the cardiac septum containing the parasympathetic cardiac ganglia from control animals were found, by high-pressure liquid chromatography, to contain significant amounts of norepinephrine (NE), epinephrine (E), dopamine (DA), and 5-HT. To allow neural elements of extraganglionic origin to degenerate, ganglia were explanted and maintained in organ culture for 8 d. Extracts from these explanted preparations had no detectable level of E, and NE was reduced, whereas DA and 5-HT levels were similar to those of control preparations. The results indicated that some of the neurons intrinsic to the cardiac septum contain DA and 5-HT and that most (greater than 70%) of the E and NE found in this tissue is of extrinsic origin. Histochemistry of control and explanted preparations showed 5-HT-immunoreactive and catecholamine-containing intrinsic neurons. A substance P-like peptide was identified by radioimmune assay in septal extracts. The peptide content diminished by one-third to one-fifth in preparations maintained in organ culture for 8–14 d, suggesting that a significant amount of the substance P-like peptide is derived from extraganglionic sources. Immunocytochemical studies demonstrated the presence of numerous long substance P-immunoreactive fibers coursing across the septum, branching over cardiac muscle fibers, and forming pericellular networks around individual parasympathetic ganglion cells and clusters of ganglion cells. In addition, numerous small intrinsic neurons exhibited immunoreactivity for substance P. Comparison of the substance P-staining patterns in control and explanted ganglia suggests that the majority of the long substance P-immunoreactive fibers innervating the mudpuppy cardiac ganglion cells are not parasympathetic preganglionic fibers. Rather, it is hypothesized that these fibers are processes of primary sensory fibers.

The present observations indicate that the mudpuppy cardiac ganglion exhibits a complex organization similar to that of mammalian sympathetic and enteric ganglia.

The vertebrate cardiac ganglion contains postganglionic neurons that contribute to the control of cardiac function (Löffelholz and Pappano, 1985). The activity of these postganglionic neurons may be determined by influences from both central nervous system projections and local reflex afferents (Schultzberg et al., 1983; Kreulen et al., 1985). Although many anatomical, histochemical, and functional characteristics of sympathetic prevertebral and paravertebral ganglia have been elucidated in recent years, less information is available about the anatomical organization and functional properties of vertebrate parasympathetic cardiac ganglia (Ehinger et al., 1983). Isolation of most vertebrate cardiac ganglia is difficult. However, the parasympathetic cardiac ganglion in *Necturus maculosus* (mudpuppy) has unique characteristics that make it a good model preparation for correlated anatomical and physiological studies. The mudpuppy parasympathetic cardiac ganglion is located on a thin septal tissue sheet that lies under the heart (McMahan and Purves, 1976). The vagus nerves containing the preganglionic parasympathetic fibers run across the septum to innervate clusters of postganglionic neurons. There are 2 neuron types in the mudpuppy cardiac ganglion: large, 35–50- μ m-diameter postganglionic parasympathetic neurons (principal cells), and smaller, 15–30- μ m-diameter intrinsic neurons. These smaller intraganglionic neurons were initially identified as catecholamine-containing small intensely fluorescent (SIF) cells by McMahan and Purves (1976). More recently, Neel and Parsons (1986b) have demonstrated that these intrinsic neurons are also immunoreactive for 5-HT and a substance P-like peptide.

The present study represents a correlated biochemical and histochemical effort to quantify the amount and determine the specific cellular origin of different biogenic amines and a substance P-like peptide within the cardiac ganglion. The results of this study demonstrate that this ganglion exhibits a complex organization, with fiber processes derived from local afferent reflex inputs as well as from parasympathetic preganglionic innervation.

Materials and Methods

Most of the experiments completed in this study were carried out on the cardiac septum containing the parasympathetic cardiac ganglion of *N. maculosus* (mudpuppy). A few immunocytochemical experiments were also carried out on the intra-atrial cardiac septum from the bullfrog, *Rana catesbeiana*. Whole-mount preparations of the septum from either

Received June 9, 1986; revised Sept. 10, 1986; accepted Sept. 25, 1986.

We thank Drs. Edith Hendley and Richard Kriebel for their helpful comments during this study and their critical review of this manuscript. We also wish to acknowledge the expert technical assistance provided by Mr. John O'Brien during much of this study. Partial support for this project was obtained from USPHS Grant NS-19880 to R.L.P.

Correspondence should be addressed to Dr. Rodney L. Parsons, Department of Anatomy and Neurobiology, College of Medicine, University of Vermont, Burlington, VT 05405.

