Presynaptic Modulation of Efferent Sympathetic and Vagal Neurotransmission in the Canine Heart by Hypoxia, High K\(^+\), Low pH, and Adenosine

Possible Relevance to Ischemia-Induced Denervation

Toshihisa Miyazaki and Douglas P. Zipes

Ischemia in the dog produces denervation of myocardium apical to the ischemic area. To investigate the mechanism(s) of the denervation, we tested the effects of hypoxia and some components of ischemia including high K\(^+\), low pH, and adenosine on efferent cardiac autonomic responses. In anesthetized, open-chest dogs, we occluded a diagonal branch of the left anterior descending coronary artery and perfused it with hypoxic Tyrode's solutions (Po\(_2<50\) mm Hg). We found that effective refractory period (ERP) shortening induced by bilateral ansae subclaviae stimulation at myocardium basal and apical to the perfusing area did not change during a 20–25-minute period of perfusion with hypoxic normal Tyrode's solution. During perfusion with hypoxic combined Tyrode's solution containing 12 mM K\(^+\), pH 6.8, and 10 \(\mu\)M adenosine, ERP shortening at basal sites induced by bilateral ansae subclaviae stimulation remained unchanged but was attenuated at apical sites (16±1 to 8±1 msec, mean±SEM, \(n=35\), \(p<0.001\)), and seven apical sites exhibited denervation (≤2-msec shortening). The maximum extracellular K\(^+\) concentration of the perfusing area, measured with a K\(^+\)-sensitive electrode, was 5.1±0.9 mM (\(N=3\) dogs) during perfusion with normal Tyrode's solution and was 11.8±0.1 mM (\(N=3\) dogs) during perfusion with hypoxic combined solution (\(p=0.017\)). In a separate group of dogs, the effects of high K\(^+\), low pH, and adenosine in the absence of ischemia were examined. Oxygenated Tyrode's solutions were instilled into the pericardial cavity to superfuse epicardial nerves. The Tyrode's solutions containing high K\(^+\) (12 mM), low pH (6.4), or adenosine (10 \(\mu\)M), individually or combined, reduced ERP shortening induced by bilateral ansae subclaviae stimulation in the ventricular intramyocardium to 46\%, 55\%, 56\%, and 33\% of each control value obtained during superfusion with normal Tyrode's solution and reduced the magnitude of ERP lengthening induced by bilateral cervical vagal stimulation to 57\%, 71\%, 61\%, and 39\%, respectively. ERP responses of the test sites to infused norepinephrine and methacholine, however, remained unaffected by superfusion with combined Tyrode's solution. Thus, high K\(^+\), low pH, and adenosine each inhibit efferent sympathetic and vagal neurotransmission presynaptically in the canine heart and may contribute to the development of denervation during early ischemia.


A cute myocardial ischemia/infarction in the dog results in efferent sympathetic and vagal denervation of nonischemic myocardium situated apically to the site of ischemia/infarction.\(^1\,\,\,2\) Since this denervation can occur within several minutes after coronary occlusion in a spatially heterogeneous manner,\(^2\) it may create regional autonomic imbalance leading to electrical heterogeneity that may facilitate the development of ventricular tachyarrhythmias. Several observations suggest a functional derangement of nerve action rather than structural neural damage. First, the early onset of denervation induced by acute ischemia\(^2\,\,\,3\) precedes by many hours

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the loss of fluorescent nerve terminals or the loss of tyrosine 3-hydroxylase and choline acetyltransferase activities in the ischemic myocardium and is reversible. Second, four 5-minute episodes of coronary occlusion, each separated by 5 minutes of reperfusion, do not attenuate efferent sympathetic and vagal responses of the apical myocardium and in fact reduce the degree of denervation produced by a subsequent 3-hour coronary occlusion. Third, even during sustained coronary occlusion, some apical sites that were designated initially as denervated spontaneously recover their response to neural stimulation. The cause of such neural dysfunction is unknown but could be due to at least two processes: the nerves themselves could become ischemic, or neural function could be affected by changes in the ischemic myocardium in which the nerves lie, where the extracellular K+ concentration often exceeds 12 mM, the pH is less than 6.8, PO2 is less than 50 mm Hg, and extracellular adenosine concentration increases. A final, but less likely, cause is that the nonischemic myocardium apical to the occlusion site could no longer respond appropriately to neurally released transmitters.

The purpose of this study was to test these possibilities by determining the effective refractory period (ERP) response of the nonischemic myocardium to 1) infused norepinephrine after coronary artery occlusion, 2) bilateral ansae subclaviae stimulation after coronary artery occlusion but during perfusion of the occluded vessel with hypoxic normal Tyrode’s solution to prevent accumulation of ischemic metabolites or with hypoxic Tyrode’s solution containing 12 mM K+, pH 6.8, and 10 μM adenosine, and 3) bilateral ansae subclaviae and vagal stimulation during epicardial superfusion with Tyrode’s solution containing high K+, low pH, or adenosine, which would expose the epicardial nerves to the effects of some substances generated by the ischemic myocardium but would avoid the effects of ischemia.

