Parasympathetic control of the heart. II. A novel interganglionic intrinsic cardiac circuit mediates neural control of heart rate

Alrich L. Gray, Tannis A. Johnson, Jeffrey L. Ardell, and V. John Massari

1Department of Pharmacology and 2Specialized Neuroscience Research Program, Howard University
College of Medicine, Washington, District of Columbia 20059; and 3Department of Pharmacology,
East Tennessee State University, James H. Quillen College of Medicine, Johnson City, Tennessee 37614

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Gray, Alrich L., Tannis A. Johnson, Jeffrey L. Ardell, and V. John Massari. Parasympathetic control of the heart. II. A novel interganglionic intrinsic cardiac circuit mediates neural control of heart rate. J Appl Physiol 96: 2273–2278, 2004. First published February 20, 2004; 10.1152/japplphysiol.00616.2003.—Intracardiac pathways mediating the parasympathetic control of various cardiac functions are incompletely understood. Several intracardiac ganglia have been demonstrated to potently influence cardiac rate [the sinoatrial (SA) ganglion], atrioventricular (AV) conduction (the AV ganglion), or left ventricular contractility (the cranioventricular ganglion). However, there are numerous ganglia found throughout the heart whose functions are poorly characterized. One such ganglion, the posterior atrial (PA) ganglion, is found in a fat pad on the rostral dorsal surface of the right atrium. We have investigated the potential impact of this ganglion on cardiac rate and AV conduction. We report that microinjections of a ganglionic blocker into the PA ganglion significantly attenuates the negative chronotropic effects of vagal stimulation without significantly influencing negative dromotropic effects. Because prior evidence indicates that the PA ganglion does not project to the SA node, we neuroanatomically tested the hypothesis that the PA ganglion mediates its effect on cardiac rate through an interganglionic projection to the SA ganglion. Subsequent to microinjections of the retrograde tracer fast blue into the SA ganglion, >70% of the retrogradely labeled neurons found within five intracardiac ganglia throughout the heart were observed in the PA ganglion. The neuroanatomic data further indicate that interganglionic neuronal circuits are found within the SA ganglion. The present data support the hypothesis that two interacting cardiac centers, i.e., the SA and PA ganglia, mediate the peripheral parasympathetic control of cardiac rate. These data further support the emerging concept of an intrinsic cardiac nervous system.

ganglia; neurocardiology; vagus; retrograde transport; atrioventricular conduction; posterior atrial ganglion

IT HAS LONG BEEN KNOWN that parasympathetic influences on cardiac function are mediated via the vagus nerves. Stimulation of the vagi results in multiple cardiac effects, including bradycardia, atrioventricular (AV) block, and reduced myocardial contractility (32). Over the past three decades, the late Walter Randall and colleagues, as well as other investigators, have shown that postganglionic neurons contained in discrete fat pads on the surface of the heart are responsible for mediating certain aspects of cardiac function (3, 11, 14). Randall’s findings have been supported in the dog by independent investigators not associated with their group (15, 17, 36), in primates (9), in humans (13, 30), and in our own laboratories in the cat (19, 20). For example, ganglion cells found in a fat pad located adjacent to the right pulmonary veins near the junction of the right atrium and superior vena cava have been shown to mediate a selective negative chronotropic effect. Thus surgical excision of this fat pad or local microinjections of ganglionic blocking agents result in blockade of the negative chronotropic effects of vagal stimulation. Due to the potent negative chronotropic nature of the ganglion cells found in this fat pad, we have referred to the ganglion cells found in this region as the sinoatrial (SA) ganglion (18, 20, 24, 25). In a similar fashion, ganglion cells found in a fat pad located at the junction of the inferior border of the left atrium and the inferior vena cava have been shown to mediate a potent reduction in the rate of AV conduction. Therefore, we have referred to the negative dromotropic ganglion cells found in this region as the AV ganglion (20, 23, 24). Furthermore, a fat pad found at the cranial margin of the left ventricle near its junction with the right ventricle has been found to contain ganglion cells that selectively mediate negative inotropic vagal effects on the left ventricle without influencing cardiac rate or AV conduction (16, 19). We refer to the ganglion cells contained within this region as the cranioventricular (CV) ganglion. Different authors have referred to the various intracardiac ganglia utilizing different names (1, 8, 14, 28, 29, 35); however, the anatomic location of functionally associated intracardiac ganglia across different species shows substantial homology [for further details about nomenclature and locations of intracardiac ganglia, see Johnson et al. (21)]. Taken as a whole, the data reviewed above are consistent with our working hypothesis that there are anatomically separated and functionally independent intracardiac ganglia found in fat pads on the surface of the heart.