Copyright © 1987 Society for Neuroscience 0270-6474/87/030837-10\$02.00/0

Figure 1. Typical chromatograms of standards (A) and septal tissue extract from control preparations (B). For the standard solution (A), 1000 pgm of each of the following were injected: norepinephrine (NE), epinephrine (E), dihydroxyphenylacetic acid (DOPAC), normetanephrine (NM), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), serotonin (5-HT). Septal tissue sample (B) was homogenized in 12 volumes of PCA. Injection volumes were 20 μ l. Chromatographic conditions and sample preparation are detailed in the text.

mudpuppy or bullfrog were removed and pinned out on Sylgard-coated petri dishes, as described previously (McMahan and Purves, 1976; Neel and Parsons, 1986a, b). All freshly dissected preparations were maintained in a cold HEPES (*N*-2-hydroxymethyl piperazine-*N*-2-ethane sulfonic acid)-buffered physiological solution (in mM: NaCl, 120; KCl, 2.5; CaCl₂, 1.8; HEPES, 1.0; pH 7.3) until used for specific experiments.

High-pressure liquid chromatographic (HPLC) analysis. Standard HPLC procedures were used to identify and quantify the presence of biogenic amines in cardiac tissue extracts from the mudpuppy cardiac septum (Kontur et al., 1984). Isolated septal tissue was pooled from 4 animals and homogenized in 10–20 volumes of 0.1 M perchloric acid (PCA) with 0.15 mM EDTA. The homogenates were centrifuged at 1000 $\times$ *g* for 20 min. The supernatant was collected and stored at -70°C until analyzed. Prior to injection into the HPLC system, the supernatant was filtered through 0.2 μ m filters.

The HPLC system used for these investigations consisted of a Waters M-45 solvent delivery module, a 7125 Rheodyne injector with a 20 or 50 μ l sample loop, and a 5 μ m Bioanalytical Systems (BAS) octadecylsilyl (ODS) reverse-phase column protected by an ODS 5 μ m Brownlee guard column. A BAS electrochemical amperometric detector (LC-4) with a glassy carbon electrode was employed. The system was operated at room temperature under the following conditions: flow rate, 1.5 ml/minute, electrode oxidation potential, +0.72 V, and 5 nA/V output sensitivity. A citric acid-phosphate buffer mobile phase was employed, with the pH adjusted to 3.85 with phosphoric acid. Sodium octyl sulfate was

used as the ion-pairing agent and acetonitrile as an organic modifier. Peaks were identified by coelution with standard compounds and by relative peak-height ratios at 2 applied potentials. Levels of norepinephrine (NE), epinephrine (E), dopamine (DA), and 5-HT were calculated on the basis of peak heights with an external standard method using a Shimadzu C-R3A Chromatopac.

Radioimmunoassay (RIA) procedures. A sensitive and specific RIA using ¹²⁵I-Tyr⁸-substance P as tracer was used to quantify the presence of the substance P-like peptide in the cardiac septum. The assay was carried out as described by Mroz and Leeman (1979). Cardiac septa from 4 animals were rapidly dissected in cold physiological saline, blotted, and weighed. The tissue was homogenized in 100–300 volumes of 0.1 N HCl, boiled for 10 min and centrifuged. The supernatant was immediately frozen and stored at -70°C ; samples were subsequently lyophilized and analyzed.

All samples were assayed in duplicate and the results calculated with a logit-log linearization method. The slopes of dilutions of tissue extracts were parallel to the substance P standard curve, indicating the presence of substance P or a substance P-like peptide.

Histochemical and immunocytochemical procedures. Whole-mount septal preparations from mudpuppy and bullfrog were prepared for either glyoxylic acid-induced fluorescence, using the procedures outlined by Furness and Costa (1975), or for immunocytochemistry, following the methods of Costa et al. (1980). To obtain glyoxylic acid-induced fluorescence, the preparations were immersed in 2% (wt/vol) glyoxylic acid in 0.1 M phosphate buffer, pH 7.0, at 4°C for 30 min. The preparations were then whole-mounted on glass slides, partially air-dried, and incubated at 100°C for 4 min. For immunocytochemistry, the isolated septal preparations were pinned on a paraffin block and incubated in cold (4°C) Zamboni fixative (0.1 M phosphate-buffered picric acid-formaldehyde mixture; pH 7.3) for approximately 18 hr (Costa et al., 1980), and rinsed in phosphate-buffered saline (PBS). The ganglia were then incubated overnight with primary antiserum at room temperature, washed with PBS, and incubated for 1 hr with a fluorescent-tagged secondary antiserum. Triton X-100 (0.3%) and normal goat serum (0.1%) were included in the incubation media. The preparations were then washed in buffer, transferred to slides, and mounted in carbonate-buffered 50% glycerol, pH 8.6. Substance P-immunoreactive neurons and fiber processes were visualized using either a rat monoclonal antiserum (Sera Labs) or a rabbit polyclonal antiserum (Immunonuclear) at dilutions of 1:100 or 1:200, followed by exposure to either (1) a fluorescein-isothiocyanate (FITC)-conjugated goat anti-rat IgG (Cappel; 1:40 dilution) or (2) a FITC-conjugated goat anti-rabbit antiserum (Cappel;

Table 1. Comparison of biogenic amine content in control and explanted cardiac septa^a

Biogenic amine ^b	Control	8 d explant	% of control
Norepinephrine	802.0 $\pm$ 88.8	230.2 $\pm$ 120.2	28.7
Epinephrine	199.6 $\pm$ 31.1	$\leq 65.5^c$	≤ 32.8
Dopamine	1633.4 $\pm$ 257.1	1867.6 $\pm$ 493.7	114.3
Serotonin	1564.0 $\pm$ 42.7	1274.2 $\pm$ 425.9	81.5

^a Tissue pooled from 4 animals for control and explanted preparations.