Materials and Methods

Surgical Procedures

One hundred twelve mongrel dogs of either sex (18–25 kg) were anesthetized with secobarbital or pentobarbital (30 mg/kg i.v.), intubated, and ventilated with room air by use of a constant volume-cycled respirator (model 607, Harvard Apparatus, South Natick, Massachusetts). To monitor arterial blood pressure a fluid-filled cannula was placed in the right femoral artery and connected to a transducer (Statham p-23Db, Gould, Cleveland, Ohio). A femoral venous cannula was used to infuse 100–200 ml/hr normal saline to replace spontaneous fluid losses. Lead II electrocardiogram was monitored throughout the study. The chest was opened through a median sternotomy. Dogs were placed on a heating pad, and the thoracotomy was covered by a plastic sheet. Epicardial temperature was monitored with a thermistor (model 400, Yellow Springs Instrument, Yellow Springs, Ohio) and was kept between 36° and 38° C by adjusting the proximity of an operating table lamp. The ansae subclaviae were isolated as they exited from the stellate ganglia, doubly ligated, and transected. The cervical vagi were also isolated, doubly ligated, and transected.

In dogs assigned to protocols 1 and 2 (see below), the heart was suspended in a pericardial cradle. A diagonal branch of the left anterior descending coronary artery was isolated carefully, and silk sutures were passed around the branch for later occlusion. Two hook electrodes were placed in midmyocardium basal to the isolated branch, and four additional electrodes were inserted apically (Figure 1, panels A and B). These electrodes served as the cathode for unipolar stimulation to determine ERP. An anodal electrode was placed in the abdominal wall. A bipolar plunge electrode was inserted in the left ventricle to record activation.

In dogs assigned to protocol 2, a bypass circuit was created between the left subclavian artery and the diagonal branch. After heparinization (3,000 IU i.v.), silicone tubing that had an internal diameter of 3 mm and a three-way stopcock was connected to the left subclavian artery. A specially designed cannula (0.8 mm i.d.) was inserted into the lumen of the diagonal branch with its tip immediately distal to the ligation and was connected to the bypass tubing to reestablish blood flow. Flow rate was measured by a flowmeter (model 613, Biotronex Laboratory, Kensington, Maryland) through an electromagnetic flow probe (model BL-2032-F25, Biotronex Laboratory) (Figure 1B). A 22G Angiocath catheter (Desseret Medical, Sandy, Utah) was inserted into the local communicating coronary vein to drain off blood during infusion of hypoxic Tyrode’s solution.

In dogs assigned to protocol 3 (see below), the pericardial cradling technique was modified to permit instillation of various solutions into the pericardial cavity to bathe the epicardial surface of the heart (Figure 1C), as reported previously. Through the pericardial opening, four hook electrodes were inserted in the anterior and posterior myocardium of the basal and apical left ventricle to a depth of 4–6 mm, and two additional electrodes were placed in the subendocardium of the right ventricular outflow and apex for ERP determination.

Measurement of Effective Refractory Period

The ERP was measured at six ventricular test sites by the extrastimulus technique, which employs a programmable stimulator (Krannert Medical Engineering, Indianapolis, Indiana) and a constant current isolator. Each ventricular test site was driven with a 2-msec rectangular cathodal stimulus twice the diastolic threshold, which was measured during each intervention. After a train of eight stimuli (S1) a late premature stimulus (S2) was introduced. The S1–S2 interval was 250 msec in dogs that received ansae subclaviae stimulation and was 280 msec in dogs that received vagal stimulation. The ventricular responses to S1 and S2 were recorded from lead II electrocar-
diagram and from a ventricular bipolar electrogram and displayed on a storage oscilloscope (model D11, Tektronix, Beaverton, Oregon). The $S_1$–$S_2$ interval was shortened in steps of 2 msec until $S_2$ failed to produce a propagated response. The $S_1$–$S_2$ interval was then increased by 5 msec and was shortened by 1-msec decrements until $S_2$ failed to produce a propagated response. The ERP was defined as the longest $S_1$–$S_2$ interval at which $S_2$ failed to produce a propagated ventricular response.

Neural Stimulation

Bilateral ansae subclaviae were stimulated with separate constant current isolators driven by a programmable stimulator (Pulsar 4, Frederick Haer, Brunswick, Maine) through shielded bipolar electrodes placed on the right and left anterior and posterior ansae. Stimuli were 4-msec rectangular pulses delivered at a frequency of 2–4 Hz and at 1.5–4 mA.

The cervical vagi were stimulated through two Teflon-coated wire electrodes embedded in the cardiac end of each vagal nerve. Stimuli were 4-msec rectangular pulses at a frequency of 20 Hz. The current strength was 0.05 mA greater than that required to produce asystole (>2 seconds) for the right vagus and asystole or complete atrioventricular block during spontaneous rhythm for the left vagus. The effects of efferent vagal stimulation on ventricular ERP were determined during intravenous infusion of norepinephrine at a rate of 0.125 or 0.25 $\mu$g/kg/min to achieve a constant background of sympathetic effect.

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

Measurement of Extracellular $K^+$ Concentration

Potassium-sensitive valinomycin electrodes and reference electrodes were prepared by following the method described by Hill et al. Both barrels (internal diameter of the shaft, 1.1 mm; tip diameter, approximately 200 $\mu$m) were fused with one to two drops of tetrahydrofuran, and a steel wire hook was attached to the distal portion. The electrodes were calibrated before and after each series of in vivo measurements in KCl-NaCl standard solutions having variable KCl concentrations (2, 4, 6, 10, and 20 mM) and a constant total ionic strength of 0.160. The potential difference between the $K^+$-sensitive electrode and a reference electrode was measured with

