Differences in the Electrophysiological Response of Canine Ventricular Subendocardium and Subepicardium to Acetylcholine and Isoproterenol

A Direct Effect of Acetylcholine in Ventricular Myocardium

Silvio H. Litovsky and Charles Antzelevitch

A prolongation of the ventricular effective refractory period in response to cholinergic agonists or vagal stimulation has been demonstrated in a number of in vivo animal models. However, exposure of isolated myocardial tissues obtained from these hearts to as much as 10^{-4} M acetylcholine has been shown to produce essentially no change in action potential duration or effective refractory period. The discrepancy between the in vivo and in vitro findings generally has been explained on the basis of accentuated antagonism, whereby parasympathetic agonists exert their influence through antagonism of the effects of β-adrenergic tone in vivo. The fact that acetylcholine exerts little if any direct effect on the electrical activity of ventricular myocardium, although well accepted, is based exclusively on studies performed using endocardial preparations. Our recent demonstration of major electrophysiological differences between canine ventricular endocardium and epicardium prompted us to examine the effects of acetylcholine and the role of accentuated antagonism in these two tissue types. Using standard microelectrode techniques, we show that acetylcholine (10^{-7}–10^{-5} M) has little if any effect in canine ventricular endocardium but a pronounced effect to either prolong or markedly abbreviate action potential duration and effective refractory period in epicardium. These effects of acetylcholine on epicardium are attended by an accentuation of the spike and dome morphology of the action potential, are readily reversed with atropine, fail to appear when epicardium is pretreated with the transient outward current blocker 4-aminopyridine, are accentuated in the presence of isoproterenol (10^{-7} to 5×10^{-6} M), and persist in the presence of propranolol. Isoproterenol-induced abbreviation of action potential duration and effective refractory period is also shown to be more pronounced in epicardium than in endocardium; equimolar concentrations of acetylcholine completely antagonize the effects of isoproterenol in endocardium and epicardium. We conclude that acetylcholine exerts important direct effects on the electrical response of canine ventricular myocardium, which are accentuated in the presence of β-adrenergic agonists. Our findings suggest the differential response of epicardium and endocardium to acetylcholine is due to the presence of a transient outward current-mediated spike and dome morphology in the epicardial action potential. Finally, the data suggest that acetylcholine may exert antiarrhythmic as well as arrhythmogenic effects through its actions to alter conduction and refactoriness. (Circulation Research 1990;67:615–627)

The parasympathetic nervous system is known to exert important influences on the electrophysiological characteristics of the heart. The specific effects and mechanisms underlying these effects have been shown to vary greatly among different animal species and cell types (see Loffelholz and Pappano and Hartzel for review). In mammalian ventricular myocardium, exogenously applied acetylcholine (ACh) has been shown to exert significant effects on contractility but little or no changes in action potential characteristics. ACh in concentrations as high as 10^{-4} M was found to produce essentially no effect on the action potential duration (APD), effective refractory period (ERP), or other action potential parameters recorded from isolated...
canine, feline, or guinea pig papillary muscles. In contrast, in vivo studies have demonstrated a clear prolongation of the ventricular ERP in response to cholinergic agonists or vagal stimulation in animal models. In addition, muscarinic blockade with atropine has been shown to produce an abbreviation of the ventricular ERP in animals as well as humans.

The discrepancy between the in vivo and in vitro results generally has been explained on the basis of accentuated antagonism. Both the electrophysiologic and inotropic effects of ACh observed in vitro and of parasympathetic stimulation in vivo are known to be accentuated in the presence of β-adrenergic agonists or sympathetic tone. The ERP prolongation observed in vivo therefore is thought to be due to a cholinergically mediated modulation of the effects of existing β-adrenergic tone.

Several studies, however, have provided evidence in support of a direct effect of the parasympathetic nervous system to prolong ventricular refractoriness. Blair and coworkers showed the persistence of the effect of atropine to abbreviate ERP in the cat ventricle after sympathectomy, and Prystowsky and coworkers demonstrated a similar effect of atropine in humans pretreated with the β-blocker propranolol.

The suggestion of a direct effect of ACh on ERP remains at odds with the lack of a demonstrated effect of ACh on APD or ERP in isolated preparations. Whereas in vivo studies of parasympathetic influence have involved assessment of ERP in both endocardial and epicardial surfaces of the ventricle, in vitro studies of the effects of cholinergic agonists have been performed exclusively on endocardial tissues. A need to evaluate these effects in isolated epicardial tissues derives from the in vivo study of Martins and Zipes in which vagal stimulation was found to exert a greater influence on the ERP of epicardium as compared with endocardium.

