Cholinergic Innervation of the Canine and Human Ventricular Conducting System

Anatomic and Electrophysiologic Correlations

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SUMMARY
Augmentation of vagal tone increases ventricular fibrillation threshold (VFT) under nonischemic and ischemic conditions and protects against spontaneous ventricular fibrillation during experimental myocardial infarction. The purpose of this study was to identify the anatomic pathways responsible for this cholinergically-mediated enhanced electrical stability and to determine whether or not these pathways are present in human hearts. Rich cholinergic innervation of the sinoatrial node, atrioventricular node, and atrial myocardium was confirmed in both canine and human hearts. Although sparse cholinergic innervation was present in ventricular myocardium, numerous cholinergic nerve fibers were present in ventricular conducting tissue of both canine and human hearts. To determine whether these cholinergic fibers mediate the protective effects of vagal stimulation, cholinergic fibers to the ventricular conducting system were ablated in dogs. The ablation procedures used resulted in histologic absence of cholinergic nerves in the ventricular conducting system; innervation of the atrium, however, was histologically intact. In these animals vagal stimulation no longer increased VFT but still caused slowing of the sinus rate. The effect of vagal stimulation on VFT was shown to be independent of adrenergic innervation in a group of catecholamine depleted animals (6-hydroxydopamine). We conclude that 1) the enhanced ventricular electrical stability produced by vagal stimulation in dogs is mediated by cholinergic nerve fibers which supply the ventricular conducting system, and 2) this anatomic pathway is present in human hearts.

Additional Indexing Words:
Acetylcholinesterase 6-hydroxydopamine
Adrenergic denervation Ventricular fibrillation threshold
Butryrylcholinesterase Cholinergic denervation

Recent Studies have demonstrated significant beneficial effects of increased parasympathetic tone on ventricular electrical stability. Vagal stimulation increases the ventricular fibrillation threshold in the presence or absence of myocardial ischemia and increased vagal tone decreases the incidence of ventricular fibrillation that occurs spontaneously during experimental acute coronary occlusion. Since each of these salutary effects of increased vagal tone can be demonstrated independent of changes in heart rate, it appears that the vagus itself has important effects on electrical stability of the ventricles. The two major goals of the present investigation were 1) to determine definitely whether the beneficial effects of vagal stimulation in dogs are in fact due to cholinergic innervation of the ventricle and 2) to determine whether cholinergic pathways exist in the human heart that could provide a basis for possible beneficial effects of increased vagal tone on the genesis of arrhythmias in man.

Methods
Human tissue was obtained at autopsy within 12 hours of death from five patients without known cardiac disease. Tissue from the sinoatrial (SA) node region, the atrioventricular (A-V) node, the His bundle, the left bundle branch, and the free wall of the left ventricle was examined. To determine whether postmortem time was a factor in the disappearance of acetylcholinesterase (AChE) activity in human tissue, fresh samples of atrial myocardium (atrial appendage removed at the time of cannulation for extracorporeal circulation) and ventricular myocardium (removed at time of myectomy for asymmetric septal hypertrophy) were obtained at the time of operation, frozen on dry ice, and analyzed immediately.

In canine experiments tissue blocks of the SA node, A-V node with bundle of His and proximal left bundle branch, and left ventricular myocardium were placed on specimen
blocks on dry ice and frozen sections of 20 to 40 μ were obtained with a cryostat. Multiple sections were made through the A-V node-His region in a plane perpendicular to the base of the aortic valve. In order to specifically identify AChE, nonspecific cholinesterases were inhibited. Thus, adjacent sections were preincubated initially with diisopropyl fluorophosphate (DFP) 10^-8 M or 10^-7 M for inhibition of nonspecific cholinesterase, i.e., butyrylcholinesterase (BuChE), following which it was incubated for 2-3 hrs at 37°C according to the thiocholine method of Koelle for the localization of acetylcholinesterase (AChE). The second part was preincubated initially without DFP and then incubated with butrylthiocholine as the substrate. The sites that reacted with butrylthiocholine will be referred to as pseudocholinesterase. The tissue slices appeared to be an important determinant of AChE activity. Tissue slices of 30 to 40 μ thickness of the tissue slices were used to effectively inhibit BuChE, as determined from additional control sections incubated with butyrylthiocholine with DFP. In human studies, however, 10^-8 M DFP was sufficient to inhibit all nonspecific cholinesterases. Incubation periods of two hours were optimal for AChE activity in dogs and two to three hours for human tissues.