FIGURE 1. Schematic diagrams of the heart. Upper panel: For protocol 1, a diagonal branch of the left anterior descending coronary artery (LAD) was occluded by injecting 0.3 ml latex. LCX, left circumflex coronary artery; LV, left ventricle; RV, right ventricle; LA, left atrial appendage; RA, right atrial appendage; PA, pulmonary artery; AO, aorta. Middle panel: For protocol 2, a bypass circuit was created between the left subclavian artery and a diagonal branch of the LAD. Then the diagonal branch was perfused with hypoxic Tyrode’s solution using an external pump. The effective refractory period was measured at six test sites (solid circles) through unipolar electrodes placed in midmyocardium. Lower panel: For protocol 3, the pericardial cradling technique was modified to permit instillation of Tyrode’s solution to bathe the epicardial surface of the heart. Through the pericardial opening, four unipolar electrodes were inserted in the anterior and posterior left ventricle to a depth of 4–6 mm, and two additional electrodes were placed in the subendocardium of the right ventricle for effective refractory period determination.
TABLE 1. Compositions of Normal Tyrode's Solution and Various Test Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>NaCl</th>
<th>NaHCO3</th>
<th>NaH2PO4</th>
<th>KCl</th>
<th>CaCl2</th>
<th>Glucose</th>
<th>Adenosine</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Tyrode's solution</td>
<td>137.0</td>
<td>12.0</td>
<td>0.9</td>
<td>4.0</td>
<td>2.0</td>
<td>5.0</td>
<td>. . .</td>
<td>7.4</td>
</tr>
<tr>
<td>Combined</td>
<td>150.0</td>
<td>5.5</td>
<td>0.9</td>
<td>12.0</td>
<td>2.0</td>
<td>5.0</td>
<td>0.01</td>
<td>6.8</td>
</tr>
<tr>
<td>High K+</td>
<td>137.0</td>
<td>12.0</td>
<td>0.9</td>
<td>12.0</td>
<td>2.0</td>
<td>5.0</td>
<td>. . .</td>
<td>7.4</td>
</tr>
<tr>
<td>Adenosine</td>
<td>137.0</td>
<td>12.0</td>
<td>0.9</td>
<td>4.0</td>
<td>2.0</td>
<td>5.0</td>
<td>0.01</td>
<td>7.4</td>
</tr>
<tr>
<td>Low pH (6.8)</td>
<td>150.0</td>
<td>5.5</td>
<td>0.9</td>
<td>4.0</td>
<td>2.0</td>
<td>5.0</td>
<td>. . .</td>
<td>6.8</td>
</tr>
<tr>
<td>Low pH (6.4)</td>
<td>150.0</td>
<td>1.5</td>
<td>0.9</td>
<td>4.0</td>
<td>2.0</td>
<td>5.0</td>
<td>. . .</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Values are millimolar concentration.

an electrometer (model FD223, WP Instruments, New Haven, Connecticut), and a calibration line for each K+-sensitive electrode was obtained by plotting the potential difference against the log K+ concentration. The electrodes used in the present study had a slope of calibration line of 57–60 mV per decade change in K+ concentration. The electrodes were inserted with an introducer and hooked into midmyocardium of the anticipated center of the perfusing area (see protocol 2). The electrodes showed a transient increase in the potential difference in response to intravenous injection of 2 mM KCl. The potential difference was recorded on a Gould Brush 2800 recorder before and during a 20-minute period of regional perfusion with hypoxic Tyrode’s solution. Myocardial extracellular K+ concentration was determined by the changes in the potential difference values from the calibration line.

**Preparation of Tyrode’s Solutions**

Normal Tyrode’s solution and altered Tyrode’s solutions of various compositions (Table 1) were used for regional perfusion and for epicardial superfusion (protocols 2 and 3, see below). In protocol 2, the solutions were deoxygenated by gassing with 95% N2–5% CO2; this procedure resulted in PO2 of less than 50 mm Hg. The solutions used for protocol 3 were gassed with 95% O2–5% CO2; the resultant PO2 was 400–500 mm Hg. The solutions were warmed to 36°–38°C on a hot plate stirrer.

**Protocols**

**Protocol 1.** The effects of ischemia on ERP responses to neural stimulation and to infused norepinephrine were examined. Eight dogs underwent occlusion of a diagonal branch with injection of 0.3 ml latex. Latex was used to produce a dense transmural myocardial infarction. Before and 10 minutes after coronary occlusion, the ERP was determined at six ventricular test sites (Figure 1A) in the baseline state and during bilateral ansae subclaviae stimulation (four dogs) or during intravenous infusion of norepinephrine at a constant rate of 0.25 μg/kg/min (separate group of four dogs).

**Protocol 2.** The effects of regional perfusion with hypoxic Tyrode’s solutions on ERP responses to ansae subclaviae stimulation were examined. After a bypass circuit (described above) was established and blood flow rate through the circuit became stabilized, the ERP was determined in the baseline state and during bilateral ansae subclaviae stimulation. Then blood flow from the subclavian artery was discontinued, and the dogs underwent regional perfusion with hypoxic solutions at a constant rate through the bypass tubing using an external peristaltic pump (model 607, Harvard Apparatus) (Figure 1B). The perfusion rate was set at a slightly higher level than the maximum spontaneous blood flow rate to minimize the effects of collateral blood flow. In the preliminary studies, we observed that methylene blue dye solution perfused at this setting stained the perfused area transmurally. To minimize the systemic effects of the hypoxic solutions, venous effluent was drained via the catheter inserted in the local communicating coronary vein (Figure 1B). Twelve dogs received hypoxic normal Tyrode’s solution, and another 12 dogs received hypoxic combined solution (12 mM K+, 6.8 pH, and 10 μM adenosine). The ERP determination was done in the same way as in the control determination, starting at 10 minutes after onset of the perfusion. Since it took 10–15 minutes for each set of ERP determinations, the perfusion was performed for 20–25 minutes. After completion of the ERP determination, the perfusion with hypoxic solution was stopped, and subclavian arterial blood perfusion was resumed. After waiting for 15 minutes, the ERP determination was repeated.