Recent work from our laboratory has delineated major differences between the characteristics of canine ventricular endocardium and epicardium. The presence of a prominent transient outward current (I_o) in epicardium but not in endocardium was shown to contribute to a number of differences in the electrophysiologic characteristics and pharmacologic responsiveness of the two tissue types.

The present study was designed to critically assess and contrast the effects of ACh in isolated endocardial and epicardial canine ventricular tissues in the presence and absence of a β-adrenergic agonist and antagonists.

Materials and Methods

Papillary muscles, right ventricular trabeculae, and right ventricular epicardial strips (approximately 2.0 × 1.5 × 0.2 cm) were isolated from hearts removed from anesthetized (sodium pentobarbital, 30 mg/kg) mongrel male dogs. The epicardial preparations were obtained by razor blade shavings (Davol Simon Dermatome Handling 3293 with cutting head 3295, Cranston, R.I.) made parallel to the fiber orientation in the right ventricular free wall. We found no major differences between the characteristics of the papillary muscles and those of trabeculae, so we have grouped these together in the presentation of the results. It might also be noted that no significant difference could be discerned between the activity of intact papillary muscles and that of strips shaved from the surface of these muscles. The use of the terms endocardial and epicardial in this paper refer to the myocardial cells on the respective surfaces of the ventricular wall, representing the outermost subendocardial and subepicardial layers.

Epicardial and endocardial preparations from the same heart were placed in a tissue bath and allowed to equilibrate for 1 hour while superfused with an oxygenated (95% O₂, 5% CO₂) Tyrode’s solution (37±0.5° C, pH 7.35) with a composition of (mM) NaCl 129, KCl 4, NaH₂PO₄ 0.9, NaHCO₃ 20, CaCl₂ 1.8, MgSO₄ 0.5, and D-glucose 5.5.

The tissues were stimulated at a basic cycle length (BCL) ranging from 250 to 2,000 msec using rectangular stimuli (1–3 msec duration, 2.5 times diastolic threshold intensity) delivered through silver bipolar electrodes insulated except at the tips.

Transmembrane potentials were recorded from one or more sites using glass microelectrodes filled with 2.7 M KCl (10–20 MΩ DC resistance) connected to a high input–impedance amplification system (WPI, New Haven, Conn.). Amplified signals were displayed on an oscilloscope (Tektronix, Beaverton, Ore.) and photographed on a 35-mm kymographic camera (Grass Instrument Co., Quincy, Mass.) or recorded on FM tape (A.R. Vetter Co., Rebersburg, Pa.). The maximal rate of rise of the action potential upstroke was measured with a differentiator adjusted for linearity with the range of 50–500 V/sec.

Care was taken to avoid transitional cells in obtaining data representative of ventricular endocardium. In the case of papillary muscles, recordings were always made from the apical region, known to be devoid of Purkinje fibers.

Restitution of action potential characteristics was determined using single test pulses (S₂) delivered after every tenth basic beat (S₁). The S₁–S₂ coupling interval was increased progressively from the end of the refractory period until the next basic beat. ERP was defined as the longest S₁–S₂ interval at which S₂ failed to elicit a propagated response.

The dose-response relations for ACh were determined over a concentration range of 10⁻⁷–10⁻⁵ M. Data were collected 20 minutes after each change in concentration. The dose-dependent effects of ACh described in Tables 1 and 2 were obtained by initial exposure of the preparations to 10⁻⁷ M ACh, followed by a washout period, during which the action potential morphology was observed to return to control, and reintroduction of ACh at the higher concentration (10⁻⁴ M). In some cases, the tissues were exposed directly to 10⁻⁶ M ACh (n=5).
Acetylcholine HCl (Sigma Chemical Co., St. Louis) was dissolved in distilled water to yield a stock solution of 1 mM. 4-Aminopyridine (4-AP) (Sigma) was dissolved in distilled water and made soluble by warming to yield a stock solution of 0.5 M. The pH of the stock solution was adjusted to 7.4 with HCl. Other agents used were atropine sulfate, dl-isoproterenol HCl, dl-propranolol, and tyramine HCl (all from Sigma).

