Modulation of Cardiac Autonomic Neurotransmission by Epicardial Superfusion
Effects of Hexamethonium and Tetrodotoxin

Toshihisa Miyazaki, Harald P. Pride, and Douglas P. Zipes

The heart contains superficial cardiac nerves whose effects may be modulated by pericardial fluid bathing the epicardium. We tested this hypothesis in open-chest dogs anesthetized with secobarbital. Oxygenated normal Tyrode's solution (NT) or NT containing hexamethonium, a ganglionic blocker (500 μM), or tetrodotoxin, a blocker of axonal neurotransmission (5 μM, TTX), was instilled into the pericardial cavity to superfuse the epicardium of the whole heart. During each superfusion, effective refractory period (ERP) was determined in deep intramycardium (≥4 mm in depth from the epicardium) of anterior and posterior left ventricle and in the subendocardium of the right ventricle in the baseline state and during bilateral cervical vagal stimulation (VS) or ansae subclaviae stimulation (SS). Lengthening of ERP induced by VS during superfusion with NT (6.9±0.3 msec, mean±SEM, n=36) was eliminated during subsequent superfusion with hexamethonium (0.9±0.5 msec, p<0.001). Hexamethonium also prevented sinus arrest induced by VS but did not affect shortening of ERP induced by SS (17.3±1.3 to 16.6±1.0 msec, n=26). TTX suppressed VS-induced changes in ERP (6.3±0.3 to 1.5±0.5 msec, n=32, p<0.001) and SS-induced changes in ERP (18.8±1.6 to 6.0±0.9 msec, n=23, p<0.001) but did not affect changes in ERP induced by intravenous administration of norepinephrine or methacholine. This indicates that the suppressive effect of TTX on neurally induced changes in ERP was due to an inhibition of neurotransmission and not on the response of the effector site to the neurotransmitter. We conclude that 1) vagal ganglia that innervate both ventricles as well as sinus node are distributed superficially and are blocked by epicardial superfusion with hexamethonium, 2) the epicardium lacks sympathetic ganglia, and 3) both vagal and sympathetic axons at some point travel superficially during their course in the heart and are blocked by TTX. These results suggest that substances in the pericardial fluid, whether normally secreted or present due to disease, have the potential to modulate autonomic neural transmission to the heart. (Circulation Research 1989;65:1212–1219)

Eff erent sympathetic and vagal neurotransmission to the canine ventricle can be interrupted by topical application of phenol to the ventricular epicardium and to the atrioventricular groove, respectively. These results suggest that efferent sympathetic and vagal nerve axons travel superficially at some point during their course in the heart. Previous anatomic studies have described the presence of numerous ganglia at the base of the heart, that is, in the atria and at the root of great vessels, in the dog and in humans. Most studies suggest that these ganglia are vagal, but anatomic differentiation of the ganglia is difficult. Recently, Randall and colleagues localized vagal ganglia that selectively innervated the sinus node and atrioventricular nodes in fatty connective tissue in the epicardium in dogs. Little is known about the localization of the ganglia that innervate the ventricles although the studies of Randall and colleagues suggest that there might be a preferential distribution of vagal ganglia to the epicardial tissues. Because vagal ganglia and both vagal and sympathetic axons at some point are in the epicardium, we postulated that cardiac neurotransmission through these superficial ganglia and postganglionic axons might be modulated by substances in pericardial fluid, whether normally secreted or present due to disease; these substances bathe the epicardium of the heart. Such epicardial superfusion in situ would...
be analogous to superfusing isolated nerves in a tissue bath.

In the present study, we tested this hypothesis by measuring the response of effrent vagal- and sympathetic-induced changes in ventricular refractoriness during epicardial superfusion with hexamethonium, a ganglionic blocker, and tetrodotoxin, a blocker of axonal neurotransmission.

**Materials and Methods**

**Surgical Preparation**

Forty-four mongrel dogs of either sex weighing 16–30 kg were anesthetized with secobarbital (30 mg/kg i.v.). Additional doses were injected as needed to maintain anesthesia. Dogs were intubated and ventilated with room air by use of constant volume-cycled respirator (model 607, Harvard Apparatus, South Natick, Massachusetts). A fluid-filled cannula placed in the right femoral artery was connected to a transducer (Statham p-23Db, Gould, Cleveland, Ohio) to monitor arterial pressure, and a femoral venous cannula was used to infuse normal saline at 100–200 ml/hr to replace spontaneous fluid losses. Lead II electrocardiogram was monitored throughout the study. In 12 dogs, right atrial pressure was measured through a venous cannula. The chest was opened through a median sternotomy, and a small incision was made on the anterior surface of the pericardium. The edges of the incision were tied with sutures at four points so that tension applied to the sutures produced a square opening approximately 2.5x2.5 cm. This provided a pericardial cavity sufficient to contain 45–120 ml (mean, 84 ml) of the solution to bathe the epicardium of the heart (Figure 1). The entire epicardial surface of the heart was supervised with the solution instilled into the pericardial cavity. This was confirmed by the staining of the entire epicardial surface with methylene blue instilled into the pericardial cavity.