In a separate group of six dogs, extracellular K+ concentration in the perfusing area was measured during hypoxic normal Tyrode’s perfusion (three dogs) or during perfusion with hypoxic combined solution (another three dogs).

**Protocol 3.** The effects of epicardial superfusion with oxygenated Tyrode’s solutions of various compositions on ERP responses to neural stimulation and to infused norepinephrine and methacholine were examined. After removal of all pericardial fluid by suction, each dog received three instillations of 40–100 ml various Tyrode’s solutions. Each solution was removed by suction after each set of ERP determinations was completed. Control (sham) dogs received three instillations of normal Tyrode’s solution. Test dogs received normal Tyrode’s solution, test solution, and then normal Tyrode’s solution again. After a 45-minute period of epicardial superfusion with each solution, the ERP was determined at six intramyocardial test sites in the baseline state and during neural stimulation. In a total of 33 dogs, the effects of a Tyrode’s
solution containing high K\(^+\) (12 mM), low pH (6.8 or 6.4), or adenosine (10 \(\mu M\)) individually and a combination of these substances (12 mM K\(^+\), 6.8 pH, and 10 \(\mu M\) adenosine) on ERP response to ansae subclaviae stimulation were examined. In another 30 dogs, the effects on ERP response to vagal stimulation of a Tyrode’s solution containing high K\(^+\) (12 mM), low pH (6.4), or adenosine (10 \(\mu M\)) individually and the combination (12 mM K\(^+\), 6.8 pH, and 10 \(\mu M\) adenosine) were examined.

In a separate group of dogs, the effects of a combined Tyrode’s solution (12 mM K\(^+\), 6.8 pH, and 10 \(\mu M\) adenosine) on the stimulus frequency–ERP response relation (four dogs, 1–4 Hz ansae subclaviae stimulation) and on the norepinephrine dose–ERP response relation (another three dogs, 0.01–0.5 \(\mu g/kg/min\) norepinephrine) were examined. The frequency-response curve and the dose-response curve were obtained during control normal Tyrode’s superfusion and during subsequent superfusion with combined Tyrode’s solution. In each dog, the ERP was determined in the baseline state then during bilateral ansae subclaviae stimulation at different frequencies or during intravenous infusion of norepinephrine at different doses. The order of stimulation frequencies or the order of doses was chosen randomly and given at 10-minute intervals.

In a separate group of four dogs, ERP responses of the test sites to bilateral vagal stimulation and to intravenous administration of methacholine (12.5 \(\mu g/kg/min\)) were examined during control normal Tyrode’s superfusion and during subsequent superfusion with combined Tyrode’s solution. The effects of vagal stimulation and methacholine on the ERP were determined during intravenous infusion of norepinephrine (0.125 or 0.25 \(\mu g/kg/min\)) to maintain a constant background level of sympathetic effect.

Analysis of Data

As reported in the previous studies, data from ventricular test sites with less than 9-msec shortening of the ERP elicited by bilateral ansae subclaviae stimulation or less than 3-msec lengthening of ERP induced by bilateral vagal stimulation during the first control determination were excluded because of insufficient effects of neural stimulation at those particular sites. A total of 35 sites out of 564 sites (6%) met these criteria. Sites were arbitrarily considered to be sympathetically denervated if bilateral ansae subclaviae stimulation shortened ERP 9 msec or more during the first control determination but 2 msec or less after an intervention. Sites were considered to be vagally denervated if bilateral vagal stimulation lengthened ERP 3 msec or more before but 1 msec or less after an intervention.

Data were expressed as mean±SEM. The difference among mean values was determined by an analysis of variance for repeated measurements. Paired and unpaired \(t\) tests were performed when two measurements were compared. When multiple comparisons were made, the \(t\) test was modified by the Bonferroni method. A statistical significance was set at a value of \(p<0.05\).

Results

Protocol 1: Effects of Ischemia on Refractory Period Responses to Neural Stimulation and to Infused Norepinephrine

Figure 2 shows that, 10 minutes after latex injection, ERP shortening induced by bilateral ansae subclaviae stimulation was unchanged at myocardium basal to the site of ischemia but was attenuated at the apical myocardium. Three of 14 apical test sites (21%) exhibited sympathetic denervation (≤2-msec shortening). In contrast, ERP shortening elicited by infused norepinephrine was preserved at apical sites as well as at basal sites.

Protocol 2: Effects of Regional Perfusion With Hypoxic Tyrode’s Solutions on Refractory Period Response to Ansae Subclaviae Stimulation

Two of 12 dogs that underwent hypoxic normal Tyrode’s perfusion and three of 12 dogs that had perfusion with hypoxic combined solution developed ventricular fibrillation during ERP determination in the absence of neural stimulation. Therefore, the
ERP response to neural stimulation could not be determined in these five dogs. Data obtained from the remaining 10 dogs of the former group and from nine dogs in the latter group are shown in Figure 3. The ERP data during blood perfusion after completion of the hypoxic perfusion were not obtained in two of the 10 dogs and in three of the 9 dogs, respectively, because these dogs failed to reestablish blood flow through the bypass circuit.

The ERP shortening induced by bilateral ansae subclaviae stimulation at basal and apical sites remained unchanged during a 20–25-minute period of regional perfusion with hypoxic normal Tyrode’s solution compared with the values obtained during blood perfusion before and after perfusion with hypoxic normal Tyrode’s solution. During perfusion with combined solution, ERP shortening induced by bilateral ansae subclaviae stimulation remained unchanged at basal sites but was attenuated at apical sites, and seven of 35 apical sites (20%) developed symptomatic denervation. Fifteen minutes after restoration of blood flow to the perfusing area, the ERP response returned in the apical sites, and denervation was reversed at all seven sites (0.7±0.6 msec shortening during perfusion with hypoxic combined solution to 15.2±3.4 msec shortening after restoration of blood flow, p<0.005).

