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Vagal control of the heart in the turtle, *Ocadia sinensis*

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ABSTRACT: The purpose of the present study was to identify the cardiac vagal nerve (CVN) of the turtle, to characterize its fiber composition, and to correlate this composition with cardioinhibitory functions. Turtles (*Ocadia sinensis*) were anesthetized with sodium pentobarbital. The CVN was identified anatomically as a thoracic vagal branch going to the heart. Transection or reversal block of this branch completely abolished the negative chronotropic and inotropic effects produced by ipsilateral cervical vagal stimulation. Electron microscopic examination of the CVN revealed that it is comprised of 500 to 1800 axon fibers. Among these, 86% were unmyelinated and 14% were myelinated fibers. Compound action potentials of the CVN consisted of A, B, and C groups. A decrease in the heart rate or a reduction of ventricular contractility was observed with electrical stimulation of the cervical vagus at an intensity which activates the B-fiber group. When the stimulus intensity increased to recruit both the B- and C-fiber groups, maximal cardioinhibitory effects were observed. The negative chronotropic effect of the right vagus was greater than that of the left vagus with low-frequency stimulation. In contrast, stimulation of the left vagus produced greater negative inotropic effect. These data indicate that the turtle heart is innervated by a single pair of CVN. The cardioinhibitory functions are subserved by small myelinated and large unmyelinated fibers. Functionally distinct vagal neurons may be distributed unevenly in the turtle brain, such that the right vagal nerve contains more chronotropic while the left more inotropic motor fibers.

KEY WORDS: Chronotropism, Compound action potential, Inotropism, Nerve fiber composition, *Ocadia sinensis*, Turtles.

INTRODUCTION

Parasympathetic innervation of the heart is one of the most important neural controls of cardiac function. Activation of the vagal nerve results in bradycardia, atrioventricular block, and reduced myocardial contractility (Cohn and Lewis, 1913; Sewall and Donaldson, 1882; Wiggers, 1916). Many studies have reported the distribution of the vagus to the heart in various species of vertebrates (Billman *et al.*, 1989; Coote, 2013; Taylor *et al.*, 2009; Taylor *et al.*, 2014; Wang, 2012). It is generally held that direct branches from the vagus to the heart are less numerous than those issuing from the sympathetic system, and there may be several on either side, which can be termed cardiac vagal nerves (CVNs) (Armour *et al.*, 1975; Nonidez, 1939; Phillips *et al.*, 1986). The precise distribution varies from species to species. Relative to sympathetic nerves, fewer investigators have studied specific distributions of the vagal efferent pathway to the heart, particularly in combination with techniques which reveal differential functional effects of vagal control (Ardell and Randall, 1986; Armour *et al.*, 1975; Billman *et al.*, 1989; Burkholder *et al.*, 1992; Lazzara *et al.*, 1973; Randall, 1994). The turtle heart-nerve preparation, owing to its large size and sturdiness, can be an excellent animal model to address these questions.

The turtle is characterized by its slow horizontal motion, low systemic blood pressure (SAP) and heart rate (HR), and its extreme tolerance to prolonged anoxia that may last from hours to even months (Berkson, 1966; Lutz *et al.*, 1985; Robin *et al.*, 1964; Stecyk *et al.*, 2004; Ultsch and Jackson, 1982) The turtle heart may stop beating for more than 1 h in response to continuous vagal stimulation (Hsieh *et al.*, 1988). Turtles are intermittent lung breathers. In these animals, brief periods of ventilation are interspersed among apneic periods of variable durations. Reciprocal changes in HR, pulmonary blood flow and intracardiac shunting are associated with intermittent lung breathing (Shelton and Burggren, 1976; Wang and Hicks, 1996). Furthermore, the heart of the turtle is characterized by a single ventricle. Proper regulation of intracardiac shunting is critical in optimizing cardiovascular function. The turtle, therefore, is expected to have a unique pattern of cardiovascular control as well as special metabolic mechanisms. The low SAP and slow HR, and special cardiorespiratory synchrony, together with the prolonged cardiac arrest on vagal stimulation, suggest that vagal mechanisms of turtles might predominate in cardiovascular integration. The purpose of this study was to identify the CVN of the turtle, determine its fiber composition, and assess some of the CVN functions in this animal, such as the chronotropic and inotropic effects and left-right functional asymmetry of the vagal cardioinhibitory effects.

MATERIALS AND METHODS

Preparation of animals

Experiments were carried out in adult turtles (*Ocadia sinensis*) of either sex, weighing 1.0~1.7 kg. In all cases, anesthesia was achieved by cooling the turtle in a freezer for sedation, followed by injection of sodium pentobarbital (25 mg/kg body weight) through a cannula in the jugular vein, which was also used for drug administration. Additional anesthetic was given during the experiment if necessary. After deep anesthetization, a thoracotomy was performed through the plastron with a saw and the anterior half of the body cavity was exposed. Blood pressure was monitored through a cannula in the carotid artery, which was connected to a pressure transducer (WPI-BLPR). The heart rate (HR) was calculated from the pressure pulse or lead I of the electrocardiogram and monitored online. The ventricular pressure (VP) was monitored via a catheter inserted into the ventricle through the left subclavian artery. The maximal rate of rise of the rising limb of the VP curve (dP/dt_{max}) under pacing conditions was used as an index of ventricular contractility. For the pacing of the heart, the ventricle was paced with a tiny hook bipolar electrode inserted into the ventral myocardium near the apex. The pacing rate was 20% above the sinus rate. Data acquisition and analysis were performed with a PowerLab System (AD Instruments). All experimental procedures adhered to American Physiological Society's *Guiding Principles in the Care and Use of Animals*, and had been approved by the IACUC of the National Taiwan University.

Isolation and identification of the CVN of the turtle

On opening the chest, branches of the vagus nerves coursing towards the heart were identified. Gross anatomical dissections demonstrated that the vagus nerves exit the skull, pass inferiorly through the neck, close to the common carotid arteries, as the cervical vagus nerves. After branching several times to innervate the structures in the neck, they run caudally into the body cavity as the thoracic vagus. One branch of each vagus arises at the level of the aortic arches, and runs towards the heart. This is likely the aortic nerve. A short distance caudally, the recurrent laryngeal nerve arises and turns back to run along the trachea. The thoracic vagus nerves continue descending and run along the pulmonary artery. A cardiac branch arises at this level, courses laterally to run parallel to the pulmonary veins and on to the dorsal surface of the heart (Fig. 1). These branches were then dissected free from connective tissues while their intactness was carefully maintained.