Cholinergic Denervation
Operative interventions were designed to destroy cholinergic ganglia that supply postganglionic fibers to the ventricles. Male mongrel dogs (18 to 20 kg) were induced with sodium thiamylal, anesthetized with halothane, and operative procedures were conducted under sterile conditions. One of three sites was exposed to vinblastine, a neurotoxic agent that destroys neuronal processes in autonomic ganglia. Site 1 was on the right side of the base of the aorta, just posterior to the right coronary artery. Vinblastine was injected into this site by one of two procedures: a) in two dogs after exposure of the aorta by circumferential dissection, vinblastine was applied to the area posterior to the right coronary artery both by injecting 10 ml of a 0.1% solution and by placing into the area a small piece of Gelfoam soaked with the vinblastine; b) in five dogs vinblastine was injected through a 25 gauge needle into the area but the aorta was not dissected and Gelfoam was not used. The amount of vinblastine injected by this method was as follows: 4 ml (0.1%) in two dogs, 1 ml (0.1%) in two dogs, and 1 ml (0.001%) in one dog. In three control dogs, 2 ml saline were injected. Site 2 was more inferior and anterior than site 1; i.e., the needle through which vinblastine was injected (1 ml of 0.1% solution) was directed inferiorly toward the central fibrous body instead of posteriorly. Site 3 was the A-V node area. This was approached through the right atrium with a 25 gauge needle and was identified when transient complete heart block was produced by the needle. One ml vinblastine (0.1%) was injected into this region. No permanent conduction disturbances occurred.

Adrenergic Denervation
Since the above dissections would be expected to interrupt adrenergic neural pathways as well as cholinergic ganglia, the effects of cardiac adrenergic denervation on the results of the present study were determined by the administration of 6-hydroxydopamine (6-OHDA) to six dogs to achieve adrenergic denervation. The animals were first given dl-propranolol (1 mg/kg) and phentolamine (5 mg). This pretreatment was found necessary to prevent death from cardiac arrhythmias and systemic hypertension induced by the initial massive catecholamine release caused by 6-OHDA. Immediately after pretreatment with propranolol and phentolamine, freshly prepared 6-OHDA hydrobromide (20 mg/kg) in saline was administered intravenously.

Experimental Procedures
Five to seven days after operation or 6-OHDA treatment, the animals were anesthetized with pentobarbital (25 to 30 mg/kg i.v.). The cervical vagi were isolated and platinum electrodes were fixed circumferentially around the two vagi. The heart was exposed through a left thoracotomy and a bipolar platinum electrode was sutured to the left ventricle. A pacing wire was sutured to the right ventricular outflow tract, and ventricular rate was held constant at 180 beats/min by right ventricular pacing during fibrillation threshold determinations under control conditions and during vagal stimulation. This rate was chosen since it was slightly faster than the heart rate of the pentobarbital-anesthetized animals. Systemic arterial pressure was monitored. Ventricular fibrillation threshold (VFT) was measured as has been described previously. Briefly, a train of 2 msec impulses at 100 Hz was delivered to the left ventricle during the vulnerable period. The train started 60 to 80 msec after ventricular activation and continued 50 msec after the T wave ended in standard lead II of the electrocardiogram. These parameters were held constant throughout the experiments. The initial intensity of the impulses was 2 mamps and the intensity was increased in approximately 4 mamp increments until ventricular fibrillation (VF) occurred. Hearts were defibrillated within 30 sec by capacitor discharge current delivered through 6 cm electrodes. Ventricular fibrillation threshold was determined under control conditions (no vagal stimulation) and during vagal stimulation. Measurements were made in an alternate manner and measured twice under both conditions. The average of the two values is reported. When vagal stimulation was used, the cervical vagi were stimulated at 20 to 40 Hz with pulses of 0.5 msec duration at an intensity necessary to achieve an atrial rate of 50 to 60 beats/min (1-5 volts). Fifteen minutes were allowed between the testing procedures. A control group of six animals was studied in an identical manner.

Tissue norepinephrine assay. Tissue norepinephrine concentration was determined by a fluorometric method.