^b Amount given as pmol/gm wet weight. Values equal mean $\pm$ SEM from 3 separate experiments.

^c The calculated limit of detectability for epinephrine.

Figure 2. Examples of glyoxylic acid-induced fluorescence in fibers and SIF cells. *A*, Long fluorescent fibers forming a pericellular network on parasympathetic postganglionic neurons. *B*, Group of fluorescent SIF cells in a control preparation. *C*, Group of SIF cells in explanted preparation maintained for 8 d in organ culture. Calibrations, 40 μ m.

1:40). Neurons and processes immunoreactive for 5-HT were visualized using an antiserum raised in rabbit against 5-HT (Immunonuclear) at dilutions of 1:100–1:500, followed by incubation in FITC-conjugated goat anti-rabbit antiserum (1:40). In a few experiments, a tetramethylrhodamine (rhodamine)-conjugated secondary antiserum (Cappel; 1:40

Figure 3. Examples of 5-HT-immunoreactive intrinsic neurons in a control preparation (*A*) and in an explanted preparation maintained in organ culture for 10 d (*B*). Calibrations, 40 μ m.

dilution) was used as the fluorophore. Control preparations were incubated with primary antisera that had been preabsorbed with excess substance P triacetate (Sigma) or a 5-HT bovine serum albumin (BSA) conjugate (Immunonuclear). These preparations exhibited no fluorescent-labeled cells or fibers.

A mouse monoclonal antiserum against synaptic vesicle antigen, a generous gift from Dr. L. A. Reichardt, was used to label synaptic terminals on the parasympathetic ganglionic cells (Matthew et al., 1981; Bowers et al., 1986). This antiserum was used at a dilution of 1:10, followed by exposure to a FITC-conjugated goat anti-mouse IgG (Cappel; 1:40). Although most experiments were performed at this dilution of primary antisera, a similar pattern of staining was obtained at a dilution of 1:100.

Each whole-mount preparation was examined with a Zeiss fluorescence photomicroscope equipped with an HBO 100 W UV light source. A 360 nm primary filter–405 nm secondary filter combination was used to visualize glyoxylic acid-induced fluorescence; a 565 nm primary filter–590 nm secondary filter combination was used to visualize rhodamine labeling; and a 485 nm primary filter–520 nm secondary filter combination was used to visualize FITC labeling. Photomicrography was performed using Ilford HP-5 black and white film push-processed 1 stop and Kodak D-19 to increase the contrast of the negative.

Organ culture of the parasympathetic ganglion. The septum containing the cardiac ganglion from either the mudpuppy or bullfrog was excised under sterile conditions, placed in tissue culture media (80% L-15, 1 mM CaCl_2 , 10 mM glucose, 7 ml fetal horse serum, 200 U/ml penicillin–streptomycin) and kept at approximately 18°C (McMahan and Kuffler, 1971). These explanted preparations were maintained in culture for 8–17 d.

Figure 4. Demonstration of substance P-immunoreactive fibers in a control mudpuppy cardiac ganglion preparation. *A*, Long immunoreactive fibers coursing across the septal tissue. *B*, Substance P-immunoreactive fibers in close apposition to 2 parasympathetic postganglionic neurons. *C*, Complex network of substance P-immunoreactive fibers surrounding a cluster of parasympathetic ganglion cells. Calibrations, 40 μ m.

Table 2. Comparison of substance P-like peptide content in control and explanted cardiac septa^a

Experiment	Condition	Amount of peptide (pmol/gm wet wt)
1	Control preparations	75.6
	8 d explanted preparations	50.0
2	Control preparations	107.0
	14 d explanted preparations	34.8
3	Control preparations	90.0
	14 d explanted preparations	58.3

^a Tissue pooled from 4 animals for control preparations and from 5 animals for explanted preparations.

Results

Biogenic amines in extracts derived from cardiac tissue containing the parasympathetic ganglion

In 3 separate trials using HPLC to analyze septal extracts, detectable quantities of NE, E, DA, and 5-HT were found (Table 1). Typical chromatograms for a solution of standards and for an extract of septal tissue are shown in Figure 1. In all 3 trials, the quantities of DA and 5-HT were comparable and markedly greater than those of either NE or E.