The maximum control blood flow rate through the bypass circuit, perfusion rate of the hypoxic solution, and venous drainage rate during hypoxic perfusion were similar in both groups (Table 2). Serum K⁺ concentration at the end of hypoxic perfusion remained within a physiological range in both groups (Table 2). Baseline heart rate, mean arterial blood pressure, and ERP values of the test sites are shown in Table 3.

Table 2. Perfusion Variables and Serum K⁺ Concentration in Dogs That Received Regional Perfusion With Hypoxic Normal Tyrode’s Solution and in Dogs That Received Regional Perfusion With Hypoxic Combined Solution

<table>
<thead>
<tr>
<th></th>
<th>Hypoxic normal Tyrode’s solution (N=10)</th>
<th>Hypoxic combined solution (N=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control blood flow rate of the diagonal branch (ml/min)</td>
<td>11.9±2.1</td>
<td>11.7±3.0</td>
</tr>
<tr>
<td>Regional perfusion rate (ml/min)</td>
<td>14.2±1.8</td>
<td>13.9±1.9</td>
</tr>
<tr>
<td>Venous drainage rate (ml/min)</td>
<td>3.9±0.9</td>
<td>5.1±0.8</td>
</tr>
<tr>
<td>Serum K⁺ concentration (mM)</td>
<td>3.6±0.2</td>
<td>3.9±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. N, number of test dogs.

Figure 3. Bar graphs showing effects of regional perfusion with hypoxic Tyrode’s solution on changes in effective refractory period (Δ ERP, ordinate) induced by bilateral ansae subclaviae stimulation at the myocardium basal and apical to the perfusing area. Left panels indicate the data from 10 dogs that received regional perfusion with hypoxic normal Tyrode’s solution, and right panels indicate the data from nine dogs receiving regional perfusion with hypoxic combined solution. Δ ERP were determined three times, that is, during subclavian arterial blood perfusion (left column), 10 minutes after the onset of perfusion with the hypoxic solution (middle column), and 15 minutes after restoration of blood flow to the perfusing area (right column). n, number of test sites. See text for details.

Protocol 2: Myocardial Extracellular K⁺ Concentration in the Perfusing Area

Within several minutes after the onset of regional perfusion with hypoxic normal Tyrode’s solution and with the combined solution, myocardial extracellular K⁺ concentration in the perfusing area reached plateau levels, approximately 4.5 mM and 11.0 mM, respectively (Figure 4). The maximum K⁺ concentration in dogs that underwent perfusion with normal Tyrode’s solution was significantly lower than in dogs that underwent perfusion with the combined solution (5.1±0.9 mM, N=3 dogs, vs. 11.8±0.1 mM, N=3 dogs, p=0.017).

Protocol 3: Effects of Epicardial Superfusion With Oxygenated Tyrode’s Solutions of Various Compositions on Refractory Period Response to Ansae Subclaviae Stimulation

Figure 5 shows that in control dogs three instillations of normal Tyrode’s solution into the pericardial space did not affect ERP shortening induced by bilateral ansae subclaviae stimulation. In contrast, oxygenated Tyrode’s solution containing high K⁺ (12 mM), low pH (6.4), or adenosine (10 μM) individually or combined (12 mM K⁺, 6.8 pH, and 10 μM...
Table 3. Baseline Heart Rate, Mean Arterial Blood Pressure, and Effective Refractory Period of the Ventricular Test Sites in Dogs That Received Regional Perfusion With a Hypoxic Tyrode's Solution

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Heart rate (beats/min)</th>
<th>MAP (mm Hg)</th>
<th>Ventricular ERP (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal sites</td>
<td>Apical sites</td>
<td>Basal sites</td>
</tr>
<tr>
<td>NT solution (N=10)</td>
<td>109±4</td>
<td>95±4</td>
<td>165±2</td>
</tr>
<tr>
<td>Arterial blood 1</td>
<td>(N=10)</td>
<td>(N=10)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>NT solution</td>
<td>112±4</td>
<td>92±4</td>
<td>167±3</td>
</tr>
<tr>
<td>Arterial blood 2</td>
<td>(N=10)</td>
<td>(N=10)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>Combined solution (N=9)</td>
<td>116±4</td>
<td>92±5</td>
<td>167±3</td>
</tr>
<tr>
<td>Arterial blood 1</td>
<td>(N=8)</td>
<td>(N=8)</td>
<td>(n=15)</td>
</tr>
<tr>
<td>Combined solution</td>
<td>123±6</td>
<td>93±4</td>
<td>164±2</td>
</tr>
<tr>
<td>Arterial blood 2</td>
<td>(N=9)</td>
<td>(N=9)</td>
<td>(n=18)</td>
</tr>
<tr>
<td>Arterial blood 2</td>
<td>125±5</td>
<td>92±3</td>
<td>168±2</td>
</tr>
<tr>
<td>Arterial blood 2</td>
<td>(N=9)</td>
<td>(N=9)</td>
<td>(n=18)</td>
</tr>
<tr>
<td>Combined solution</td>
<td>125±7</td>
<td>76±6*</td>
<td>163±2</td>
</tr>
<tr>
<td>Arterial blood 2</td>
<td>(N=6)</td>
<td>(N=6)</td>
<td>(n=12)</td>
</tr>
</tbody>
</table>

Values are mean±SEM for the 10 dogs that received hypoxic normal Tyrode's (NT) solution and the nine dogs that received combined solution. MAP, mean arterial blood pressure; ERP, effective refractory period; arterial blood 1, measurement during subclavian arterial blood perfusion; arterial blood 2, measurement after restoration of subclavian arterial blood flow to the perfusing area after NT or combined solution; N, number of test dogs; n, number of ventricular test sites.