Statistical methods. Comparison of the response to vagal stimulation in the same animal (paired data) or between groups of animals (unpaired data) was performed using the Student’s t-test.

Results
Cholinergic Innervation: Specific AChE Localization
Rich cholinergic innervation of the SA node, atrial myocardium, and A-V node, as identified by specific histochecmical stains for AChE, was present in both human and canine hearts. Abundant innervation of human and canine atrial tissues was found. Cholinergic innervation of ventricular myocardium, however, was limited to perivascular terminals, axonal.
trunks, and sparse branches that originate from the larger trunks in both canine and human myocardium (fig. 1). Human ventricular myocardium obtained at operation demonstrated the same sparse AChE activity, indicating that the paucity of cholinergic innervation of the ventricle was not an artifact due to obtaining the human tissue 6-12 hours postmortem.

In contrast to the scant innervation of ventricular myocardium, the ventricular conducting system of human and canine hearts was richly innervated. In human hearts, cholinergic innervation took the form of delicate nerve terminals that ran parallel to the Purkinje fibers in the left bundle (fig. 2). In the left bundle of canine hearts the cholinergic terminals appeared to envelop the Purkinje fibers (fig. 3). Even after repeated attempts to reorient the tissue blocks of both species, it is difficult to be certain that this difference in alignment of nerve fibers is a real species difference or an artifact of tissue sectioning. In humans, the ventricular myocardium adjacent to the left bundle contained some cholinergic nerves (fig. 2). This was the only area of ventricular myocardium that contained this degree of AChE activity.

Pseudocholinesterase Activity

Canine ventricular conducting tissue was rich in BuChE, as indicated by dense staining in the bundle of His, left bundle branch and peripheral Purkinje tissue when incubated with butyrylthiocholine (fig. 4), and complete absence of this enzyme when tissues were preincubated with DFP 10⁻⁷M. No “breakthrough” of the enzyme was found after appropriate control incubations; thus, BuChE activity did not influence specific AChE activity (see below and fig. 5). Sections of proximal human conducting tissue obtained after death contained little or no BuChE. Since no conducting tissue was obtained in any of the patients at operation, we do not know with certainty whether the absence of BuChE was a function of the time elapsed before the necropsy specimens were obtained; however, hearts obtained from dogs several hours after death demonstrated intense BuChE activity. Thus, the absence of BuChE in human hearts appears to be due to a difference in BuChE content of the human and canine proximal conducting systems.

Figure 1

Acetylcholinesterase (AChE) containing nerves in ventricular myocardium (canine). Most nerve fibers with AChE activity are found in the perivascular tissue with an occasional branch into ventricular myocardium as seen in this plate (black arrow). This appears to be the pattern in the myocardium of both ventricular free walls and septum. (330 X)

Figure 2

AChE activity in ventricular conducting system (human). The left bundle branch shows abundant nerve fibers (arrows). In contrast to myocardium elsewhere in the ventricles, the myocardium adjacent to the ventricular conducting system also contains nerve fibers. (130 X)
Cholinergic Denervation

In the six animals in which vinblastine was injected at the base of the aorta just posterior to the right coronary artery (site 1), vagal stimulation caused no change in VFT; this is in contrast to the increase in VFT produced by vagal stimulation in control animals (table 1). This impaired electrophysiologic effect correlated with morphologic findings in that cholinergic fibers of the ventricular conducting system in the vinblastine treated dogs were absent or markedly reduced (table 1, fig. 5). In one of the control animals in which saline was injected into site 1, ventricular fibrillation threshold did not increase with vagal stimulation although ventricular cholinergic innervation was intact anatomically. (One possible explanation for this finding was that operative dissection interrupted preganglionic vagal fibers but did not destroy postganglionic fibers). In the remaining control animals, vagal stimulation increased ventricular fibrillation threshold, and cholinergic innervation was intact. In contrast to the marked decrease in cholinergic innervation of the ventricular conducting system induced by this selective denervation technique, supraventricular cholinergic innervation was intact. This was manifest both physiologically (vagal stimulation produced atrial slowing) and morphologically (abundant innervation of the SA node and atrial muscle was present). Vinblastine injected into the A-V node region (sites 2 and 3) altered neither the effects of vagal stimulation on VFT nor cholinergic innervation of the ventricles.