There are numerous ganglia found throughout the heart for which little or no functional role(s) have been ascribed (1, 4, 12, 21, 37). One such ganglion is found in a fat pad on the rostral dorsal surface of the right atrium (26, 31). To maintain consistency with our nomenclature scheme, we refer to this ganglion as the posterior atrial (PA) ganglion. Preliminary studies conducted by David Randall and colleagues utilizing a discriminative classical Pavlovian conditioning procedure have provided evidence suggesting that this ganglion may be involved in attenuating sympathetic cardioacceleration in the dog (31); however, little is known about the potential effects of the PA ganglion on cardiac rate or AV conduction. We have investigated these issues in the present report with both physiological and neuroanatomic methods.

Address for reprint requests and other correspondence: V. J. Massari, Dept. of Pharmacology, Howard Univ. College of Medicine, 520 W St., N.W., Washington, DC 20059 (E-mail: vmassari@howard.edu).

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MATERIALS AND METHODS

Physiological studies. The Institutional Animal Care and Use Committee of Howard University reviewed and approved the experimental design of all animal experiments. Acute physiological experiments were performed on five mongrel cats weighing 2.8–4.0 kg. Baseline vital signs such as temperature, respiration rate, and heart rate were recorded. Cats were anesthetized with 25 mg/kg iv pentobarbital sodium. Supplemental doses of 1.25–1.5 mg/kg iv pentobarbital sodium were administered as needed. The cats were assessed for depth of anesthesia by testing for the absence of the palpebral, ocular, and pedal reflexes both before the beginning of surgery and every half hour thereafter. Each animal’s core temperature was maintained at 37–38°C using a servocontrolled heating pad. Cats were prepared for the experiment by inserting an intravenous catheter into the brachial vein and intubating with a cuffed endotracheal tube. Limb leads were attached to appropriate appendages to record lead II of the ECG. All electrical leads were connected to a Gould TA 6000 thermal array recorder. A Pulse-Oximeter (Vet-Ox) clamp was placed on the tongue and was used to monitor blood oxygen concentration throughout the experiment. Once the surgical plane of anesthesia was reached, the right cervical vagus was isolated, a 0.5-mm platinum-stimulating electrode was placed on it, and it was bathed in mineral oil. The left femoral artery was also isolated, and a Millar probe (Millar Instruments, Houston, TX) was inserted into it to record blood pressure. Blood pressure readings served as a very sensitive indicator of depth of anesthesia. A thoracotomy was performed via the right fifth intercostal space. Cats were artificially respirated with 95% O 2 -5% CO 2 on a positive-pressure respirator cycling at 12 breaths/min with a tidal volume setting between 50 and 100 ml. A longitudinal incision was made in the pericardium, and the heart was removed from the pericardial sac. A pacing wire was sutured onto the right atrial appendage. Atrial pacing was performed at a rate 10% greater than resting sinus rate. The right vagus was stimulated over 20-s intervals at 20 Hz, 15 V, and 0.1-ms duration. These parameters were found to result in maximal vagal stimulation. Heart rates were obtained from the R-R interval. AV conduction measurements were obtained from the stimulus artifact-R interval during atrial pacing. Heart rate and AV conduction were recorded before and during the stimulation interval. Heart rate and AV conduction measurements were calculated from beats that occurred during the middle of the stimulation interval. Baseline AV conduction and heart rate responses to three consecutive vagal stimulations were recorded with and without atrial pacing, respectively. The PA ganglion was identified by its gross anatomic location (21), and 10 μl of the ganglionic blocker hexamethonium (C6) (5 μg/μl) was injected into it. Five minutes later, the vagus was stimulated three consecutive times with and without the heart being paced, and C6-induced changes in heart rate and AV conduction were recorded. Thirty minutes was allowed for the ganglion to recover from the effects of the ganglionic blocker. After this time, the right vagus nerve was again stimulated three consecutive times with and without the heart being paced, and effects on heart rate and AV conduction were recorded again. If the condition of the cat permitted, the process was repeated.

At the end of the experimental protocol, a series of controls was performed. Because in the experimental animals the heart was removed from the pericardial sac, potential leakage of the ganglionic blocker from the injection site would result in its deposition in the chest cavity. To control for potential effects produced by such leakage, 10 μl of C6 (5 μg/μl) was injected into the chest cavity of all five experimental animals, and the effects of vagal stimulation on heart rate and AV conduction before and after C6 injection were compared.