*p<0.05 vs. the first arterial blood perfusion.

Baseline heart rate, mean arterial blood pressure, and the ERP values were constant throughout three periods of superfusion in all groups (Table 5).

Protocol 3: Effects of Epicardial Superfusion With Combined Solution on Stimulus Frequency–and Norepinephrine Dose–Refractory Period Response Relations

Figure 6 shows that the extent of ERP shortening induced by bilateral ansae subclaviae stimulation increased as the frequency level of stimulation increased (p<0.001) during normal Tyrode's superfusion of the epicardium and during superfusion with the hypoxic solutions.
combined solution. However, less shortening of ERP was induced during superfusion with the combined solution than during superfusion with normal Tyrode's solution at all three frequency levels tested (p<0.001). The change in ERP elicited by infused norepinephrine increased as the dose increased (p<0.001) during superfusion with normal Tyrode's solution and with the combined solution. The dose-response curves for the two mediums were not different (p=0.966).

**Protocol 3: Effects of Epidcardial Superfusion With Oxygenated Tyrode's Solutions of Various Compositions on Refractory Period Response to Vagal Stimulation**

Figure 7 shows ERP response to vagal stimulation. Three instillations of normal oxygenated Tyrode's solution into the pericardial space in the control dogs did not affect ERP lengthening induced by bilateral vagal stimulation. Instillation of oxygenated Tyrode's solutions containing high K+ (12 mM), low pH (6.4), or adenosine (10 μM) individually or combined (12 mM K+, 6.8 pH, and 10 μM adenosine) significantly reduced ERP lengthening of the intramyocardium to 57%, 71%, 61%, and 39%, respectively, of each control value obtained during the first normal Tyrode's superfusion. These effects were reversed after removal of the solutions and instillation of normal oxygenated Tyrode's solution, except for the effect of pH 6.4. The frequency of vagally denervated sites during superfusion with each solution is shown in Table 4. Although the suppressive effect of epidcardial superfusion with high K+ solution (12 mM) on vagally induced ERP lengthening was reversed for the group after removal of the solution and instillation of normal Tyrode's solution, four of 33 sites remained denervated.

Baseline heart rate, mean arterial blood pressure, and the ERP values were constant throughout three periods of superfusion in all groups (Table 6).

**Protocol 3: Effects of Epidcardial Superfusion With Combined Solution on Refractory Period Responses to Vagal Stimulation and to Methacholine**

Figure 8 shows that the ERP lengthening induced by bilateral vagal stimulation during control superfusion with normal Tyrode's solution was suppressed by subsequent superfusion with combined Tyrode's solution. However, lengthening of ERP induced by intravenous infusion of methacholine remained constant.

**Discussion**

**Major Observations**

The major observations from the present study were that 1) acute myocardial ischemia produced by latex injection into a diagonal branch attenuated the ERP shortening of myocardium apical to the ischemic area.
induced by efferent sympathetic nerve stimulation but did not affect the ERP response to intravenous norepinephrine, 2) occlusion of a diagonal branch and perfusion with hypoxic normal Tyrode’s solution did not affect ERP shortening induced by efferent sympathetic nerve stimulation at basal and apical sites, while perfusion with hypoxic Tyrode’s solution containing high K⁺ (12 mM), low pH (6.8), and adenosine (10 μM) promptly attenuated the ERP response at apical myocardium, 3) instillation into the pericardial cavity of oxygenated Tyrode’s solution containing high K⁺ (12 mM), low pH (6.4), or adenosine (10 μM) individually or the oxygenated combined solution (12 mM K⁺, 6.8 pH, and 10 μM adenosine) suppressed ERP responses of the intramyocardium to efferent sympathetic and vagal nerve stimulation without affecting the ERP responses to intravenous norepinephrine and methacholine, and 4) the effects of high K⁺ (12 mM) and

**Table 5.** Baseline Heart Rate, Mean Arterial Blood Pressure, and Ventricular Effective Refractory Periods in Dogs From Each Group That Received Epicardial Superfusion and Ansae Subclaviae Stimulation