Adrenergic Denervation

Aortic dissection and injection of vinblastine into the paraaortic region resulted in some degree of adrenergic denervation, as demonstrated by loss of norepinephrine stores from the myocardium (table 1). The more extensive dissection of site 1 was associated with the greatest loss of myocardial norepinephrine. To be certain that loss of norepinephrine was not influencing the responses to vagal stimulation, ventricular fibrillation threshold was determined in five dogs in which norepinephrine stores were depleted by pretreatment with 6-OHDA. In these dogs ventricular norepinephrine was less than 0.01 μg/g of tissue. Ventricular fibrillation threshold in the absence of vagal stimulation was 73 ± 14 mamps, a value significantly higher than that obtained in the absence of vagal stimulation in control animals (24 ± 8; P < 0.005). However, with vagal stimulation VFT increased in the norepinephrine-depleted animals to 108 ± 21 (P < 0.001), a value not significantly different than that obtained in control animals during vagal stimulation (108 ± 21 mamps vs 86 ± 10 mamps, NS). Thus, adrenergic denervation appeared to increase baseline levels of VFT, however, the presence or absence of an increase in VFT during vagal stimulation was independent of whether or not adrenergic innervation to the ventricle was intact.

Discussion

In contrast to the profound inotropic and electrophysiologic alterations that vagal stimulation produces in atrial tissue, its effects on ventricular function are much smaller. This finding correlates with anatomic evidence of rich cholinergic innervation of atrial tissue but scant innervation of ventricular myocardium. However, recent studies have shown that vagal stimulation has profound effects on ventricular electrical stability. The present investigation, therefore, was undertaken to determine whether or not functionally important ventricular cholinergic pathways exist. Our results demonstrate that cholinergic innervation of ventricular myocardium is sparse, which correlates with the small effects of vagal stimulation on ventricular contractility; however, in-

Figure 3

AChE activity in the ventricular conducting system (canine). Left bundle branch outlined by large arrows. In contrast to the human, the nerve fibers (small arrow) appear to encapsulate the individual Purkinje fibers. (33 X)
nervation of the ventricular conducting system is abundant and appears to be the anatomic basis for the effects of vagal stimulation on ventricular electrical stability.

Precise identification of cholinergic pathways was made possible by the development of histochemical techniques capable of specifically identifying AChE. These techniques demonstrated that AChE activity was abundant in cholinergic neurons, but sparse or absent in adrenergic or sensory neurons. Thus, histochemical identification of AChE could be used as a marker for cholinergic nerve fibers.

Utilizing this approach, histochemical studies in several animal species (guinea pigs, rabbits, sheep and ox) revealed scant AChE activity in ventricular myocardium but high activity in the ventricular conducting system. The results of the present investigation confirm and extend these studies. We found that in contrast to the few cholinergic fibers present in ventricular myocardium, an abundant supply of cholinergic nerves is present in the ventricular conducting system of both man and dog. Moreover, these nerves are intimately related to Purkinje fibers: in the dog, cholinergic nerves surround the Purkinje fibers; in man, cholinergic nerves run parallel to them. That these anatomic pathways have functional significance is indicated by the observation that when cholinergic innervation of the Purkinje system (as assessed histochemically) is destroyed by injecting vinblastine into the paraaortic area, vagal stimulation no longer increases ventricular fibrillation threshold. It is of interest that although this denervation technique abolishes anatomic and functional cholinergic innervation of the ventricular conduction system, cholinergic innervation of the atrium is unaffected, as demonstrated histochemically and physiologically (vagal stimulation continues to cause slowing of the atrial rate).