A piece of the cervical vagus about 3~4 cm long was isolated from the carotid sheath and cut centrally. The peripheral end was placed on a pair of silver wire electrodes (with a separation of 3 mm) and covered with liquefied Vaseline. The cervical vagus was then stimulated electrically (0.5 ms, 400 μA, 10 Hz) to elicit a maximal decrease in the heart rate or a reduction in dP/dt_{max} of the ventricular pressure in non-paced or paced hearts, respectively. The electrical stimulation was held constant for a period to allow steady-state responses to be measured. Transection or reversible lidocaine (0.02%) block of the ipsilateral cardiac branches, described above, was performed. The effects of transection or lidocaine block were observed to identify the CVN. The process was then repeated for the cardiac branches on the other side.

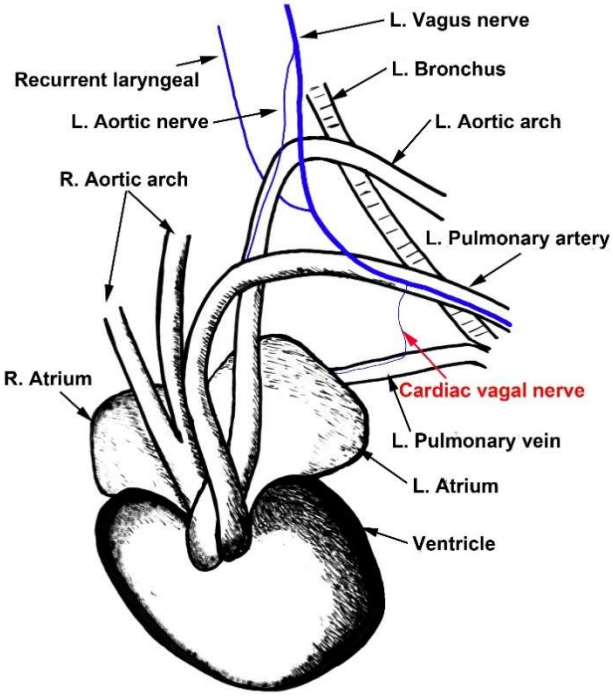

Fig. 1. Ventral view of the left cardiac vagal nerve and related thoracic vagal branches.

Electron microscopic morphometric analysis of the CVN

The CVNs, identified above, were removed from 3 turtles and immersed in a fixative of 4% paraformaldehyde and 1% glutaraldehyde for 1 h. Subsequently, each nerve segment was cut into several pieces (2~3 mm long) and washed with 0.1 M phosphate buffer (PB, pH 7.4). The tissue blocks were osmicated (1% osmium tetroxide in 0.1 M PB) for 2 h and washed in 0.1 M PB. After dehydration in a graded ethanol series, the tissue blocks were infiltrated and embedded in Spurr's resin, baked at 70 °C for 24 h for polymerization. After being placed in a box dryer for several days, the tissue block was cut in cross-section on an ultramicrotome (Richert-Jung Ultracut E). Ultrathin sections (~70 nm) were mounted on formvar–coated copper grids and stained with uranyl acetate and lead citrate. Sections were examined and photographed under

a transmission electron microscope (JEOL: JEM-1200EXII). The fiber composition of the CVN was analyzed with the montage of constructed electron micrographs (×4000). The number, diameter, and area of myelinated and unmyelinated fibers were measured using IMAQ Vision Builder (National Instruments). A cross correlation analysis of the frequency distributions of fiber diameters between the left and right CVNs was performed according to the following equation:

$$R = \frac{\sum(Yi \times Yj)}{\sqrt{\sum Yi^2} \times \sqrt{\sum Yj^2}};$$

where R is the correlation coefficient, and Yi and Yj are the numbers of fibers in each fiber size category of the two nerves, respectively.

Analysis of turtle vagus compound action potentials

A piece of the cervical vagus about 3~4 cm long was isolated from the carotid sheath and cut centrally. The peripheral end was placed on a pair of bipolar silver wire stimulation electrode (with an inter-electrode separation of 3 mm) and covered with liquefied Vaseline. The recording electrode was placed at the thoracic vagus or the CVN. The cervical vagus was then stimulated electrically through an isolation unit (Grass PSIU6) connected to a Grass S44 stimulator at room temperature (20~25°C). The stimulation intensity (μA) was gradually increased in order to excite the various fiber groups in the cervical vagus. By observing the compound action potential (CAP), we were able to determine the strength of the threshold and maximum responses of various fiber components and correlate these with cardiac functions.

Comparison of the cardioinhibitory effects of the left and right vagi

The cervical vagi of both sides were isolated as described above, and each was separately stimulated with increasing intensities (μA) at various frequencies. The negative chronotropic or inotropic effects were observed in non-paced or paced hearts, respectively. The cardioinhibitory effects of the left and right cervical vagi were compared at different stimulus intensities and frequencies.

Data analysis

Percentage changes in HR and dP/dt_{max} in response to stimulating the vagus were obtained from the ratio which was calculated from the difference between the pre-stimulus value and the maximum response versus the pre-stimulus value. Data, where not specified, are presented as the mean ± S.E. For comparison of the effects of electrical stimulation of the vagus on either side, data were analyzed using two-way repeated-measures ANOVA. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Identification of the CVN of the turtle

Gross anatomical dissections revealed one CVN on each side. Figure 1 shows the relative position of the left CVN diagrammatically. Electrical stimulation of left (n=5) or right (n=4) cervical vagus elicited cardiac arrest. Transection or lidocaine block of this CVN branch completely abolished the negative chronotropic effect (Figs. 2 and 3). After washing out the lidocaine with saline, the turtle heart stopped again during vagal stimulation. During ventricular pacing, the dP/dt_{max} of the ventricle significantly decreased (37.8% ± 4.0%) with left (n=7) or right (n=5) cervical vagal stimulation. Local lidocaine anesthesia of the CVN completely blocked the negative inotropic effect. After washing out the lidocaine, the reduction in ventricular force returned (38.9% ± 5.2%) (Figs. 4 and 5). These data indicate that the turtle heart is innervated by a single pair of CVNs, one each from the left and right sides.

Fiber composition of the CVN

Electron microscopic examination (Fig. 6) of the CVN in 3 turtles revealed that it is comprised of 926 ± 206 (range, 463~1853) fibers. Among these, 86.2% ± 3.0% were unmyelinated fibers (UMFs) and 13.8% ± 3.0% were myelinated fibers (MFs). The ratio of UMFs to MFs was 8.3 ± 2.0 on average (Table 1). Figure 7 shows the frequency distribution of fiber diameters of the CVN in a single turtle. The mean fiber diameter of UMFs was 1.1 (range, 0.5~2) μm and that of MFs was 2.4 (range, 2~4) μm. Cross correlation analysis in 3 pairs of CVNs showed that the mean correlation coefficient was 0.84. This suggests that the diameter-frequency distribution is very similar between individuals despite large differences in fiber number.