<table>
<thead>
<tr>
<th>Heart rate (beats/min)</th>
<th>Superfusion</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N=5)</td>
<td>116±3</td>
<td>116±2</td>
<td>115±1</td>
</tr>
<tr>
<td>Combined (N=6)</td>
<td>115±11</td>
<td>115±11</td>
<td>115±9</td>
</tr>
<tr>
<td>High K⁺ (N=6)</td>
<td>108±3</td>
<td>111±3</td>
<td>112±5</td>
</tr>
<tr>
<td>Adenosine (N=6)</td>
<td>108±4</td>
<td>107±4</td>
<td>112±4</td>
</tr>
<tr>
<td>Low pH (6.8) (N=4)</td>
<td>110±9</td>
<td>111±7</td>
<td>112±6</td>
</tr>
<tr>
<td>Low pH (6.4) (N=6)</td>
<td>118±6</td>
<td>116±6</td>
<td>117±7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>Superfusion</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N=5)</td>
<td>116±1</td>
<td>117±3</td>
<td>113±2</td>
</tr>
<tr>
<td>Combined (N=6)</td>
<td>111±4</td>
<td>107±2</td>
<td>102±3</td>
</tr>
<tr>
<td>High K⁺ (N=6)</td>
<td>108±11</td>
<td>107±8</td>
<td>102±6</td>
</tr>
<tr>
<td>Adenosine (N=6)</td>
<td>106±10</td>
<td>103±6</td>
<td>93±9</td>
</tr>
<tr>
<td>Low pH (6.8) (N=4)</td>
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<td>97±3</td>
<td>84±5</td>
</tr>
<tr>
<td>Low pH (6.4) (N=6)</td>
<td>106±5</td>
<td>107±5</td>
<td>100±3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ventricular ERP (msec)</th>
<th>Superfusion</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=25)</td>
<td>160±1</td>
<td>160±1</td>
<td>163±3</td>
</tr>
<tr>
<td>Combined (n=32)</td>
<td>173±2</td>
<td>170±2</td>
<td>170±2</td>
</tr>
<tr>
<td>High K⁺ (n=33)</td>
<td>171±3</td>
<td>167±3</td>
<td>168±2</td>
</tr>
<tr>
<td>Adenosine (n=32)</td>
<td>178±2</td>
<td>174±3</td>
<td>177±3</td>
</tr>
<tr>
<td>Low pH (6.8) (n=23)</td>
<td>169±2</td>
<td>167±2</td>
<td>166±2</td>
</tr>
<tr>
<td>Low pH (6.4) (n=32)</td>
<td>169±3</td>
<td>167±3</td>
<td>165±3</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Superfusion 1, normal Tyrode’s solution; superfusion 2, superfusion with each test solution; superfusion 3, normal Tyrode’s solution; ERP, effective refractory period; N, number of test dogs; n, number of ventricular test sites.

**FIGURE 6.** Graphs showing stimulus frequency of bilateral ansae subclaviae stimulation—effective refractory period (ERP) response curves (left panel) and intravenous norepinephrine dose–ERP response curves (right panel) obtained during epicardial superfusion with oxygenated normal Tyrode’s solution and with oxygenated combined solution. n, number of test sites.
adenosine were reversible, while low pH (6.4) caused sustained depression of the autonomic responses.

Effects of Regional Ischemia With and Without Ischemic Metabolite Accumulation

The response to ischemia is complex and includes deprivation of oxygen as well as the accumulation of ischemic metabolites from loss of cellular integrity. To examine the effect of regional ischemia without accumulation of ischemic metabolites, we perfused the occluded diagonal branch with hypoxic normal Tyrode’s solution. Extracellular K⁺ concentration in the perfused area remained within a physiological range for 20 minutes; this occurrence suggested that hypoxic normal Tyrode’s perfusion largely prevented metabolite buildup. We realize that this conclusion for other metabolites is by inference only. During this perfusion, ERP shortening in response to efferent

Table 6. Baseline Heart Rate, Mean Arterial Blood Pressure, and Ventricular Effective Refractory Periods During Norepinephrine Infusion in Dogs That Received Epicardial Superfusion and Vagal Stimulation

<table>
<thead>
<tr>
<th></th>
<th>Control (N=6)</th>
<th>Combined (N=6)</th>
<th>High K⁺ (N=6)</th>
<th>Adenosine (N=6)</th>
<th>Low pH (6.4) (N=6)</th>
<th>Superfusion 1</th>
<th>Superfusion 2</th>
<th>Superfusion 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>149±10</td>
<td>151±8</td>
<td>153±9</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>144±6</td>
<td>140±8</td>
<td>142±7</td>
</tr>
<tr>
<td>Ventricular ERP (msec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>145±1</td>
<td>146±1</td>
<td>146±2</td>
</tr>
</tbody>
</table>

Values are mean±SEM and were obtained during intravenous infusion of norepinephrine at a rate of 0.125 or 0.25 µg/kg/min to achieve a constant background of sympathetic effect. Superfusion 1, normal Tyrode’s solution; superfusion 2, superfusion with each test solution; superfusion 3, normal Tyrode’s solution; ERP, effective refractory period; N, number of test dogs; n, number of ventricular test sites.
sympathetic stimulation was preserved in apical as well as basal myocardium. These results indicate that short-term regional ischemia in the absence of significant metabolite buildup does not interrupt the response to efferent sympathetic stimulation. In contrast, perfusion with a hypoxic Tyrode’s solution containing high K⁺, low pH, and adenosine suppressed ERP shortening of the apical myocardium in response to efferent sympathetic stimulation. Although it is possible that the solution entered the systemic circulation and affected the sympathetic response of the test sites, this is unlikely because the response at the basal myocardium remained unaffected. The logical conclusion is that ischemic metabolites accumulate in the ischemic myocardium and affect presynaptic transmission in the nerves traveling through the ischemic area.

We cannot exclude the possibility that adenosine injected into the coronary artery was trapped by the vascular endothelium and inactivated by adenosine deaminase, at least to some extent, before it reached the extracellular space. Also the hypoxic combined solution used in the present study simulates only a part of the extracellular environment of early ischemia. There may be several other substances that potentially contribute to ischemia-induced denervation. For example, prostaglandin E₂, which modulates sympathetic neurotransmission in the heart via a presynaptic receptor, is known to be released from the ischemic myocardium.