To determine which of these biogenic amines were derived from fiber processes of extraganglionic neurons and which from cells intrinsic to the ganglion, a similar analysis was undertaken using septal tissue sheets that were maintained, as an explant, in organ culture for 8–10 d. The extent of the septum and surrounding sinus venosus tissue excised was matched as closely as possible to tissue taken from control animals. The results obtained for extracts of cultured preparations are summarized in Table 1. After 8–10 d in culture, the levels of both NE and E were dramatically decreased. In contrast, the levels of DA and 5-HT were not significantly different from those obtained in control preparations.

Histochemical identification of cells and fibers containing biogenic amines

Two approaches were used to identify the cell types and fiber processes containing biogenic amines. First, glyoxylic acid-fixed preparations were used to determine the general distribution of amine-containing fibers and cells. Second, immunocytochemical procedures were used to test specifically for the presence of 5-HT-containing cells and fibers. The results obtained with each of these procedures were compared for freshly dissected preparations and for preparations maintained in organ culture for 8–10 d.

In glyoxylic acid-fixed preparations, numerous bundles of long, brightly fluorescent varicose fibers were observed coursing throughout the septum. Individual axons were seen forming complexes over strands of cardiac muscle, blood vessels, and individual parasympathetic ganglion cells (Fig. 2*A*). These long fluorescent fibers were not observed after 8 d in culture. It has been demonstrated previously that these long fluorescent fibers are sympathetic postganglionic axons (Neel and Parsons, 1986a). No parasympathetic ganglion cells exhibited catecholamine fluorescence; however, there were numerous SIF cells interspersed among the clusters of parasympathetic ganglion cells (Fig. 2*B*).

Figure 5. Examples of substance P-immunoreactive intrinsic neurons present in a control septal preparation (*A*) and an explanted septal preparation maintained in organ culture for 10 d (*B*). Calibrations, 25 μ m.

The general appearance and distribution of these SIF cells did not change after 8–10 d in culture (Fig. 2*C*).

In tissues prepared for 5-HT-immunocytochemistry, there was no evidence of any 5-HT-immunoreactive long fibers or parasympathetic postganglionic neurons; however, numerous small intrinsic neurons exhibited immunoreactivity for 5-HT (Fig. 3*A*). These neurons were similar in size, shape, and number of cellular processes to neurons exhibiting glyoxylic acid-induced fluorescence. These results confirmed those obtained in a previous study of the histochemical characteristics of the intrinsic neurons in the cardiac ganglion (Neel and Parsons, 1986b). The morphology of these 5-HT-immunoreactive intrinsic neurons was similar in both freshly dissected preparations and preparations maintained in culture for 8–10 d (Fig. 3*B*).

Substance P-like peptide in tissue extracts from mudpuppy myocardium

Freshly dissected mudpuppy cardiac septal tissue was found to contain a significant amount of immunoreactive substance P-like peptide (89.2 ± 10.4 pmol/gm wet wt; mean $\pm$ SEM of 6 assays using 2–4 septa/trial) and, after 8–14 d in organ culture, the level of the substance P-like material was reduced to 30–50% (Table 2). These results suggest that a considerable amount

Figure 6. Demonstration of the presence of substance P-immunoreactive fibers in explanted septal preparations maintained in organ culture for 14–17 d. *A*, Bundle of substance P-immunoreactive fibers coursing across the septum (14 d in culture). *B*, Complex fiber network over a group of parasympathetic ganglion cells (17 d in culture). *C*, Long immunoreactive fibers in close apposition to an isolated parasympathetic ganglion cell (17 d in culture). Calibrations, 30 μ m.

Figure 7. Comparison of the immunoreactivity to substance P antiserum (*A, C*) or antivesicle membrane antiserum (*B, D*) in control bullfrog septal preparations (*A, B*) with explanted bullfrog septal preparations maintained in organ culture for 10 d (*C, D*). *A*, Substance P-immunoreactive fibers and preganglionic fiber terminations on individual parasympathetic ganglion cells. *C*, Marked reduction in substance P immunoreactivity in an explanted preparation. *B*, the pattern of immunoreactivity seen in a control preparation with Antivesicle antiserum. Each parasympathetic ganglion cell has numerous brightly fluorescent spots on its soma and axon hillock regions. *D*, These immunoreactive fluorescent spots are no longer present after 10 d in organ culture. Calibrations, 30 μ m in *A* and *D*; and 40 μ m in *B* and *C*.

of the substance P-like peptide is contained in processes of cells that are extraseptal in origin.

Distribution of substance P-like peptide containing cells and fiber processes

Numerous substance P-reactive fibers were found in bundles coursing across the cardiac septum (Fig. 4*A*) and were also present in the vagal trunks. Some of these fibers formed a network over strands of cardiac muscle, made pericellular appositions to individual ganglion cells (Fig. 4*B*), or formed a complex fiber network over clusters of ganglion cells (Fig. 4*C*). The arborizations seen around individual ganglion cells ranged from loose spirals to more closely confined apparent contacts. Individual substance P-immunoreactive fibers most often appeared to contact multiple cells within a cluster and not to be restricted to an individual ganglion cell. Not all ganglion cells were contacted by substance P-like fiber processes. In areas of the septum where individual ganglion cells or groups of ganglion cells were readily visualized, we estimated that 40–50% of these cells received close contact from substance P-immunoreactive fibers. In the

main portion of the ganglion near the ventricle, the density of substance P-reactive fibers often made it impossible to quantitate the relationship between these fibers and ganglion cells.