These results not only indicate that the cholinergic nervous system exerts an important effect on ventricular electrical stability, but also suggest that the ventricular conduction system plays an important role in the genesis of arrhythmias. Reentrant pathways have been demonstrated experimentally in depressed Purkinje fibers. In addition, although electrotonic interactions are capable of maintaining the
homogeneity of transmembrane potentials of distal Purkinje fibers, artificial interruption of contiguous cells (as might result from ischemia) could prevent this electrotonic interaction, produce disparate action potential durations and refractory periods, and thereby promote reentrant arrhythmias. Moreover, although the results of most previous investigations have suggested a minimal effect of the cholinergic system on ventricular function, other studies have shown that acetylcholine and vagal stimulation may cause electrophysiologic alterations of the ventricular conduction system. Bailey et al. demonstrated that acetylcholine decreases the rate of diastolic depolarization (Phase IV) of the action potential of Purkinje cells located in the proximal ventricular conducting system; consequently, the rate of rise of Phase 0 is enhanced. These changes would presumably lead to depressed automaticity and enhanced conduction velocity. Other studies compatible with such a concept demonstrated that acetylcholine is capable of decreasing the idioventricular rate following destruction of the A-V node region in isolated perfused rabbit hearts and in intact dogs. The effect of acetylcholine on idioventricular rate in both studies was found to be enhanced by phystostigmine or neostigmine and blocked by atropine. Finally, Spear and Moore demonstrated that vagal stimulation slowed the rate of firing of unifocal ventricular pacemakers in dogs in which A-V block was induced by extremely small lesions in the His bundle.

The possibility was considered that the effects of vagal stimulation we observed might be due to alterations in adrenergic rather than cholinergic tone. This was considered because of the existence of sympathetic fibers in the vagal trunk, as well as the demonstration of adrenergic nerve fibers running parallel to, although apparently not entering, the ventricular conduction system. Several findings, however, make this possibility unlikely. First, even when the adrenergic neurotransmitter, norepineph-
Table 1

Anatomic-Physiologic Correlations of Cholinergic Innervation of the Ventricular Conducting System

<table>
<thead>
<tr>
<th>Site 1</th>
<th>Dose VB (mg/ml)</th>
<th>Cholinergic fibers</th>
<th>Ventricular conducting system</th>
<th>NE (µg/g) IV septum</th>
<th>Ventricular fibrillation threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Circumferential dissection around base of aorta Vinblastine right side</td>
<td>10 ml  (0.1%)</td>
<td>++++*</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>b) Needle passed to right parasortic area, 4 ml Vinblastine</td>
<td>4 ml  (0.1%)</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>0→+</td>
</tr>
<tr>
<td>Same as above except 1 ml Vinblastine</td>
<td>1 ml  (0.1%)</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>0→+</td>
</tr>
<tr>
<td>Same as above 1 ml Vinblastine</td>
<td>1 ml  (0.001%)</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Normal saline injected, 2 ml</td>
<td>—</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Site 2 Needle directed inferiorly toward central fibrous body 1 ml Vinblastine (mg/ml)</td>
<td>1 ml  (0.1%)</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Site 3 Transatrial to AVN region Nonoperated control animals (6)</td>
<td>1 ml  (0.1%)</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>no histology</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Abbreviations: VB = vinblastine; SAN = sinoatrial node; AVN = atrioventricular node; NE = norepinephrine; IV septum = interventricular septum.
*A grading system of AChE activity of 0 to 4+ was made for each section. 0 was complete absence of AChE activity and 4+ was dense innervation as always found in the sinoatrial node region.
rine, had been depleted by prior administration of 6-OHDA, vagal stimulation still resulted in an increase in ventricular fibrillation threshold. Moreover, several investigators have demonstrated that the adrenergic nervous system has an effect on ventricular electrical stability opposite to that of the cholinergic system, i.e., adrenergic neural stimulation decreases electrical stability.\(^{18,19}\) Our findings are compatible with such a role for the adrenergic system. Ventricular fibrillation threshold is higher in animals in which neural stores of norepinephrine have been depleted by pretreatment with 6-OHDA than in control dogs. The high control values for VFT in animals after vinblastine injection appear to correlate with the degree of adrenergic depletion (Table 1). Furthermore, in one animal in which paraaortic vinblastine injection resulted in loss of cholinergic fibers but did not cause total catecholamine depletion, vagal stimulation decreased ventricular fibrillation threshold; one explanation for this finding is that the decrease in ventricular electrical stability resulted from adrenergic neural stimulation that was not countered by the stabilizing effects of cholinergic stimulation.

In conclusion, it is clear that there is abundant cholinergic innervation of the ventricular conducting system of many species, including man. In addition, it appears that the effects of vagal stimulation on electrical stability of the canine ventricle are mediated by these cholinergic fibers. The precise role of the ventricular cholinergic system in man remains to be elucidated.

**Acknowledgment**

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**References**

2. **Coo RL, Gillis RA**: Role of the vagus nerves in the cardiovascular changes induced by coronary occlusion. Circulation 49: 86, 1973
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