![FIGURE 1. A diagram showing pericardial cradling technique for epicardial superfusion. Through the pericardial opening, six electrodes were inserted into the right and left ventricles to determine the effective refractory period, and the Tyrode's solution was instilled into the pericardial cavity to bathe the entire epicardial surface of the heart.](http://circres.ahajournals.org/)

**Measurement of Effective Refractory Period**

The ERP was determined at six test sites by the extrastimulus technique with a programmable stimulator (Kranert Medical Engineering, Indianapolis, Indiana) and a constant current isolator. Each ventricular test site was driven with a 2-msec rectangular stimulus twice the diastolic threshold, which was measured during each intervention. A train of eight stimuli (S₈) was followed by a late premature stimulus (S₉) that produced a propagated response. The S₈-S₉ interval was 240–250 msec in dogs tested for sympathetic response and 280–300 msec in dogs tested for vagal response and was kept constant throughout each experiment. The ventricular response to S₉ was recorded in lead II electrocardiogram and from the bipolar ventricular electrode and displayed on a storage oscilloscope. The S₈-S₉ interval was shortened in 2-msec steps until S₉ failed to produce a propagated response. The S₈-S₉ interval was then increased by 5 msec and was shortened by 1-msec decrements until S₉ failed to produce a propagated response. The ERP was defined as the longest S₈-S₉ interval at which S₉ failed to produce a propagated response. The ERP was determined twice at each test site. If the values were not within 1 msec of each other, the data were discarded and the determination was repeated.

**Neural Stimulation**

Decentralized bilateral ansae subclaviae were stimulated with shielded bipolar electrodes placed on the anterior and posterior ansae; separate constant current isolators driven by a programmable stimulator (Pulsar 4, Frederick Haer, Brunswick, Maine)
were used. Stimuli were rectangular 4-msec pulses at a frequency of 2-4 Hz and 2-4 mA.

Both cervical vagi were stimulated through two Teflon-coated wire electrodes embedded in the cardiac end of each vagal nerve surgically interrupted in the neck. Rectangular pulses of 4-msec duration were delivered at a frequency of 20 Hz by use of separate constant-current isolators. The current strength was 0.05 mA greater than that required to produce sinus arrest (>2 seconds) for the right vagus and complete atrioventricular block for the left vagus. The effects of vagal stimulation were determined during intravenous infusion of norepinephrine at a constant rate of 0.125 μg/kg/min to achieve a constant background of sympathetic effect. The ERP determined during norepinephrine infusion served as control for the determination of the efferent vagal effect on ventricular refractoriness.

The conditions of neural stimulation were kept constant in each experiment.

Solutions

Normal Tyrode’s solution (NT) was used for control epicardial superfusion. The composition of the solution was (mM) MgCl₂ 0.5, NaH₂PO₄ 0.9, CaCl₂ 2.0, NaCl 137.0, NaHCO₃ 12.0, KCl 4.0, and glucose 5.0. This resulted in a pH of 7.35. Test solution was prepared by adding hexamethonium (5x10⁻⁴ M; Sigma Chemical, St. Louis, Missouri) or tetrodotoxin (5x10⁻⁶ M; Sigma Chemical) to the NT. The solutions were prewarmed on a hot plate stirrer to 36°-38° C and gassed with 95% O₂-5% CO₂; oxygen tension reached about 400 mm Hg before they were instilled into the pericardial cavity.

Experimental Protocols

In each dog, the solution was instilled into the pericardial cavity, allowed to remain 60 minutes, and then removed by suction. Control dogs received three instillations of NT; test dogs received NT, test solution, and then NT again, separated by 10-minute intervals. The dogs were randomized to control and test groups. Each group contained two subgroups in which either the efferent sympathetic or efferent vagal response was tested. After a 45-minute period of each epicardial superfusion, the ERP was determined at six test sites before and during neural stimulation. It took about 15 minutes to determine each set of ERPs. In a separate group of six dogs, the effect of intravenous administration of norepinephrine (0.25 μg/kg/min) or methacholine (12.5 or 25 μg/kg/min) on the ERP was examined to determine whether the ventricular myocardium was normally responsive during epicardial superfusion with tetrodotoxin. In these dogs, changes in ERP induced by stimulation of ansae subclaviae or cervical vagi were also measured during epicardial superfusion with NT and during subsequent superfusion with tetrodotoxin.