In addition to the long fiber processes, there were numerous small intrinsic neurons that also exhibited substance P immunoreactivity (Fig. 5*A*) (Neel and Parsons, 1986*a*). These cells were similar in their morphological characteristics to those identified as SIF cells. Processes from some of these cells appeared to also come into close apposition with adjacent ganglion cells. However, the processes derived from the intrinsic substance P-immunoreactive neurons extended for much shorter distances than those of the majority of the long substance P-immunoreactive fibers. As reported previously, no ganglion cells were found that exhibited substance P immunoreactivity (Neel and Parsons, 1986*b*).

Substance P-immunocytochemical experiments were also carried out, using septal preparations cultured for 8–17 d. The distribution of the substance P-immunoreactive fibers and cells in cultured preparations was very similar to that in freshly dissected preparations. Further, the morphological characteristics

of substance P-immunoreactive intrinsic neurons were not noticeably different in cultured preparations (Fig. 5*B*) from those observed in freshly dissected preparations. Numerous long substance P-reactive fiber bundles were present, coursing across the septum (Fig. 6*A*) and forming fiber networks on strands of cardiac muscle. Individual ganglion cells, as well as clusters of ganglion cells, were seen surrounded by complex networks of substance P-immunoreactive fibers (Fig. 6, *B*, *C*). Although the pattern of substance P-immunoreactive fiber distribution appeared quite similar in explanted and normal preparations, the fluorescence in the long substance P-immunoreactive fibers was less intense in the cultured preparations, especially those maintained longer than 8 d. Thus, the decrease in substance P-like material, determined biochemically, probably resulted from the early stages of long substance P-immunoreactive fiber degeneration.

Origin of the long substance P-immunoreactive fibers

We hypothesized that the long extraganglionic substance P-immunoreactive fibers in the mudpuppy cardiac ganglion were processes of primary afferent fibers, as seen in mammalian sympathetic ganglia (Hökfelt et al., 1977; Gamse et al., 1981; Matthews and Cuello, 1982), rather than preganglionic terminals, as found in the bullfrog cardiac ganglion (Bowers et al., 1987). To test this hypothesis, we compared the change in immunoreactivity observed when using antisera against synaptic vesicle membrane and substance P in control ganglia with cultured ganglia. Parallel experiments were performed using mudpuppy and bullfrog cardiac ganglion preparations. Previously, Bowers et al. (1987) showed that a substance P-like peptide is colocalized with acetylcholine in the preganglionic terminals in the bullfrog cardiac ganglion and that both the antivesicle antiserum and substance P antiserum effectively label preganglionic terminals in bullfrog preparations. It was assumed that the degeneration of the preganglionic synaptic terminations would have a similar time course in bullfrog and mudpuppy preparations under comparable culture conditions. Therefore, it was further assumed that the bullfrog preparation could be used to indicate the time course of degeneration of amphibian preganglionic terminals.

In freshly dissected bullfrog cardiac ganglia, every ganglion cell exhibited immunoreactive staining for both substance P and antivesicle membrane on its axon hillock and soma (Fig. 7, *A*, *B*). In addition, substance P-immunoreactive fibers were seen within the vagal trunk and coursing across the septal tissue sheet. Fibers within the vagal trunk and out in the septum also exhibited immunoreactivity to the antivesicle antisera. After 8 d in organ culture, only a few ganglion cells exhibited immunoreactive staining on their somas or axon hillocks using either the anti-substance P or antivesicle antisera. In addition, the intensity of the staining of fibers within the vagus was less than that in freshly dissected preparations. After 10–14 d, no ganglion cells were observed displaying spotted staining on their axon hillocks or somata (Fig. 7, *C*, *D*). These results demonstrated that amphibian preganglionic terminals degenerated within 8–10 d in culture. By contrast, in these same preparations some long fiber processes, immunoreactive for either substance P or synaptic vesicle antigens, were evident coursing across the septum, over strands of cardiac muscle, and within the vagal nerve trunks. However, in these cultured bullfrog preparations, the substance P-immunoreactive long fibers exhibited less intense fluorescence than those in control preparations.

Figure 8. Demonstration of the loss of immunoreactivity to the antivesicle antiserum in mudpuppy cardiac ganglia following 10 d in organ culture. *A*, Pattern of immunoreactivity observed with the antivesicle antiserum in a control mudpuppy preparation. Every parasympathetic ganglion cell exhibits brightly fluorescent spots concentrated on the somata and axon hillock regions. Also evident is an immunoreactive intrinsic neuron indicated by the arrow. *B*, Marked reduction in immunoreactivity to the antivesicle antiserum in an explanted preparation organ cultured for 10 d. Calibration, 30 μ m in *A*; 40 μ m in *B*.