Compound action potential (CAP) of the turtle vagus and its function

Peripheral electrical stimulation of the cervical vagus elicited conducted CAPs at the thoracic vagus and its branches including the aortic, recurrent laryngeal, and cardiac vagal nerves. CAPs of the thoracic vagus and CVN consisted of the A, B, C1, and C2 groups (Fig. 8A). Table 2 shows the conduction velocities of various components of the potentials recorded at the thoracic vagus. Conduction velocities of the left and right thoracic vagi did not significantly differ ($p > 0.05$). The various components of the CAP derived from the CVN were distributed more diffusely (Fig. 8B) and were then referred to as the A, B, C1, and C2 groups according to the potentials of the thoracic vagus and the stimulus intensity. A decrease in the HR or a reduction in ventricular contractility was observed on electrical stimulation of the cervical vagus with an intensity which activated the B-fiber group.

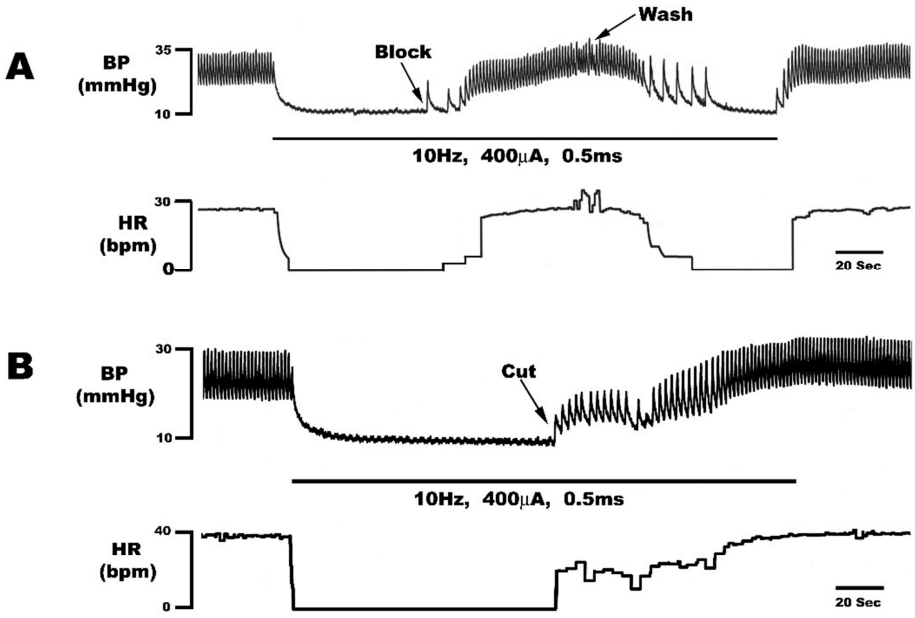

Fig. 2. Representative example of the effect of blocking the cardiac vagal nerve on the negative chronotropic effect of the right cervical vagus. The turtle heart was totally arrested during electrical stimulation of the right cervical vagus (10 Hz, 400 μA, 0.5 ms). Note that both a reversible lidocaine block (0.02%) (**A**) and transection (**B**) of the ipsilateral cardiac vagal nerve respectively abolished this negative chronotropic effect.

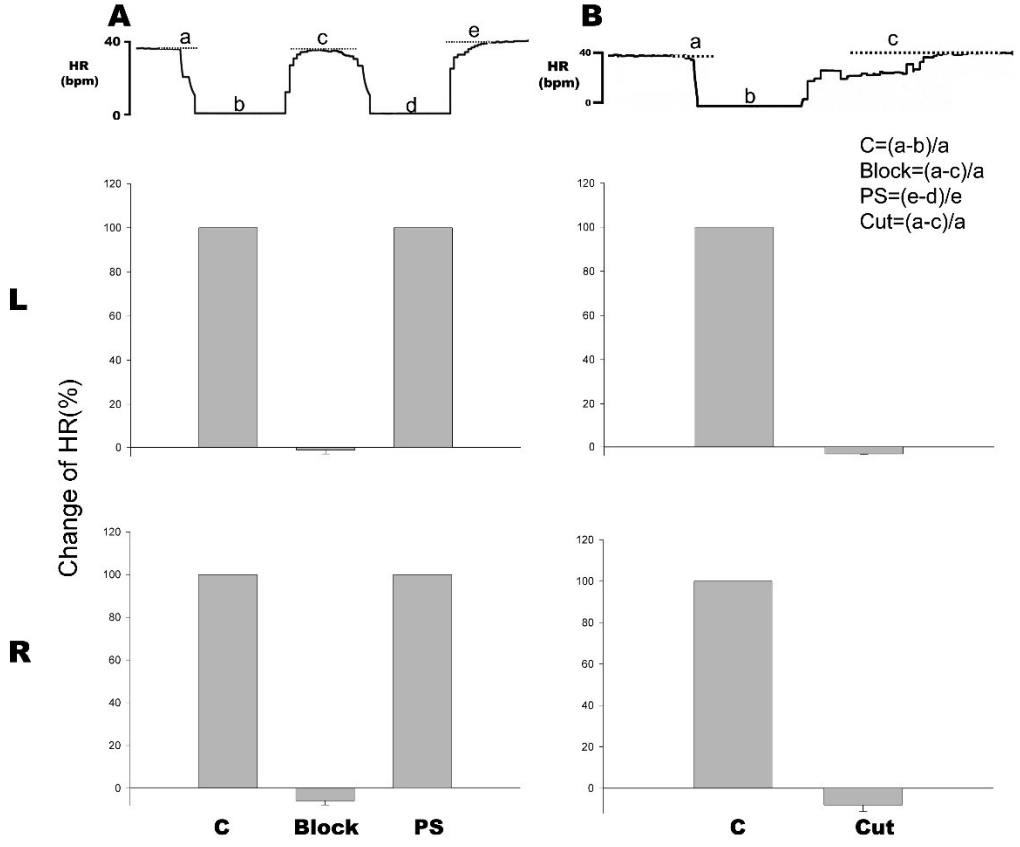

Fig. 3. Respective quantitative analysis of the reversible lidocaine block (**A**) and transection (**B**) of the cardiac vagal nerve on the negative chronotropic response to ipsilateral cervical vagal stimulation. L, left side (n = 5), middle panel; R, right side (n = 4), lower panel; a, pre-stimulating heart rate (HR); b, minimal HR value during stimulation; c, steady-state HR value after lidocaine application or nerve transection; d, minimal HR value during stimulation after washing out the lidocaine; e, post-stimulation HR value.