Statistical analysis was performed using analysis of variance coupled with the least significant difference procedure or a paired t test, as appropriate, and nonlinear regression curve-fitting techniques.24

The terminology used to describe the various phases of the epicardial and endocardial action potential is as defined in a previous publication.19

**Results**

Figure 1 illustrates the concentration-dependent effects of ACh on the action potential characteristics of isolated epicardial and endocardial tissues obtained from the same heart. At a BCL of 300 msec, ACh (10⁻⁷–10⁻⁵ M) produced little change in endocardium (upper right panel). In contrast, similar concentrations of ACh produced pronounced changes in the morphology of the responses recorded from epicardium (upper left panel). With ACh concentrations of 10⁻⁷ and 10⁻⁶ M, the epicardial APD was increased by 11 and 19 msec (7.1% and 12.3%), respectively. These changes were attended by a more accentuated phase 1, a slowing of the second action potential upstroke, and a negative shift of the peak plateau. The slope of phase 3 was largely unaffected. When [ACh] was raised to 10⁻⁵ M, further accentuation of phase 1 resulted in suppression of the action potential plateau, thus leading to a marked abbreviation of the action potential (54 msec decrease in APD₉₀ versus control; 35%). The lower panel of Figure 1 shows the concentration-dependence of ACh effects on the action potential of epicardial tissues from the same heart.

**TABLE 1. Effects of 10⁻⁷ M Acetylcholine on Action Potential Parameters of Canine Ventricular Epicardium**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Acetylcholine (10⁻⁷ M)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>−82.2±0.9</td>
<td>−81.8±0.8</td>
<td>−0.4±0.2</td>
</tr>
<tr>
<td>Amplitude</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 0 (mV)</td>
<td>97.2±3.4</td>
<td>96.3±3.8</td>
<td>−0.8±0.7</td>
</tr>
<tr>
<td>Phase 1 (mV)</td>
<td>71.6±5.5</td>
<td>68.6±5.7</td>
<td>−3.0±1.3†</td>
</tr>
<tr>
<td>Phase 2 (mV)</td>
<td>97.6±4.5</td>
<td>96.6±5.1</td>
<td>−0.9±1.0</td>
</tr>
<tr>
<td>Magnitude</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1 (mV)</td>
<td>26.7±6.7</td>
<td>28.8±7.3</td>
<td>2.2±1.0†</td>
</tr>
<tr>
<td>APD₉₀ (msec)</td>
<td>138.5±12.7</td>
<td>142.8±13.1</td>
<td>4.3±3.2*</td>
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<tr>
<td>APD₉₀ (msec)</td>
<td>167.2±16.2</td>
<td>173.5±17.4</td>
<td>6.3±3.1†</td>
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<tr>
<td>ERP (msec)</td>
<td>171.7±15.9</td>
<td>177.9±17.1</td>
<td>6.2±3.0†</td>
</tr>
</tbody>
</table>

Values are mean±SD measured at a basic cycle length of 500 msec; n=6. APD₉₀, action potential duration measured at 50% full repolarization; APD₉₀, action potential duration measured at 90% full repolarization; ERP, effective refractory period. Amplitudes of phases 1 and 2 were measured from resting membrane potential to the end of phase 1 and peak plateau, respectively.

*p<0.05, †t test for paired data.

**TABLE 2. Effect of 10⁻⁶ M Acetylcholine on Action Potential Parameters of Canine Ventricular Epicardium**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Acetylcholine (10⁻⁶ M)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>−82.8±0.8</td>
<td>−82.1±0.9</td>
<td>−0.7±0.2</td>
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<tr>
<td>Amplitude</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Phase 0 (mV)</td>
<td>94.6±4.1</td>
<td>92.8±4.8</td>
<td>−1.7±6.0</td>
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<tr>
<td>Phase 1 (mV)</td>
<td>69.2±5.9</td>
<td>63.9±6.4</td>
<td>−5.3±3.5†</td>
</tr>
<tr>
<td>Phase 2 (mV)</td>
<td>95.4±6.4</td>
<td>93.2±7.5</td>
<td>−2.2±4.2</td>
</tr>
<tr>
<td>Magnitude</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1 (mV)</td>
<td>25.5±5.8</td>
<td>28.9±6.2</td>
<td>3.5±1.7†</td>
</tr>
<tr>
<td>APD₉₀ (msec)</td>
<td>136.2±11.3</td>
<td>146.7±11.8</td>
<td>10.5±5.3†</td>
</tr>
<tr>
<td>APD₉₀ (msec)</td>
<td>166.2±12.9</td>
<td>179.2±14.3</td>
<td>13.0±5.9†</td>
</tr>
<tr>
<td>ERP (msec)</td>
<td>170.5±13.1</td>
<td>183.6±13.9</td>
<td>13.1±5.8†</td>
</tr>
</tbody>
</table>