In freshly dissected mudpuppy preparations, similar to that seen in the bullfrog ganglion, all parasympathetic ganglion cells exhibited antivesicle immunoreactive fluorescent spots on the cell soma and axon hillock (Fig. 8*A*). In addition, immunoreactivity was evident in many nerve fibers in the mudpuppy preparation, both within the ganglion and on the septum. Many individual intrinsic neurons also exhibited an intense cytoplasmic immunoreactivity (Fig. 8*A*). There was a marked decrease in fluorescent staining on mudpuppy ganglion cells maintained in organ culture for 8–13 d. In most instances, fluorescent sites were not present on individual ganglion cells (Fig. 8*B*). However, the intrinsic neurons in these cultured preparations showed immunoreactivity similar to that in freshly dissected preparations. Many fibers coursing over the septum and branching over strands of muscle also retained immunoreactivity in the cultured mudpuppy preparations.

Figure 9. Schematic diagram summarizing the organization of the mudpuppy cardiac ganglion. Two cell types are shown. Parasympathetic postganglionic projection cells (*principal cells*) innervate the cardiac muscle and *intrinsic neurons*. Each principal cell is innervated by *parasympathetic preganglionic axons*, as are some of the interneurons. Processes of the intrinsic neurons are intermingled between the clusters of postganglionic neurons. In addition, catecholamine-containing *sympathetic postganglionic axons* and collateral processes of substance P-immunoreactive primary afferent fibers appear to be in close apposition to individual parasympathetic ganglion cells. Drawing modified from that by McMahan in Hartzell et al., 1976.

Figure 10. Schematic diagram illustrating an anatomical circuit possibly subserving local reflex control of the parasympathetic cardiac ganglion cells. Collateral processes of primary sensory fibers are shown in close apposition to individual *principal cells* as they course through the cardiac ganglion towards the spinal cord or cranial nerve nuclei. These *afferent fiber processes* could provide a sensory input to postganglionic neurons, which in turn innervate *cardiac muscle*.

Discussion

Figure 9 presents a diagrammatic representation of the basic features of the mudpuppy cardiac ganglion, incorporating the findings presented in this report. There are 2 neuron types in the mudpuppy cardiac ganglion: principal projection neurons, which innervate cardiac muscle, and smaller intrinsic neurons, which are dispersed between the ganglion cells. These small intrinsic neurons form complexes with adjacent principal cells and blood vessels. McMahan and Purves (1976) have previously shown that the preganglionic cholinergic fibers primarily innervate the principal cells, although a few intrinsic neurons also receive vagal input. The present results indicate, as do those published previously (Neel and Parsons, 1986a), that these intrinsic cells may contain a catecholamine (primarily DA), 5-HT, a substance P-like peptide, or some combination of these. Many other long substance P-immunoreactive fibers come in close apposition with both the principal cells and smaller intrinsic neurons as they course through the cardiac ganglion. Some ganglion cells also come into close apposition or pericellular contact with sympathetic postganglionic axons (Neel and Parsons, 1986b).

We have postulated that most of the substance P-immunoreactive fibers coming into close apposition to individual cells or clusters of ganglion cells are not parasympathetic preganglionic axons, but, rather, are processes of primary sensory fibers. This conclusion is based on the following observations: First, the pattern of substance P-immunoreactive fibers, in close approximation with ganglion cells, does not resemble the type of synaptic contact demonstrated previously for preganglionic fiber terminals (McMahan and Purves, 1976). In many cases, the substance P fibers appeared to make loose pericellular con-

nections around individual cells, whereas the preganglionic fibers form a close spiral around the axon hillock and soma of each ganglion cell. Second, the immunoreactivity against the synaptic vesicle membrane, producing a pattern similar to that reported for preganglionic terminals stained with zinc iodide (McMahan and Purves, 1976), was reduced much more dramatically than the immunoreactivity for substance P after 8–10 d in culture. This difference demonstrates that the terminal portions of the preganglionic fibers synapsing on individual ganglion cells degenerated more rapidly than the substance P-immunoreactive fiber processes. The results, using the bullfrog cardiac ganglion, support our view that preganglionic parasympathetic terminals in amphibians degenerate within 8–10 d in culture. The presence of substance P-immunoreactive fibers making contact with mudpuppy ganglion cells after 17 d in culture supports the view that these fibers are not parasympathetic preganglionic axon terminals. Last, substance P-like immunoreactive fibers have been found in the hearts of several species and it is generally thought that these fibers are primary sensory nerves (Weihi et al., 1984; Urban and Papka, 1985).