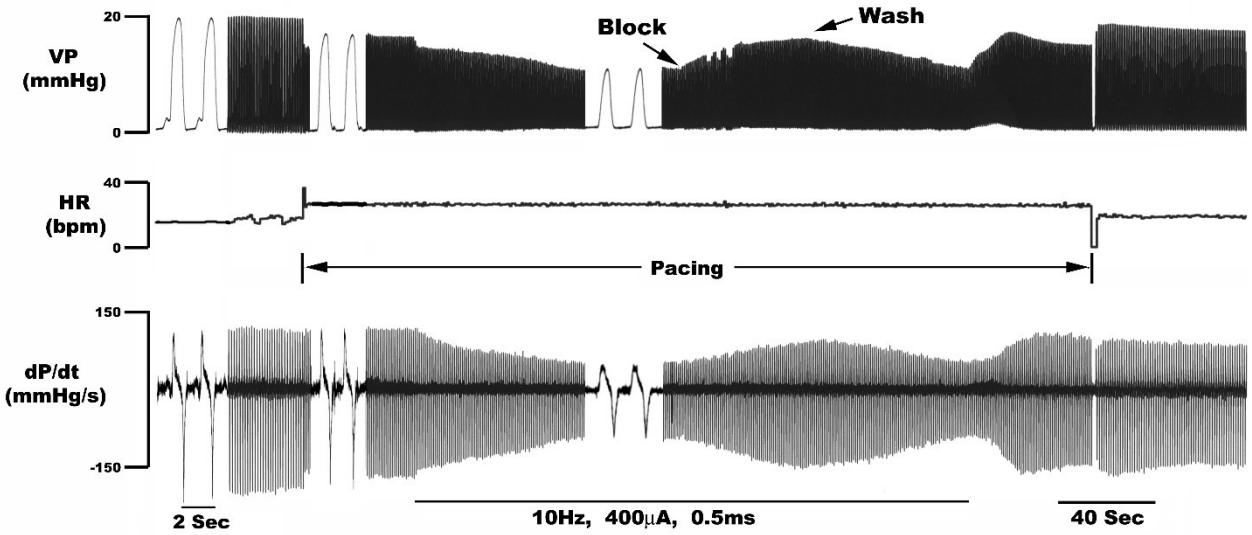

Fig. 4. Representative example of the effect of blocking the cardiac vagal nerve on the negative inotropic effect of left cervical vagal stimulation in a paced heart. The dP/dt_{max} of the ventricle (lower panel) was reduced during the electrical stimulation of the cervical vagus (10 Hz, 400 μA, 0.5 ms). Note that reversible lidocaine block (0.02%) of the ipsilateral cardiac vagal nerve (arrow with "Block") abolished the negative inotropic effect, and the negative inotropic effect returned after washing away the lidocaine from the cardiac vagal nerve.

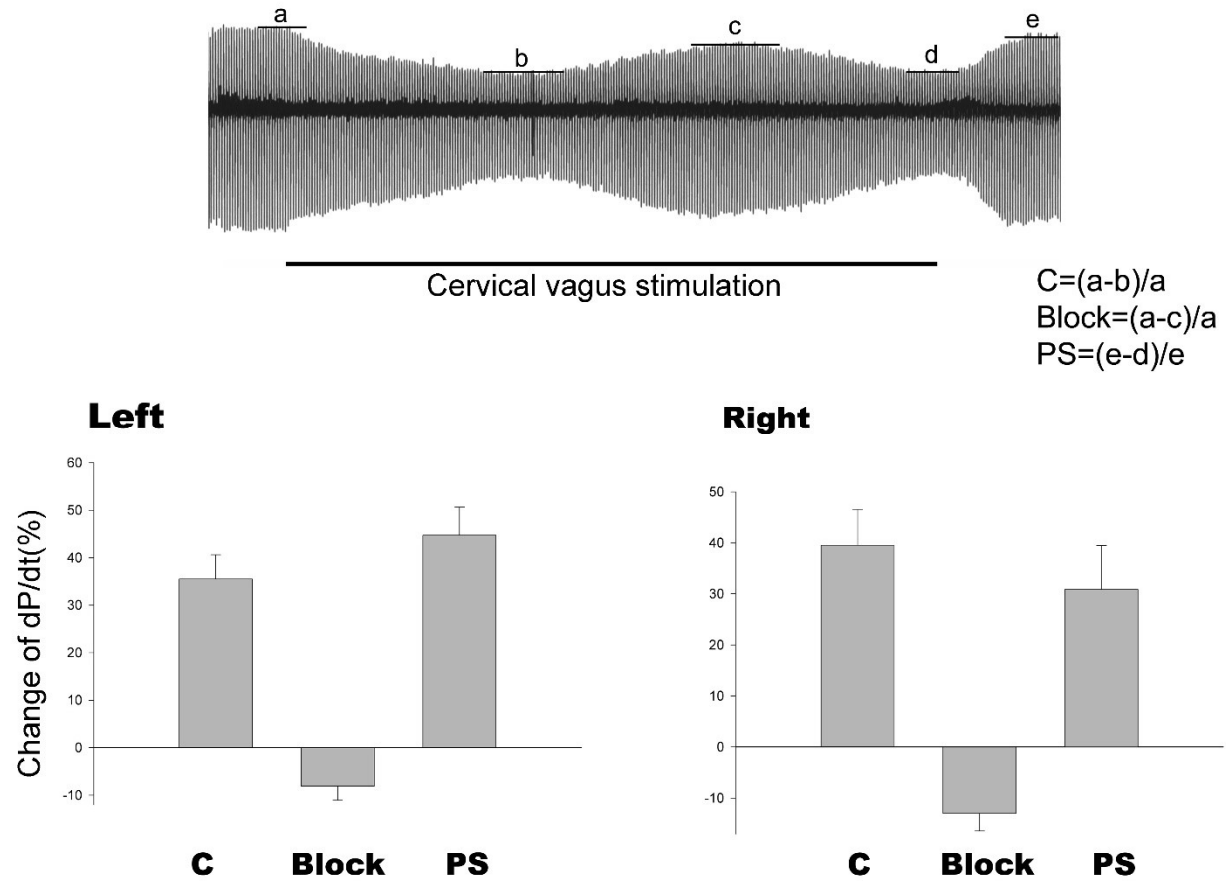

Fig. 5. Quantitative analysis of the reversible lidocaine blockade of the cardiac vagal nerve on the negative inotropic response to ipsilateral cervical vagal stimulation. For abbreviations, see Figure 3.