Values are mean±SD measured at a basic cycle length of 500 msec; n=11. APD₉₀, action potential duration measured at 50% full repolarization; APD₉₀, action potential duration measured at 90% full repolarization; ERP, effective refractory period. Amplitudes of phases 1 and 2 were measured from resting membrane potential to the end of phase 1 and peak plateau, respectively.

*p<0.05, †t test for paired data.
response relations for the effects of ACh on APD$_{90}$ in epicardium and endocardium. The biphasic relation in epicardium contrasts sharply with the lack of ACh effect in endocardium. Qualitatively similar results were obtained in five other experiments.

The effects of ACh were also rate dependent in some preparations. At a BCL of 500 msec, moderate levels of ACh ($10^{-7}$–$10^{-6}$ M) invariably produced a prolongation of the epicardial action potential (Tables 1 and 2). Under these conditions, the ACh-induced prolongation of APD at 50% repolarization (APD$_{90}$) and APD$_{90}$ was attended by an increase in the magnitude of phase 1 (measured from the beginning to the end of phase 1 of the action potential) and a corresponding decrease in the amplitude of phase 1 (measured from the resting membrane potential to the end of phase 1). All of these parameters were significantly different after ACh when compared with control. ACh did not cause a significant change in resting membrane potential or phase 2 amplitude (measured from the resting potential to the peak of the action potential plateau). Phase 0 amplitude was slightly but insignificantly decreased with $10^{-6}$ M ACh. In most experiments, we also measured ERP before and after ACh. In all cases, the changes in ERP paralleled those of APD.

The rate dependence of the effects of ACh on APD is illustrated in Figure 2. Under control conditions, the APD-rate relation was significantly more pronounced in epicardium than in endocardium. This phenomenon has been described previously and shown to be due to an accentuation of the spike and dome morphology of the epicardial action potential with deceleration.\textsuperscript{20} ACh in concentrations as high as $10^{-5}$ M produced no change in the APD of endocardium at any stimulation rate (panel B). In epicardium, increasing concentrations of ACh produced a progressive upward shift of the APD-rate relation. The prolongation of APD was slightly more pronounced at the faster stimulation rates. With $10^{-5}$ M ACh, APD$_{90}$ prolonged at BCLs greater than or equal to 500 msec but shortened dramatically at a BCL of 300 msec. The ACh-induced upward shift of the relation was in large part due to an accentuation of phase 1 and a slowing of the second upstroke. The marked abbreviation of APD at the more rapid stimulation rates (BCLs less than 400 msec) was due to an all or none repolarization at the end of phase 1, resulting in a loss of the action potential dome.

The data thus far presented were obtained under steady-state stimulation conditions. In some cases, epicardium exposed to ACh required more than a hundred beats to achieve a steady state after an abrupt change in the stimulation rate. This phenomenon was observed under conditions giving rise to an abbreviation of the action potential secondary to the loss of the plateau, as illustrated in Figure 3. Under control conditions (panel C), a steady-state value for APD was achieved within four beats after a sudden acceleration from a BCL of 2,000 to 250 msec and within seven beats on return to a BCL of 2,000 msec. In the presence of $2 \times 10^{-6}$ M ACh, abrupt accelera- 

\textbf{FIGURE 1.} Dose-dependent effects of acetylcholine (ACh) in isolated canine ventricular epicardial and endocardial tissues. Upper panels: Transmembrane recordings obtained before (solid lines) and after (dotted lines) exposure to $10^{-7}$–$10^{-5}$ M ACh. Lower panel: Action potential duration measured at 90% repolarization (APD$_{90}$) plotted as a function of ACh concentration. In epicardium (Epi), ACh produced an accentuation of phase 1 magnitude and slowing of the second action potential upstroke, resulting in prolongation of APD at ACh concentrations of $10^{-7}$ and $10^{-6}$ M. ACh in a concentration of $10^{-5}$ M suppressed the plateau, thus causing a marked abbreviation of the action potential. Endocardium (Endo) was largely unresponsive to ACh. Basic cycle length = 300 msec. C, control. Action potential traces were reconstructed with illustration tape from enlarged projection of 35-mm film.
Figure 2. Effects of acetylcholine (ACh) on the relation between action potential duration at 90% repolarization (APD90) and basic cycle length (BCL). Insets depict the transmembrane responses recorded in control (solid line) and after ACh (10^-5 M) at BCLs of 300 and 2,000 msec. Action potential traces were reconstructed with illustration tape from enlarged projection of 35-mm film.