We further suggest that the substance P-immunoreactive fibers may represent an anatomical circuit underlying local reflex control of cardiac function (Fig. 10). These fibers could make direct synaptic contact on ganglion cells, as occurs in some mammalian sympathetic ganglia (Matthews and Cuello, 1982), or exhibit their influence from a distance (Jan and Jan, 1982). One possibility is that the released substance P-like peptide promotes vasodilation via interaction with these nervous elements and/or mast cells or via direct effects upon vascular smooth muscle. Synaptic profiles containing large granular vesicles, typical of peptidergic synaptic contacts, have been observed in a recent ultrastructural analysis of the synaptic profiles of individual ganglion cells (R. Kriebel and R. L. Parsons, unpublished observations). However, the frequency of this type of synaptic terminal is quite small. Numerous cell processes containing large dense granular vesicles have also been seen coursing throughout the neuropil between individual ganglion cells and adjacent small intrinsic neurons.

Numerous biogenic amine-containing fibers are present within the mudpuppy cardiac ganglion (McMahan and Purves, 1976; Neel and Parsons, 1986a, b). These are derived from both extraganglionic and intrinsic neurons. The results of the present study confirm that most of the catecholamine within the intrinsic neurons is DA (Hartzell et al., 1977). However, a smaller amount of NE may also be contained in some SIF cells. Previously, we demonstrated that many of the small intrinsic neurons exhibited 5-HT immunoreactivity (Neel and Parsons, 1986b). The results obtained in the present study demonstrate that the 5-HT content is comparable to that of DA. Therefore, some of the small intrinsic neurons may use 5-HT as a transmitter substance either in addition to, or in place of, a catecholamine. Hartzell et al. (1977) indicated that the direct application of catecholamines to mudpuppy cardiac ganglion cells produced no measurable alteration of the membrane potential. However, the inability to demonstrate any direct effect of the catecholamines does not rule out more indirect influences that they may exert within the ganglion. For instance, it is well established that the catecholamines have presynaptic sites of action in many autonomic ganglia through which they modify transmitter release from preganglionic nerve terminals (Christ and Nishi, 1971a, b). Serotonin is also known to exert both pre- and postsynaptic actions at different synaptic sites (Hirai and

Koketsu, 1980; North et al., 1980). In view of the high endogenous levels of DA and 5-HT reported here, the action of these biogenic amines on ganglionic transmission within the cardiac ganglion should be further investigated.

In higher vertebrates, such as birds and mammals, the parasympathetic cardiac cells are quite small and the ganglion is often diffuse, embedded in the myocardial tissue or contained in connective tissue complexes on the entrance of the major vessels of the heart (Ellison and Hibbs, 1976; Kirby et al., 1980; Löffelholz and Pappano, 1985). Difficulties in isolating the cardiac ganglion in these species have limited previous studies of the synaptic mechanisms that integrate information from the central nervous system and from local reflex afferents. Therefore, the anatomical and histochemical organization of the mudpuppy parasympathetic cardiac ganglion makes it an excellent model system for undertaking correlative anatomical and physiological studies of integration within vertebrate cardiac ganglia. The neural elements of the mudpuppy cardiac ganglion are not organized in the fashion expected of a simple relay system. Rather, the complex morphological and histochemical organization supports the possibility of a more sophisticated role for the cardiac ganglion in the regulation of heart function. The complexity of the mudpuppy cardiac ganglion is similar to that exhibited by many mammalian sympathetic and enteric autonomic ganglia (Llewellyn-Smith et al., 1983; Schultzberg et al., 1983). For example, both mudpuppy cardiac ganglia and mammalian ganglia have intrinsic as well as postganglionic neurons and a variety of peptides or amine transmitters, in addition to the preganglionic transmitter, acetylcholine.

The results of the present study have demonstrated the presence of a number of putative transmitter substances, as well as a complex organization of the mudpuppy cardiac ganglion. They provide a framework for future studies concerning the possible role of identified neuroactive substances within a vertebrate parasympathetic cardiac ganglion.

References

- Bowers, C. W., L. Y. Jan, and Y. N. Jan (1986) A substance P-like peptide in bullfrog autonomic nerve terminals: Anatomy, biochemistry and physiology. *Neuroscience* 19: 343–356.
- Christ, D. D., and S. Nishi (1971a) Effects of adrenaline on nerve terminals in the superior cervical ganglion of the rabbit. *Br. J. Pharmacol.* 41: 331–338.
- Christ, D. D., and S. Nishi (1971b) Site of adrenaline blockade in the superior cervical ganglion of the rabbit. *J. Physiol. (Lond.)* 213: 107–117.
- Costa, M., R. Buffa, J. B. Furness, and E. Solcia (1980) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. *Histochemistry* 65: 157–165.
- Ehinger, B., E. Sundler, and R. Uddman (1983) Functional morphology in two parasympathetic ganglia: The ciliary and pterygopalatine. In *Autonomic Ganglia*, L.-G. Elfvin, ed., pp. 97–123, Wiley, New York.
- Ellison, J. P., and R. G. Hibbs (1976) An ultrastructural study of mammalian cardiac ganglia. *J. Mol. Cell. Cardiol.* 8: 89–101.
- Furness, J. B., and M. Costa (1975) The use of glyoxylic acid for the fluorescence histochemical demonstration of peripheral stores of noradrenaline and 5-hydroxytryptamine in whole mounts. *Histochemistry* 41: 335–352.
- Gamse, R., A. Wax, R. E. Zigmond, and S. E. Leeman (1981) Immunoreactive substance P in sympathetic ganglia: Distribution and sensitivity towards capsaicin. *Neuroscience* 6: 437–441.
- Hartzell, H. C., S. W. Kuffler, R. Strichgold, and D. Yoshikami (1977) Dynoptic excitation and inhibition resulting from direct action of acetylcholine on two types of chemoreceptors on individual amphibian parasympathetic neurons. *J. Physiol. (Lond.)* 271: 817–846.
- Hirai, K., and K. Koketsu (1980) Presynaptic regulation of the release