Hemodynamic data analysis. The heart rates obtained before vagal stimulation and during the peak response to vagal stimulation (maximum R-R interval during the stimulation period) each were averaged over three consecutive trials to provide a mean heart rate before and during vagal stimulation. Mean AV intervals were calculated in an analogous fashion (maximal stimulus-R interval during the stimulation period) during the trials while the heart was being paced. These results were obtained before blockade of the PA ganglion (baseline), during blockade of the PA ganglion, and after recovery of the PA ganglion from that blockade. The mean peak response to vagal stimulation was normalized to percent change from the mean prestimulation levels. The percent change in heart rate and AV conduction during right cervical vagal stimulation before pharmacological blockade and during pharmacological blockade of the PA ganglion was analyzed with a paired t-test. A P value of <0.05 was considered statistically significant.

Retrograde tracing studies. Experiments were performed on eight mongrel cats weighing 2.8–4.0 kg. Baseline vital signs of temperature, respiration rate, and heart rate were recorded. Cats were pretreated with 0.05 mg/kg of atrovent to reduce secretions followed by 22 mg/kg of ketamine and 0.2 mg/kg of acepromazine for induction of anesthesia. Cats were prepared for surgery by inserting an intravenous catheter into the brachial vein and intubating with a cuffed endotracheal tube. Isoflurane gas anesthesia was then used to bring the animal to a surgical plane of anesthesia. Cardiac rate and blood oxygen concentration were monitored as described above. A thoracotomy was performed via the right fifth intercostal space. At this point, the cat was artificially respirated on a positive-pressure respirator with a tidal volume setting between 50 and 100 ml and cycling at 12 breaths/min. An incision was made into the pericardium that was large enough to expose the heart and allow for neuroanatomic identification and access to the SA ganglion (21). One microliter of a 2% solution of the retrograde tracer fast blue (27, 33) was injected into the SA ganglion in five cats and into the pericardial sac in an additional three cats. After injections were made, the pericardium was closed, the muscles and skin were sutured in layers, spontaneous respiration was reestablished, fluids along with the potent analgesic butorphanol (0.2 mg/kg) and the antibiotic penicillin procaine G (30,000 IU/kg) were administered, and the animal was awakened from anesthesia. Four days after the injection, the cats were killed under deep barbiturate anesthesia (50 mg/kg ip pentobarbital sodium) by intravenous perfusion with a phosphate-buffered solution containing fixatives as previously described in detail (21). The hearts were removed and then postfixed in the same solution for 2 h. Hearts were then cryoprotected by placing them into a 20% sucrose phosphate-buffered solution for 3 days followed by a 30% sucrose phosphate-buffered solution for 4 days. After cryoprotection, hearts were flash frozen, and transverse serial sections 50 μm thick were cut as previously described (21). Every third section was examined via fluorescence microscopy using UV optics. All intrinsic cardiac ganglia were inspected for the presence of retrogradely labeled neurons. Neuronal counts that are reported were adjusted by multiplication by a factor of three.

RESULTS

Physiological studies. Stimulation of the right cervical vagus, before pharmacological blockade of the PA ganglion, resulted in a decrease in heart rate from a baseline of 183 ± 6 to 110 ± 12 beats/min, a 40 ± 5% change (P < 0.001; n = 5) (Figs. 1 and 2). After hexamethonium was injected into the PA ganglion, the vagally induced negative chronotropic effects were attenuated, and stimulation of the right cervical vagus resulted in a decrease in heart rate from 186 ± 12 to 144 ± 14 beats/min, a 23 ± 4% change (P < 0.001; n = 5) (Figs. 1 and 2). After time for drug-induced ganglionic blockade was allowed to wear off, recovery of function was observed (Fig. 1).
During atrial pacing, vagal stimulation before blockade of the PA ganglion prolonged the rate of AV conduction from a baseline of 111 ± 6 to 149 ± 14 ms, a 35 ± 10% change ($P = 0.01; n = 5$) (Fig. 2). During blockade of the PA ganglion, stimulation of the right cervical vagus did not significantly change the effects of vagal stimulation on AV conduction time ($P > 0.05$) (Fig. 2). Injecting C6 at the same dose into the chest cavity or intravenously had no effect on vagally induced changes in heart rate or AV conduction.