Figure 3. Effects of abrupt acceleration and deceleration on the action potential characteristics of epicardium in the absence and presence of acetylcholine (ACh) (2 × 10^-6 M). Panel A: Traces depict the last action potential recorded at a basic cycle length (BCL) of 2,000 msec (last) and the fifth, 100th, 115th, and 140th beats recorded after the change to a BCL of 250 msec in epicardium exposed to ACh. Panel B: After the 400th beat at a BCL of 250 msec, BCL was changed back to 2,000 msec. Action potentials shown represent the last beat recorded at a BCL of 250 msec and the first, third, fifth, and 10th beats after deceleration. Panel C: Action potential durations at 90% repolarization (APD90) of epicardial responses recorded in control and after ACh are plotted as a function of the beat number immediately after acceleration of the stimulation rate from a BCL of 2,000 to 250 msec (left arrow) and on return to a BCL of 2,000 msec (right arrow). Action potential traces were reconstructed with illustration tape from enlarged projection of 35-mm film.
tion caused an abbreviation of APD during the first 10 beats, followed by a slight but progressive prolongation over the next 90 beats. A second phase of abbreviation started 100 beats after the change in rate, and APD continued to shorten over the next 40 beats. A steady-state APD was not achieved until the 140th beat. On return to a BCL of 2,000 msec, a rebound prolongation of APD occurred followed by progressive abbreviation until a steady state was achieved seven beats later. Panels A and B illustrate the action potentials recorded during these transitional periods. With acceleration (panel A), the initial abbreviation of APD appeared to be in part due to the drop in the voltage of the peak plateau. The progressive prolongation that followed was in large part secondary to a slowing of the second upstroke. Finally, the second abbreviation phase was due to a gradual loss of the action potential plateau. With deceleration (panel B), the rebound prolongation of APD was attended by a reappearance of the plateau, initially with a markedly delayed second upstroke. The delay diminished with each successive beat, thus leading to progressive abbreviation of APD until a steady state was achieved after the seventh beat.

Suppression of the plateau after acceleration was observed in three out of six preparations exposed to 2 to 5×10⁻⁶ M ACh. The number of beats required to achieve a steady-state abbreviation in these experi-ments ranged between 10 and 170. In the other three preparations, a depression of the plateau could not be achieved with ACh concentrations as high as 10⁻⁵ M; the spike and dome in these preparations was less prominent than in the others.

To obtain a better understanding of the time-dependent changes in action potential morphology after ACh-induced depression of the plateau, we examined the characteristics of restitution in the two epicardial preparations in which abbreviation of the action potential was rate independent after exposure to ACh (5×10⁻⁶ M). Figure 4 illustrates a representative example. The APD₉₀ of premature beats elicited once after every 10th basic beat (BCL=2,000 msec) is plotted as a function of the diastolic interval (interval between full repolarization of the basic potential and the upstroke of the premature beat). The first beat in each panel is the last of a train of 10 basic beats. Subsequent beats represent responses elicited with stimuli applied progressively later in diastole. Action potential traces were reconstructed with illustration tape from enlarged projection of 35-mm film.
Figure 5. Modulation by 4-aminopyridine (4-AP) of acetylcholine (ACh)-induced effects on epicardium and endocardium. Panel A1: Superimposed transmembrane recordings obtained before and after 20 minutes exposure of epicardium to ACh (10^{-6} M). Panel A2: Recordings obtained after exposing the preparation to the transient outward current blocker 4-AP (3 mM) (after washout of ACh) and after the reintroduction of ACh. Panel B1: Endocardium in the presence of 4-AP (1 mM) and the combination of ACh and 4-AP. In epicardium, 4-AP abolished the spike and dome and prevented the ACh-induced prolongation of action potential duration. In endocardium, 4-AP did not significantly alter the ACh-induced changes. Basic cycle length=500 msec. Action potential traces were reconstructed with illustration tape from enlarged projection of 35-mm film.