Table 1. Numbers of axon fibers in the cardiac vagal nerves in 3 turtles

Turtle #		Total no.	Unmyelinated fibers (UMF)		Myelinated fibers (MF)		UMF/MF Ratio
			Number	(%)	Number	(%)	
#1	Left CVN	1853	1711	92.3	142	7.7	12.0
	Right CVN	1138	936	82.2	202	17.8	4.6
#2	Left CVN	736	687	93.3	49	6.7	14.0
	Right CVN	463	427	92.2	36	7.8	12.0
#3	Left CVN	590	456	77.3	134	22.7	3.4
	Right CVN	1035	826	79.3	209	20.2	4.0
	Average	926	840	86.2	129	13.8	8.3
	SE	206	192	3.0	30	3.0	2.0

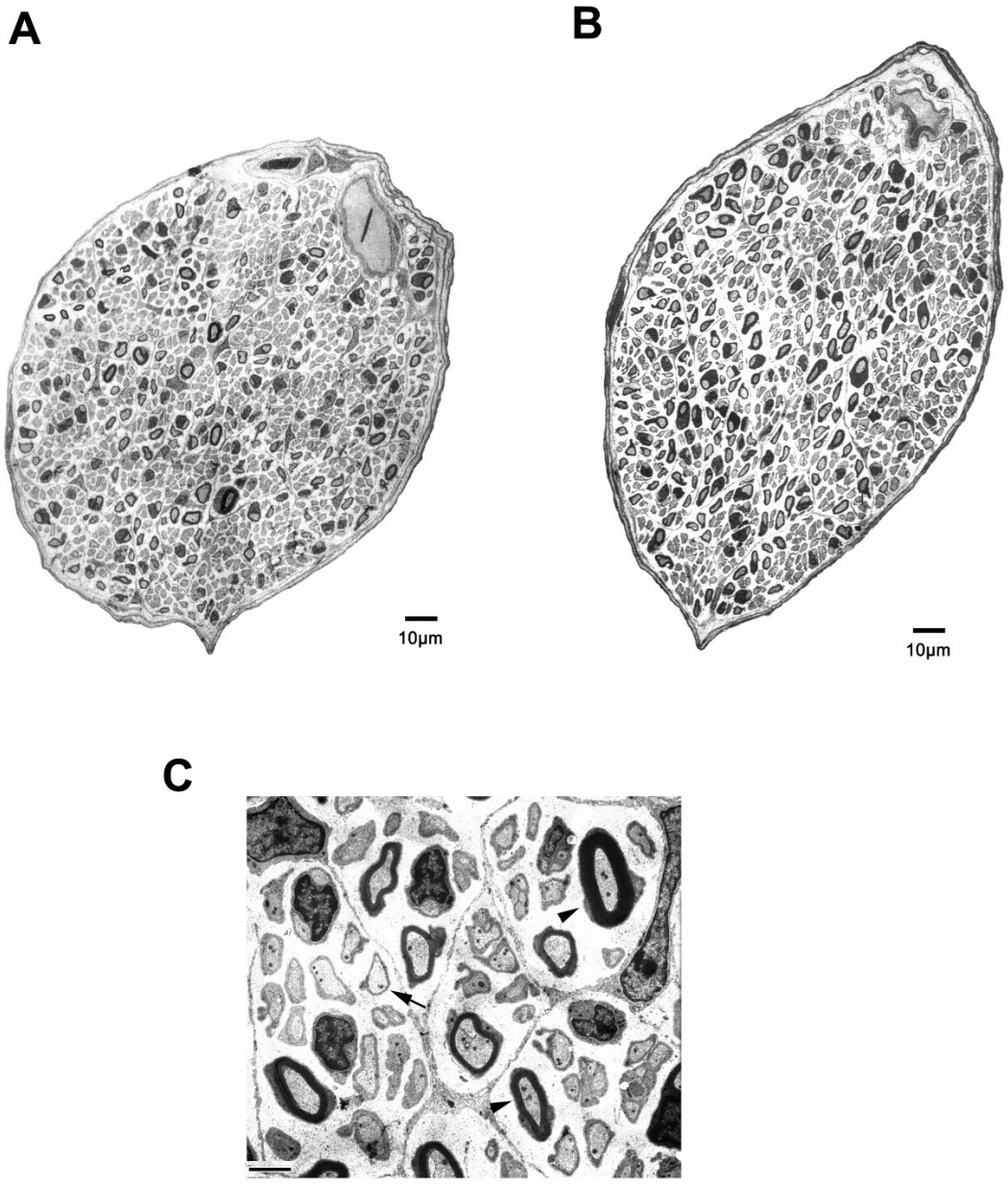

Fig. 6. Representative photomicrographs showing cross-sections of the left cardiac vagal nerve (**A**) and the right cardiac vagal nerve (**B**) in a turtle. The electron micrograph (**C**) shows the unmyelinated (arrow) and myelinated fibers (arrowheads) in the cardiac vagal nerve. The calibration bar in C represents 2 μm.