Also, the effect of epicardial superfusion with tetrodotoxin on the fast component of local bipolar electrograms recorded from subepicardial muscle (<1 mm in depth from the epicardium) and intramyocardium (4-6 mm) of the left ventricle was determined in three dogs.

In three other dogs, NT containing fluorescein (10⁻⁴ or 10⁻³ M) was instilled into the pericardial cavity, and plasma concentration of fluorescein at 45 minutes after the onset of superfusion was measured by fluorescence spectrophotometer (model MPF-66, Perkin-Elmer, Norwalk, Connecticut).

Analysis of Data

Data from the test site with less than 9 msec shortening of ERP induced by bilateral ansae subclaviae stimulation or less than 3 msec lengthening of ERP induced by bilateral vagal stimulation during the first control superfusion with NT were discarded because of possibly insufficient neural effect at that particular site for the accurate detection of alterations in neurotransmission by the following epicardial superfusion. This resulted in exclusion of data from a total of 15 sites. Data are expressed as mean±SEM. Repeated-measures ANOVA was used. When multiple comparisons were made, a modified t test by Bonferroni was used. A statistical significance was set at a value of p<0.05.

Results

Effects of Epicardial Superfusion on Hemodynamic Parameters and Ventricular Refractoriness

Heart rate and mean arterial blood pressure were not changed 15 minutes after instillation of NT into the pericardial cavity (Table 1). Mean right atrial pressure was increased slightly. Baseline ERP, measured at 20 ventricular test sites in
TABLE 2. Baseline Effective Refractory Period of the Ventricular Test Sites

<table>
<thead>
<tr>
<th>Study Type</th>
<th>Group</th>
<th>First Superfusion</th>
<th>Second Superfusion</th>
<th>Third Superfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vagal Study</td>
<td>Control group (n=35)</td>
<td>145±1</td>
<td>146±1</td>
<td>146±2</td>
</tr>
<tr>
<td></td>
<td>Hexamethonium group (n=36)</td>
<td>142±2</td>
<td>145±2</td>
<td>142±2</td>
</tr>
<tr>
<td></td>
<td>Tetrodotoxin group (n=32)</td>
<td>154±1</td>
<td>159±2*</td>
<td>153±2</td>
</tr>
<tr>
<td>Sympathetic Study</td>
<td>Control group (n=25)</td>
<td>160±1</td>
<td>160±1</td>
<td>163±3</td>
</tr>
<tr>
<td></td>
<td>Hexamethonium group (n=26)</td>
<td>162±2</td>
<td>165±2</td>
<td>167±2*</td>
</tr>
<tr>
<td></td>
<td>Tetrodotoxin group (n=23)</td>
<td>159±3</td>
<td>159±3</td>
<td>158±3</td>
</tr>
</tbody>
</table>

Values are mean±SEM in msec. In test groups, the second epicardial superfusion was done with test solution containing hexamethonium or tetrodotoxin. n, number of test sites.

*p<0.05 vs. the values during the first superfusion with normal Tyrode’s solution.

Effects of Epicardial Superfusion with Tetrodotoxin on Changes in Ventricular Refractoriness Induced by Vagal Stimulation

Lengthening of ERP induced by bilateral vagal stimulation during the second superfusion with tetrodotoxin in six dogs tested for vagal response and during the third superfusion in five dogs from the hexamethonium group tested for sympathetic response (Table 2).

Effects of Epicardial Superfusion with Hexamethonium and Tetrodotoxin (Figure 2). Although the baseline ERP during superfusion with tetrodotoxin was prolonged as a whole compared with that during the first superfusion with NT (Table 2), it was constant (≈2-msec change) at 10 of 32 sites (151±3 to 151±2 msec). At these 10 sites, lengthening of ERP induced by vagal stimulation was suppressed by superfusion with tetrodotoxin (5.6±0.4 to 1.3±0.7 msec, p<0.001) as well as at the other 22 sites (6.7±0.3 to 1.6±0.7 msec, p<0.001). Significant recovery of vagal-induced lengthening of ERP was noted after removal of either test solution from the pericardial cavity and a 45-minute period of subsequent superfusion with NT. However, the changes in ERP did not quite reach the control values obtained during the first superfusion with NT. In six control group dogs, vagal-induced lengthening of ERP was not changed through three 45-minute periods of epicardial superfusion with NT.