**Retrograde tracing studies.** We inspected six intrinsic cardiac ganglia for the presence of neurons retrogradely labeled from the microinjection of fast blue into the SA ganglion. Three of these ganglia were associated with the atria, i.e., the PA ganglion, the SA ganglion, and the AV ganglion. The other three ganglia were distributed on or within the ventricles, i.e., the CV ganglion on the surface of the left ventricle, the right ventricular (RV) ganglion on the surface of the right ventricle, and the interventriculoseptal (IVS) ganglion within the interventricular septum (for details, see Ref. 21).

This study involved the injection of 1 μl of fast blue fluorescent tracer into two different sites. In the experimental group of animals, the tracer was injected directly into the SA ganglion (Fig. 3), whereas in the control group of animals, the tracer was injected into the pericardial sac. This control group was exceptionally conservative insofar as it presumes that as much as 100% of the tracer could potentially leak from the injection site into the SA ganglion onto surrounding structures in the heart.

Four days after the injection of fluorescent tracer into the SA ganglion, the extent of diffusion of the tracer was limited to the SA fat pad. A total of 78.6 ± 2.7% of the neurons in the SA ganglion, which were identifiable, were labeled; however, not all labeled neurons could be unequivocally identified due to the intense fluorescence that was seen at the center of the injection site (Fig. 3A). While many of the neurons of the SA ganglion appeared to be nonspecifically labeled by their close apposition to high concentrations of the tracer near the center of the injection site (Fig. 3A), multiple neurons within the SA ganglion were clearly labeled but were not surrounded by detectable levels of fast blue (Fig. 3B) in each of the animals examined.

Retrogradely labeled neurons containing the tracer were also readily identifiable within intracardiac ganglia outside of the SA ganglion (Fig. 4). Statistical analysis of the percentage of labeled cells found within each ganglion in the experimental group utilizing a one-way ANOVA test (Fig. 5) showed that there was a statistically significant difference in the number of labeled neurons found within different intrinsic cardiac ganglia ($F(4,15) = 45.62 (P < 0.001)$). A Dunnett’s T3 post hoc test showed that the percentage of labeled cells found in the PA ganglion was significantly larger than that found in all other ganglia, with all $P$ values of ≤0.01. All other permutations of comparisons between ganglia, however, were statistically nonsignificant. Furthermore, when tested with an independent two-sample $t$-test assuming unequal variances, there was a significant difference in the mean percentage of labeled cells found in the PA ganglion in the experimental group of animals (71.4 ± 6.8%) compared with that found in the PA ganglion in the control group of animals (26.8 ± 4.2%) ($P = 0.001$) (Fig. 5).

In one of the five animals utilized in this study, the injection of the fluorescent tracer missed the SA ganglion by ~2 mm. In this animal, only 29.2% of the retrogradely labeled neurons were found in the PA ganglion, which is similar to that found in the experimental group. The results from this animal were excluded from analysis but served as a negative control. It was
not possible to identify the exact location of the axonal pathway interconnecting the PA and SA ganglia in the tissues examined using fast blue as a tracer, due both to low levels of the tracer in axons and problems with fluorescence quenching during microscopic observation of the heart.

**DISCUSSION**

In the classical tradition of Langley (22), neurons within the heart have been considered to be exclusively postganglionic parasympathetic efferents. This concept of the neuroanatomic organization of the heart has persisted for almost a century. In more recent times, however, an increasing body of primarily physiological data has been consistent with the hypothesis that neurons within intracardiac ganglia do not serve as simple relays between the central nervous system and their effectors (2, 4, 6, 7, 31, 34). Rather, it has been postulated that individual cardiac ganglia may serve as complex integrative centers within which considerable processing of autonomic signals may occur. These physiological data have resulted in the proposal of several relatively elaborate schematic descriptions of the organization of what has been referred to as an “intrinsic cardiac nervous system” (1, 5). It has been suggested that the intrinsic cardiac nervous system is composed of a hierarchy of nested feedback control loops that include parasympathetic efferent neurons, afferent neurons, local circuit intraganglionic neurons, interganglionic neurons, sympathetic postganglionic neurons, and the terminals of cardiac neurons projecting from higher centers (1, 4, 5).

While the available physiological data have provoked this radical reinterpretation of the autonomic innervation of the heart, morphological evidence in support of many proposed physiological hypotheses has been absent. Numerous physiological studies have shown that the SA ganglion is responsible in large part for mediating parasympathetic effects on cardiac rate. Ablation of this ganglion, either by surgical excision or topical application of phenol, results in complete inhibition of

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**Fig. 3.** A: intense fast blue fluorescence was observed in the right atrium in close contiguity to a cluster of sinoatrial (SA) ganglion neurons (arrows) that are presumptively nonspecifically labeled. B: some fluorescent neurons were also found in loci within the SA ganglion that were not surrounded by detectable fast blue fluorescent labeling. These neurons were presumptively retrogradely labeled from adjacent areas within the injection site.