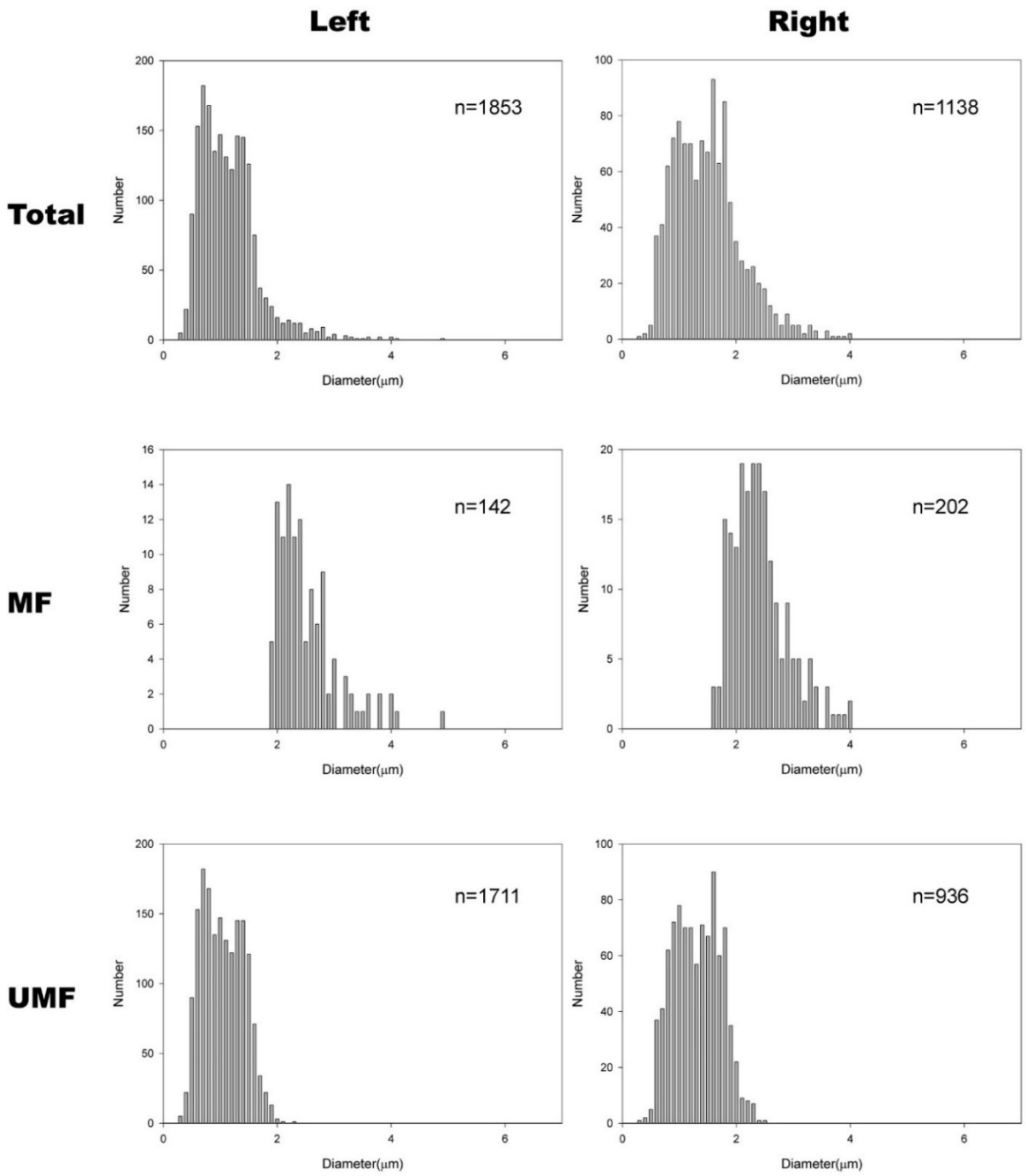

Fig. 7. Histograms of the distribution of fiber diameter-frequency of cardiac vagal nerves in the same animal as in Figure 6. MF, myelinated fibers; UMF, unmyelinated fibers.

When the stimulus intensity increased to recruit both the B- and C-fiber groups, the maximal cardioinhibitory effects were observed.

Comparison of the cardioinhibitory effects of the left and right vagi

Electrical stimulation (with a 0.5-ms pulse duration for a total duration of 10 s) of the left or right cervical vagus with increasing stimulus intensities elicited stronger cardioinhibitory effects. At a 0.5-Hz stimulation frequency, the negative chronotropic effect of the right vagus was greater than that of the left vagus with low-frequency stimulation (p = 0.005, n = 6) (Fig. 9); in contrast, the negative inotropic effect was stronger on the left side (p = 0.008, n = 6). There were no differences in the slope or the maximal effect of either the negative chronotropic or the inotropic effects of the left and right vagi at a 10-Hz stimulation frequency (Fig. 10).

Table 2. Conduction velocity of the components in compound action potential (m/s).

Components	Left	Right
Aδ	4.60± 0.19 (n=23)	4.40± 0.30 (n=8)
B	2.37± 0.14 (n=14)	2.19± 0.24 (n=8)
C1	0.87± 0.05 (n=15)	0.84± 0.12 (n=8)
C2	0.43± 0.01 (n=22)	0.39± 0.03 (n=8)