Effects of Epicardial Superfusion with Hexamethonium on Changes in Ventricular Refractoriness at Each Test Site Induced by Vagal Stimulation

Vagal-induced lengthening of ERP during epicardial superfusion with NT at test sites located in the anterior and posterior left ventricle and right ventricle was eliminated or attenuated during subsequent superfusion with hexamethonium (Figure 3).

Effects of Epicardial Superfusion with Hexamethonium on Sinus Arrest Induced by Vagal Stimulation

Sinus arrest (>2 seconds) induced by bilateral vagal stimulation during the first epicardial superfusion with NT was prevented by subsequent superfusion with hexamethonium in all six test dogs. After removal of the hexamethonium solution and a 45-minute period of subsequent superfusion with NT, sinus arrest was induced again by vagal stimulation in five of six test dogs. A representative example is shown in Figure 4.
Effects of Epicardial Superfusion With Hexamethonium and Tetrodotoxin on Changes in Ventricular Refractoriness Induced by Ansae Subclaviae Stimulation

Shortening of ERP induced by bilateral ansae subclaviae stimulation during the first epicardial superfusion with NT was suppressed after a 45-minute period of subsequent superfusion with tetrodotoxin (Figure 5). Significant recovery was noted after removal of the tetrodotoxin solution from the pericardial cavity and a 45-minute period of subsequent superfusion with NT. Three 45-minute periods of epicardial superfusion with NT in five dogs and with NT-hexamethonium-NT in five test dogs did not alter shortening of ERP induced by ansae subclaviae stimulation.

Effects of Epicardial Superfusion With Tetrodotoxin on Changes in Ventricular Refractoriness Induced by Intravenous Administration of Norepinephrine and Methacholine and on Ventricular Electrograms

In three dogs, shortening of ERP induced by intravenous infusion of norepinephrine (0.25 μg/kg/min) during epicardial superfusion with NT was not changed by subsequent superfusion with tetrodotoxin despite a significant attenuation of the response to bilateral ansae subclaviae stimulation (Figure 6). Also, lengthening of ERP induced by methacholine (12.5 or 25 μg/kg/min) remained unchanged by superfusion with tetrodotoxin despite a significant attenuation of the response to vagal stimulation in three other dogs (Figure 6).

Superfusion with tetrodotoxin for 45 minutes did not affect the rate of change (dV/dt) of the fast component of bipolar electrograms recorded from subepicardial muscle (903±259 to 916±262 mV/sec, n=3) and deep intramyocardium of the left ventricle (1,597±247 to 1,606±247 mV/sec, n=3).

Systemic Absorption of Fluorescein From the Pericardial Cavity

Plasma level of fluorescein at 45 minutes after instillation of NT containing fluorescein (10^-4 or 10^-3 M) is shown in Table 3.

Discussion

Major Findings

The present data indicate that epicardial superfusion with hexamethonium prevented lengthening of ERP in deep ventricular myocardium and sinus arrest induced by cervical vagal stimulation. Hexamethonium did not suppress shortening of ERP elicited by ansae subclaviae stimulation. Epicardial superfusion with tetrodotoxin suppressed both vagal- and sympathetic-induced changes in ventricular ERP without affecting changes in ERP induced by intravenous administration of norepinephrine and methacholine. These effects of hexamethonium and tetrodotoxin were reversible after washout of the solution from the pericardial cavity.

Consideration of the Experimental Model

Using the present model, we attributed attenuation of the neurally induced responses to the effects of epicardial superfusion on neurotransmission through superficial cardiac nerves. Although it is possible that the hexamethonium and tetrodotoxin might have been absorbed into systemic circulation from the pericardial cavity via the pericardium and/or subepicardial capillaries and affected the deep myocardial test sites directly, this does not seem likely. Studies of pericardial transport function have shown very slow rates of absorption, about 1.3 ml/hr from the rabbit pericardial cavity. It took more than 24 hours for 2.0 ml phenolsulfonphthalein dye, whose molecular weight (354 MW) was comparable with that of hexamethonium (273 MW) and tetrodotoxin (319 MW), to be absorbed completely from the pericardial cavity in a
Figure 4. A representative example of recordings showing the effect of epicardial superfusion with hexamethonium on vagal-induced sinus arrest. Bipolar electrograms recorded from the right atrial appendage (RA) and right ventricle (RV) and electrocardiogram lead II (ECG II) are shown. During the first control superfusion with normal Tyrode's solution (panel A), bilateral cervical vagal stimulation (VS) with stimulation parameters set to produce sinus arrest (>2 seconds) for the right vagus (20 Hz, 4-msec duration, 0.25 mA) and complete atrioventricular block for the left vagus (20 Hz, 4-msec duration, 0.72 mA) caused sinus arrest. After 45 minutes of superfusion with hexamethonium (panel B), neither sinus arrest nor second degree or complete atrioventricular block was induced by VS at the same stimulation parameters. After a 45-minute period of the third superfusion with normal Tyrode's solution (panel C), sinus arrest was again induced by VS.