**Fig. 4.** A: arrows identify 4 of the multiple neurons in the PA ganglion that were retrogradely labeled after microinjections of fast blue into the SA ganglion using a fast blue filter set. B: the same section utilizing an FITC filter set. Note that fast blue-labeled neurons in A comprise a subset of the total number of neurons contained within the PA ganglion as illustrated in B.
vaguely induced bradycardia (3). Similar but reversible physiological effects result from the injection of ganglionic blocking agents (20, 35). Furthermore, injecting fluorescent tracers into the SA ganglion results in retrogradely labeling neurons in the SA ganglion (26). This evidence clearly indicates that the SA ganglion mediates its negative chronotropic effect by modulating SA nodal activity.

On the other hand, little has been known about the functional properties and intracardiac projections of the PA ganglion. Previous studies have indicated a potential role for the PA ganglion in mediating in part sympathetic-parasympathetic interactions for control of chronotropic function (31). In the present report, we have performed a coordinated series of related physiological and neuroanatomic studies. We have demonstrated that microinjections of a ganglionic blocking drug into the PA ganglion attenuates the negative chronotropic effects of vagal stimulation on the heart without significantly affecting vagal effects on AV conduction. Therefore, these data and other physiological data indicate that there are at least two intracardiac ganglia that mediate the vagal control of cardiac rate, i.e., the SA ganglion and the PA ganglion.

The SA ganglion controls cardiac rate via parasympathetic postganglionic axons that project to the pacemaker cells of the SA node (26). However, neuroanatomic data clearly indicate that neurons in the PA ganglion do not project to the SA node (26). We hypothesized, therefore, that the negative chronotropic effects mediated by the PA ganglion might be due to an interganglionic projection to the SA ganglion. We now report that subsequent to microinjections of a retrograde neuronal tracer into the SA ganglion, the vast majority of retrogradely labeled neurons found throughout the heart were found in the PA ganglion. Several lines of evidence indicate that failure to find retrogradely labeled neurons within other intracardiac ganglia was not due to an inadequate survival time or relative distance from the injection site. Thus, for example, the PA and AV ganglia are approximately equidistant from the SA ganglion (21); however, retrograde labeling in these two ganglia was quite different. Furthermore, the 4-day survival time that was used in the present report has also been demonstrated to provide ample staining of cardioinhibitory vagal preganglionic neurons in the nucleus ambiguus of the brain stem after microinjections into the SA or AV ganglia (10), a distance that is substantially greater than that separating the SA ganglion from other intracardiac ganglia. These neuroanatomic data are therefore consistent with the hypothesis that the PA ganglion may affect the parasympathetic control of cardiac rate through an interganglionic neuronal circuit, which thereby modulates the actions of the SA ganglion on the SA node. The present data, to our knowledge, are the first neuroanatomic demonstration of an interganglionic circuit between any two intracardiac ganglia.

Microinjections of a retrograde neuronal tracer into the SA ganglion labeled ~80% of the neurons in this ganglion. Although many of these neurons are probably nonspecifically labeled due to their contiguity to high concentrations of the tracer, in all animals multiple neurons also appear to be labeled by retrograde transport within the SA ganglion because the tissues surrounding these neurons did not contain detectable levels of tracer. This morphological conclusion is consistent with recent physiological data that indicate that there is a functional interdependence of spontaneous and reflex-evoked activity between neurons within the SA ganglion much of the time (34). These morphological and physiological data suggest that intraganglionic neuronal circuits may be found in the SA ganglion; however, an ultrastructural analysis is urgently needed to confirm this hypothesis.

In summary, we have presented both physiological and neuroanatomic data to support the hypothesis that the peripheral autonomic control of cardiac rate is controlled by two interacting cardiac centers, i.e., the SA and PA ganglia. An interganglionic neuronal circuit connects the PA ganglion to the SA ganglion. The SA ganglion then projects to the SA node to mediate parasympathetic effects on cardiac rate. Our neuroanatomic data indicate that intraganglionic neuronal circuits are also found within the SA ganglion. These circuits may also function in a coordinated fashion to modulate cardiac rate within the feline heart. Taken as a whole, the present data support the emerging concept of an intrinsic cardiac nervous system (1, 32, 34).

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