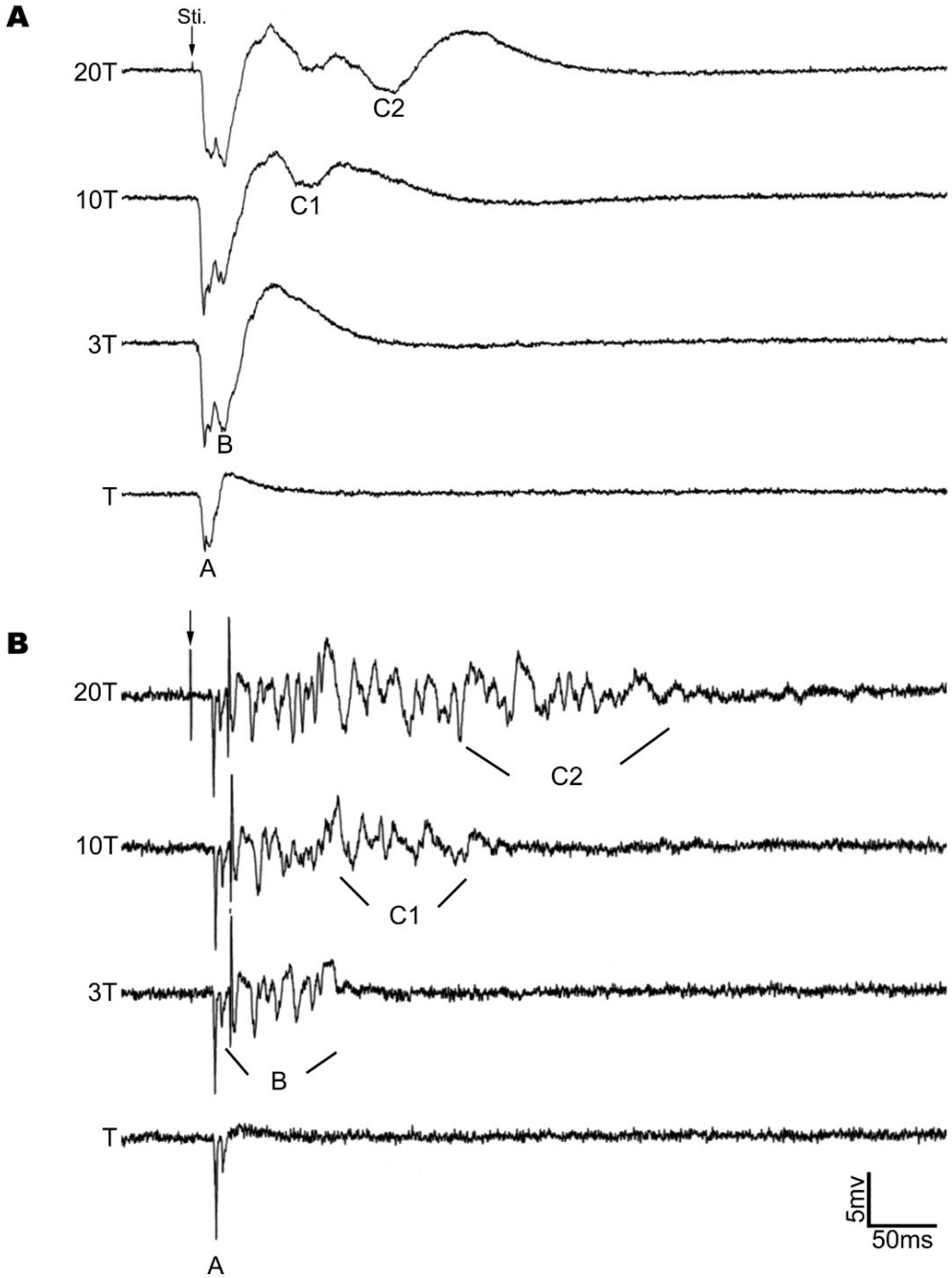

Fig. 8. Representative examples of compound action potentials recorded from the thoracic vagal trunk (**A**) and cardiac vagal nerve (**B**) of the same turtle after electrical stimulation of the cervical vagal trunk on the same side. The arrow above the top trace indicates initiation of stimulation. The threshold stimulus for group A is T (20 μA). Following an increase in the stimulus intensity, B-, C1-, and C2-fiber groups appeared at 3T, 10T, and 20T, respectively. The distance between the stimulating and recording electrodes was 60 mm for the thoracic vagus and 90 mm for the cardiac vagal nerve.

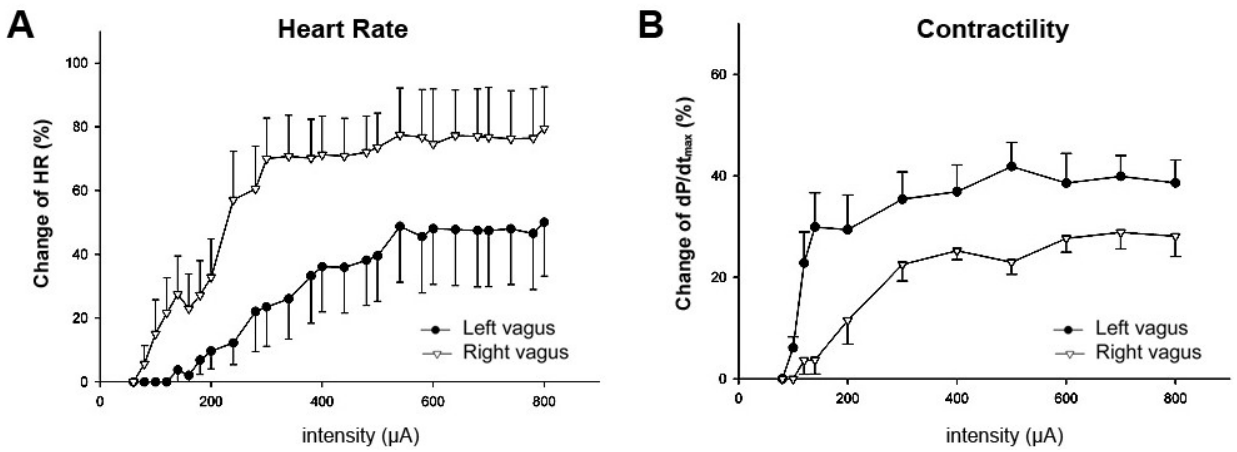

Fig. 9. Electrical stimulation (0.5 ms at 0.5 Hz for 10 s) of the left and the right cervical vagi with increasing stimulus intensities which elicited stronger cardioinhibitory effects. The negative chronotropic effect of the right vagus was greater than that of the left vagus (**A**). The negative inotropic effect of the left vagus was greater than that of the right vagus (**B**). *n* = 6 for both experiments.

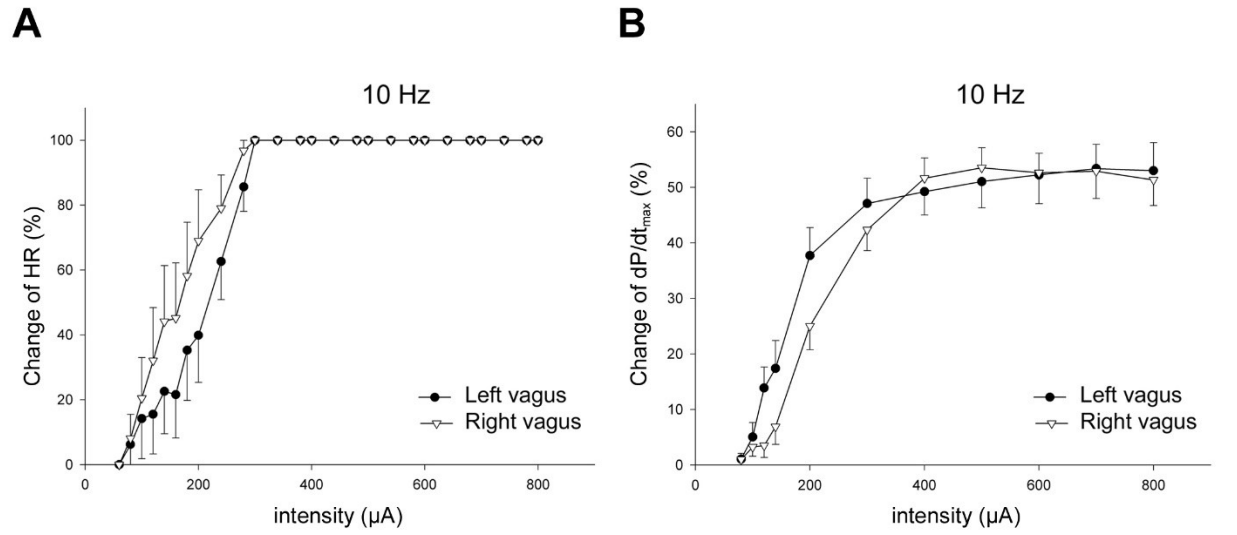

Fig. 10. Comparison of negative chronotropic (**A**) and inotropic (**B**) effects produced by left and right vagus nerves at 10Hz electrical stimulation.