**Effects of High K⁺, Low pH, and Adenosine and Their Relevance to Ischemia-Induced Denervation**

To examine whether high K⁺, low pH, or adenosine individually modulated efferent cardiac sympathetic and vagal neurotransmission by acting at pre-synaptic sites, we used an epicardial superfusion model. Using this model, we have shown that instillation into the pericardial space of Tyrode’s solution containing tetrodotoxin (5 μM), a blocker of axonal neurotransmission, attenuates the refractory period responses of the ventricular intramyocardium to efferent sympathetic and vagal stimulation without affecting postjunctional responsiveness to norepinephrine and methacholine. These data indicate that both vagal and sympathetic axons are distributed superficially at some point in the heart, and their function is subjected to the effects of substances located in the pericardial fluid. Thus, using this model, we can examine the effects of certain substances on cardiac neurotransmission separately from their effects on the effector sites. Further, this model allows us to control precisely the concentration of the substances to be tested, eliminating the variables of blood flow and streaming inherent in the coronary perfusion approach. Steady-state conditions, however, might not necessarily have been met during epicardial superfusion with adenosine solution, since adenosine is known to be removed over time from pericardial infusate. Importantly, however, this technique totally avoids the influence on the results of any reduction in coronary blood flow produced by a cannulation model.

The present data demonstrate that 12 mM K⁺, low pH of 6.4, and 10 μM adenosine individually inhibit both efferent sympathetic and vagal responses. Since the responses of the test sites to norepinephrine and methacholine were unaffected, the effects of these substances can be attributed to presynaptic modulation. Lorenz and Vanhoutte have shown that increasing the K⁺ concentration up to 20 mM inhibits norepinephrine release from nerve terminals and relaxes canine venous smooth muscle constricted by supramaximal sympathetic stimulation. Puig and Kirpekar have reported that perfusion of spleen at pH 6.2–6.4 for 30 minutes inhibits norepinephrine release evoked by nerve stimulation. These investigators suggest a reduction of Ca²⁺ entry into the nerve terminals, an essential process in evoking neurotransmitter release, as a possible explanation for the effects of high K⁺ and low pH. Presynaptic action potentials trigger neurotransmitter release from the nerve terminals indirectly by increasing Ca²⁺ entry through voltage-dependent calcium channels. Alterations in action potential characteristics of the neurons may modulate Ca²⁺ entry, thereby reducing neurotransmitter release. Thus, suppression of efferent sympathetic and vagal neurotransmission by high K⁺ and low pH in the previous studies and in the present study may be explained, in part, by their potential effects on action potential characteristics of the neurons. In the present model, it seems more likely that the high K⁺ and low pH milieu affected action potential propagation in the postganglionic axons at some point proximal to the nerve terminals, thereby inhibiting efferent cardiac autonomic responses.
In the ischemic area, extracellular K+ concentration reaches 8–12 mM within 10 minutes after coronary occlusion.7–9 In preliminary studies, we observed that perfusion of an occluded diagonal branch with hypoxic normal Tyrode’s solution did not prevent efferent sympathetic-induced increase in norepinephrine output in the effluent from the local communicating coronary vein in one dog (1,356 pg/min without stimulation and 6,720 pg/min during stimulation), whereas perfusion with hypoxic Tyrode’s solution containing 12 mM K+ eliminated the increase in another two dogs (804 pg/min without stimulation and 684 pg/min during stimulation) (authors’ unpublished data). These preliminary data also support the possibility that accumulation of K+ in the extracellular space causes a failure of neurotransmission within the ischemic area.

The effect of low pH in the ischemic area is more complex. In addition to inhibiting neurotransmitter release, low pH also affects binding and storage of norepinephrine within the nerve terminals24 and inhibits neuronal reuptake.25 In the present study, epicardial superfusion with the Tyrode’s solution having a pH of 6.8, a degree of acidosis that can occur within a 10-minute period of ischemia,10 did not modulate efferent sympathetic and vagal responses. Therefore, it seems unlikely that a reduction in pH alone to 6.8 or higher causes denervation during early ischemia. On the other hand, the Tyrode’s solution with a pH of 6.4, a level of acidosis that occurs after ischemia exceeding 15 minutes,10 caused sustained depression of efferent sympathetic and vagal responses after removal of the solution. Ciuffo et al26 have observed a prolonged depression in response to nerve stimulation in the myocardium that had been subjected to 25-minute ischemia. They found that augmentation in segment shortening in the postischemic myocardium induced by efferent sympathetic nerve stimulation remained depressed, while the responsiveness to systemic norepinephrine was maintained. Reduction of pH to a critical level during prolonged ischemia may contribute to this postischemic neural disturbance.

Adenosine inhibits neurotransmitter release from the sympathetic and parasympathetic nerve terminals in many species and tissues.21–32 Also it exerts postjunctional adrenergic antagonism.33,34 In the present study, we observed an inhibitory effect of adenosine on efferent sympathetic and vagal neurotransmission. Although the effect of adenosine in our model seems to be due to a presynaptic modulation, the precise mechanism responsible for its action remains uncertain. Adenosine in the solution might have acted by a presynaptic A1-receptor–mediated inhibition of neurotransmission32,35 and/or by changing membrane potential or conduction properties of the neurons.36 Whatever mechanism operates, it is likely that adenosine can cause denervation by interrupting neurotransmission presynaptically in the ischemic myocardium, since it accumulates in micromolar concentrations.11,12 Finally, the results from the present study that suggest a presynaptic action of adenosine do not preclude the possibility that adenosine might have a significant postjunctional effect also in different experimental settings, as suggested by several studies37,38 performed in dogs.

Implications of the Study
During the early period of ischemia, it is likely that efferent sympathetic nerve input to the heart increases.39,40 The suppressive effects of high K+, low pH, and adenosine on cardiac efferent sympathetic neurotransmission may help protect the ischemic myocardium from excessive catecholamine stimulation and modulate afferent reflexes. However, because metabolite concentration in the ischemic myocardium is spatially heterogeneous,7,8 it is likely that neural interruption is not uniform. The resultant autonomic imbalance may contribute to arrhythmia development during acute myocardial ischemia.

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