Patient. Consistent with these studies, no pericardial solution was lost during a 45-minute period of epicardial superfusion in the present model. Plasma level of fluorescein (332 MW) was approximately 1/1,000 of its concentration in the pericardial solution 45 minutes after instillation of the solution. Based on this finding, it seems unlikely that the plasma level of tetrodotoxin and hexamethonium had been sufficiently high to affect the test sites directly. In fact, epicardial superfusion with tetrodotoxin did not reduce the rate of change of the fast component of local ventricular electrograms. Also, superfusion with hexamethonium did not blunt the response to ansae subclaviae stimulation. Finally, tetrodotoxin-induced suppression of ERP changes during neural stimulation but not during infusion of norepinephrine and methacholine suggests that the attenuation of the response to the former is not due to an alteration in the reactivity of the test sites to the neurotransmitter but due to an inhibition by tetrodotoxin of neurotransmission through the sympathetic and vagal axons.

Effects of Epicardial Superfusion With Hexamethonium

Hexamethonium impairs ganglionic transmission by competing with acetylcholine for ganglionic cholinergic sites and prevents the development of postganglionic depolarization.9 It has no effect on nerve conduction or on the release of transmitter substance from the nerve terminals.9 Therefore, suppression, by epicardial superfusion with hexamethonium, of lengthening of ventricular refractoriness and sinus arrest elicited by vagal stimulation is likely due to a blockade of cardiac vagal ganglia. This suggests that vagal ganglia that innervate the ventricles are distributed superficially in a manner similar to the ganglia that selectively innervate the sinus and AV nodes.7-8 Recently, Blomquist et al13 presented data indicating that postganglionic vagal axons, arising from supraventricular ganglia, cross the AV groove to innervate the canine ventricles. Our data suggest that these ganglia must be located in the epicardial tissues. More precise localization requires further studies.

The fact that epicardial superfusion with hexamethonium did not block the efferent sympathetic response elicited by ansae subclaviae stimulation suggests that the epicardium lacks sympathetic gan-
TABLE 3. Plasma Fluorescein Level Determined by Fluorescence Spectrophotometry

<table>
<thead>
<tr>
<th></th>
<th>Fluorescein in the pericardial solution</th>
<th>Plasma fluorescein level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>$10^{-4}$ M</td>
<td>$9.9 \times 10^{-8}$ M</td>
</tr>
<tr>
<td>Dog 2</td>
<td>$10^{-4}$ M</td>
<td>$9.7 \times 10^{-8}$ M</td>
</tr>
<tr>
<td>Dog 3</td>
<td>$10^{-3}$ M</td>
<td>$1.1 \times 10^{-6}$ M</td>
</tr>
</tbody>
</table>

FIGURE 6. Bar graphs showing effects of epicardial superfusion with tetrodotoxin (TTX) on changes in ventricular effective refractory period (ERP) induced by neural stimulation and by intravenous administration of norepinephrine (0.25 µg/kg/min) and methacholine (12.5 or 25 µg/kg/min). The effects of vagal stimulation and methacholine on the ERP were determined during intravenous infusion of norepinephrine (0.125 µg/kg/min). NT, normal Tyrode's solution; n, number of test sites.

Implications of the Study

In the present study, tetrodotoxin was used to block the neurotransmission in the axons, which it does by blocking the inward sodium current at doses that do not alter muscle depolarization or contractility. The suppressive effects of epicardial superfusion with tetrodotoxin on efferent autonomic responses in the ventricles suggest that both sympathetic and vagal efferent fibers are distributed superficially at some point in their course to the heart where they must come in contact with the pericardial fluid. These conclusions are concordant with previous studies using epicardial phenol applications, which established that vagal efferent fibers cross superficially at the atrioventricular groove and are then located subendocardially, whereas sympathetic efferent fibers are located in the superficial subepicardium of the ventricles.

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