DISCUSSION

In the present study, we identified a single pair of CVNs in turtles, one on each side. Each CVN is comprised of 500~1800 axon fibers. Among these, 86% were unmyelinated and 14% were myelinated fibers. CAPs of the CVN consisted of A, B, and C groups. A decrease in the HR or a reduction of ventricular contractility was observed on electrical stimulation of the cervical vagus at an intensity which activated the B-fiber group. When the stimulus intensity increased to recruit both the B- and C-fiber groups, the maximal cardioinhibitory effects were observed. In addition, bilateral asymmetry was found for control of the HR and cardiac contractility, such that the right CVN had greater control over the HR and the left CVN was more sensitive toward contractility control.

Numbers of nerve fibers of the cervical vagus nerve are about 23,000 in rabbits (Evans and Murray, 1954), 30,000~35,000 in cats (James O. Foley, 1937), and 20,000 in pigeons (Schwaber and Cohen, 1978). Ratios of myelinated to unmyelinated fibers are 1/3~1/7 in cats and rabbits (Agostoni *et al.*, 1957; Evans and Murray, 1954; James O. Foley, 1937) and 2/1~1.7/1 in pigeons. The number of fibers of the CVN in cats is about 3000, consisting predominately of unmyelinated and small myelinated (2~7 μm in diameter) ones (Heinbecker and O'Leary, 1933), and there are about 2500 in pigeons, consisting predominately (63%) of myelinated (1.5~6 μm in diameter) ones (Schwaber and Cohen, 1978). In this study, we show that the CVN of the turtle is composed predominantly (86%) of unmyelinated fibers. Its composition is close to that of cats and rabbits, but contains fewer fibers, averaging slightly more than 900

in number.

In the present study, we electrically stimulated the cervical vagus and recorded the CAPs of three branches of the thoracic vagus: the recurrent laryngeal nerve, aortic nerve, and CVN. The main induced spike in the recurrent laryngeal nerve was the A component, which corresponds to the fact that the recurrent laryngeal nerve contains many large myelinated motor fibers (Murray, 1957). The main components of the CAP of the aortic nerve are A and C. The CAP of the CVN could be differentiated into A, B, and C components, which corresponded to myelinated (3~7 μm), small myelinated (2~3 μm), and unmyelinated (< 1 μm) fibers, respectively. It has been proposed that the B-fiber group of the vagus, originating from the nucleus ambiguus, controls chronotropy while the C-fiber group, originating from the dorsal motor nucleus, controls inotropy (Geis and Wurster, 1980). However, using a modified form of the anodal block technique, it was demonstrated in cats, rats, and rabbits that stimulation of either B or C fibers alone can produce chronotropic effects (Jones *et al.*, 1995; Nosaka *et al.*, 1979; Woolley *et al.*, 1987), and their effects are not additive (Jones *et al.*, 1995). Nonetheless, previous studies on cats suggested that vagal control of the HR is mediated mainly by B fibers, which also appear to mediate reductions in atrial contraction and the slowing of atrioventricular conduction (Heinbecker and Bishop, 1935; Middleton *et al.*, 1950). Recruitment of C fibers produced no further cardioinhibitory effect over and above that evoked by B-fiber stimulation alone in cats (Jones *et al.*, 1995). This could have been due to occlusion of convergent myelinated and unmyelinated preganglionic input to postganglionic neurons, or the production of a near-maximal postganglionic response at the effector organ. In turtles, we demonstrated that a decrease in the HR or a reduction in ventricular contractility was observed with electrical stimulation of the cervical vagus with an intensity which activated the B-fiber group. When the stimulus intensity increased to recruit both the B- and C-fiber groups, the maximal cardioinhibitory effects were observed.

Electrical stimulation of the vagus nerve induced cardiac arrest in the turtles. If stimulation was given continuously, the cardiac arrest could last for more than an hour with no sign of vagal escape. This is consistent with the finding that the turtle has a great capacity for tolerating anoxia and of undergoing anaerobic metabolism (Berkson, 1966; Lutz *et al.*, 1985; Robin *et al.*, 1964; Ultsch and Jackson, 1982). The great tolerance to anoxia has been attributed to a remarkable ability to use anaerobic glycolysis as an energy source (Chih *et al.*, 1989; Sick *et al.*, 1982). During diving, cardiovascular adjustment including bradycardia, increased right to left (R-L) intracardiac shunting and redistribution of the cardiac output are also involved (Hicks, 1994; Shelton and Burggren, 1976; Wang and Hicks, 1996). Bradycardia or cardiac arrest slows the circulation and hence reduces the energy expenditure of the entire body. Thus in a turtle, the predominant vagal mechanism responsible for cardiac inhibition and development of R-L shunting is an important component of cardiorespiratory synchrony that prolongs the anoxia tolerance during diving (Hicks and Malvin, 1992). Whether turtles are provided with a reflex baroreceptor mechanism and whether a turtle heart stops beating completely or continues beating at a slower rate during diving remain to be investigated.

The mammalian heart is usually endowed with many pairs of cardiac vagal nerves (Phillips *et al.*, 1986). Selective stimulation of the individual cardiac branches of the vagus can induce highly selective changes in cardiac functions, including HR, contractility, and conduction (Armour *et al.*, 1975). A selective parasympathectomy in the dog heart blocks specific cardiac functions without influencing others (Bluemel *et al.*, 1990; O'Toole *et al.*, 1986; Randall and Ardell, 1985; Randall *et al.*, 1986; Randall *et al.*, 1987). Selective stimulations of specific discrete parasympathetic ganglia in the heart can influence specific cardiac functions (Furukawa *et al.*, 1990). Other investigators have reported the same conclusions in the cat (Gatti *et al.*, 1995; Gatti *et al.*, 1997), rat (Burkholder *et al.*, 1992), monkey (Billman *et al.*, 1989), and human (Carlson *et al.*, 1992). In the present study, transection or reversal block of the CVN completely abolished the negative chronotropic and inotropic effects produced by ipsilateral cervical vagal stimulation. Our data indicate that the turtle has the simplest cardiac vagal branching pattern, although the number and distribution of ganglia in the turtle heart and their control of cardiac function remain to be investigated.

Evidence for differentiation of function of the left and right vagi indicates a dominant heart rate effect by the right vagus and a preferential ventricular contractility effect by the left vagus (Hondeghem *et al.*, 1975; Thompson *et al.*, 1987) Some investigators (Burkholder *et al.*, 1992; Hamlin and Smith, 1968), however, were unable to show these functional asymmetries in the mammalian heart. Our present data indicate that this functional asymmetry is prominent and significant in the turtle vagus-heart preparation if a low stimulation rate is used. Therefore, vagal preganglionic neurons in the medullary oblongata of the turtle may be an interesting animal model for the study of central asymmetry in cardiac chronotropic and inotropic controls.

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