- of acetylcholine by 5-hydroxytryptamine. *Br. J. Pharmacol.* 70: 499–500.
- Hökfelt, T., L.-G. Elfvin, M. Schultzberg, M. Goldstein, and G. Nilsson (1977) On the occurrence of substance P-containing fibers in sympathetic ganglia: Immunohistochemical evidence. *Brain Res.* 132: 29–41.
- Jan, L. Y., and Y. N. Jan (1982) Peptidergic transmission in sympathetic ganglia of the frog. *J. Physiol. (Lond.)* 327: 219–246.
- Kirby, M. L., T. A. Weidman, and J. W. McKenzie (1980) An ultrastructural study of the cardiac ganglia in the bulbar plexus of the developing chick heart. *Dev. Neurosci.* 3: 174–184.
- Kontur, P., R. Dawson, and A. Monjan (1984) Manipulation of mobile phase parameters for the HPLC separation of endogenous monoamines in rat brain tissue. *J. Neurosci. Methods* 11: 5–18.
- Kreulen, D. L., J. H. Szurszewski, M. Schultzberg, Z. J. Bosnjak, and J. Krier (1985) Symposium: Sensory mechanisms in sympathetic ganglia. *Soc. Neurosci. Abstr.* 11: 363.
- Llewellyn-Smith, I. G., J. B. Furness, A. J. Wilson, and M. Costa (1983) Organization and fine structure of enteric ganglia. In *Autonomic Ganglia*, L.-G. Elfvin, ed., pp. 145–182, Wiley, New York.
- Löffelholz, K., and A. J. Pappano (1985) The parasympathetic neuroeffector junction of the heart. *Pharmacol. Rev.* 37: 1–24.
- Matthew, W. D., L. Tsavaler, and L. F. Reichardt (1981) Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J. Cell Biol.* 91: 257–269.
- Matthews, M. R., and A. C. Cuello (1982) Substance P-immunoreactive peripheral branches of sensory neurons innervate guinea pig sympathetic neurons. *Proc. Natl. Acad. Sci. USA* 79: 1668–1672.
- McMahan, U. J., and S. W. Kuffler (1971) Visual identification of synaptic boutons on living ganglion cells and varicosities in postganglionic axons in the heart of the frog. *Proc. R. Soc. Lond. [Biol.]* 177: 485–508.
- McMahan, U. J., and D. Purves (1976) Visual identification of two kinds of nerve cells and their synaptic contacts in a living autonomic ganglion of the mudpuppy (*Necturus maculosus*). *J. Physiol. (Lond.)* 254: 405–425.
- Mroz, E. A., and S. E. Leeman (1979) Substance P. In *Methods of Hormone Radioassay*, B. M. Jaffe and H. R. Behrman, eds., pp. 121–137, Academic, New York.
- Neel, D. S., and R. L. Parsons (1986a) Anatomical evidence for the interaction of sympathetic and parasympathetic neurons in the cardiac ganglion of *Necturus*. *J. Auton. Nerv. Syst.* 15: 297–308.
- Neel, D. S., and R. L. Parsons (1986b) Catecholamine, serotonin, and substance P-like peptide containing intrinsic neurons in the mudpuppy parasympathetic cardiac ganglion. *J. Neurosci.* 6: 1970–1975.
- North, R. A., G. Henderson, Y. Katayama, and S. M. Johnson (1980) Electrophysiological evidence for presynaptic inhibition of acetylcholine release by 5-hydroxytryptamine in the enteric nervous system. *Neuroscience* 5: 581–586.
- Schultzberg, M., T. Hökfelt, J. M. Lundberg, C. J. Dalsgaard, and L.-G. Elfvin (1983) Transmitter histochemistry of autonomic ganglia. In *Autonomic Ganglia*, L.-G. Elfvin, ed., pp. 205–233, Wiley, New York.
- Urban, L., and R. E. Papka (1985) Origin of small primary afferent substance P-immunoreactive nerve fibers in the guinea-pig heart. *J. Auton. Nerv. Syst.* 12: 321–331.
- Weihi, E., M. Reinecki, and W. G. Forssman (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.