Effect of Acetylcholine in the Presence of Isoproterenol

The effect of ACh to antagonize the actions of β-adrenergic agonists was examined in a series of experiments in which epicardial and endocardial preparations were pretreated with isoproterenol (10^{-7} to 5×10^{-6} M). In the example illustrated in Figure 6, isoproterenol (5×10^{-7} M) abbreviated the epicardial action potential by 20 msec (152 versus 172 msec) and that of endocardium by 11 msec (161 versus 172 msec). These changes were accompanied by a diminution of the spike and dome morphology of the epicardial response and an elevation of the plateau of the endocardial action potential. The addition of ACh (1 μM) resulted in a complete reversal (11 msec prolongation) of the isoproterenol-induced changes in the endocardial response. The action potential morphology of endocardium in the presence of ACh and isoproterenol was indistinguishable from that of control. In epicardium, the addition of 1 μM ACh completely reversed the effects of isoproterenol on the spike and dome, as well as the effects of isoproterenol on APD. APD increased beyond control with 1 μM ACh. APD prolonged further (15 msec; 187 versus 172 msec) when the ACh concentration was increased to 5 μM.

The reintroduction of 4-AP after pretreatment of the preparation with 4-AP caused only a slight drop in the plateau phase with no other changes in the action potential. In endocardium, 4-AP (1 mM) produced only a prolongation of APD with little change in the early phases of the action potential. The addition of ACh (10^{-6} M) caused only a slight negative shift in the plateau voltage in both the absence and presence of 4-AP; APD_{90} remained unchanged. Thus, the effects of ACh on epicardium pretreated with 4-AP are similar to those of ACh on endocardium.

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TABLE 3  Effects of Isoproterenol (8 x 10^{-7} M) in the Absence and Presence of Acetylcholine (8 x 10^{-7} M) on Action Potential Parameters of Canine Ventricular Epicardium and Endocardium

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<th>Control</th>
<th>Isoproterenol</th>
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<tr>
<td><strong>Epicardium (n=6)</strong></td>
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</tr>
<tr>
<td>Resting potential (mV)</td>
<td>-82.9±1.0</td>
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<td>-83.0±1.1</td>
</tr>
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<td>Amplitude</td>
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<tr>
<td>Phase 0 (mV)</td>
<td>95.3±4.3</td>
<td>95.2±3.7</td>
<td>94.0±2.5</td>
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<tr>
<td>Phase 1 (mV)</td>
<td>66.2±6.4</td>
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<td>66.9±6.1†</td>
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<tr>
<td>Phase 2 (mV)</td>
<td>97.0±4.4</td>
<td>95.5±3.1</td>
<td>96.5±3.0</td>
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<tr>
<td>Magnitude</td>
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<tr>
<td>Phase 1 (mV)</td>
<td>29.2±7.7</td>
<td>14.5±2.6*</td>
<td>27.2±6.1†</td>
</tr>
<tr>
<td>APD_{50} (msec)</td>
<td>144.0±10.9</td>
<td>126.2±7.7*</td>
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<tr>
<td>APD_{90} (msec)</td>
<td>173.7±9.2</td>
<td>154.7±8.8*</td>
<td>171.3±10.3†</td>
</tr>
<tr>
<td>ERP (msec)</td>
<td>178.0±9.5</td>
<td>156.7±9.1*</td>
<td>175.5±10.0†</td>
</tr>
<tr>
<td><strong>Endocardium (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting potential (mV)</td>
<td>-83.4±1.2</td>
<td>-82.8±1.4</td>
<td>-83.6±1.1</td>
</tr>
<tr>
<td>Amplitude</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 0 (mV)</td>
<td>110.0±4.7</td>
<td>110.5±4.4</td>
<td>109.0±4.6</td>
</tr>
<tr>
<td>Phase 1 (mV)</td>
<td>100.5±5.8</td>
<td>102.0±5.3‡</td>
<td>100.0±6.1§</td>
</tr>
<tr>
<td>APD_{50} (msec)</td>
<td>144.0±11.3</td>
<td>132.0±11.1*</td>
<td>144.2±10.6†</td>
</tr>
<tr>
<td>APD_{90} (msec)</td>
<td>178.0±14.4</td>
<td>164.5±16.1*</td>
<td>178.2±14.7†</td>
</tr>
<tr>
<td>ERP (msec)</td>
<td>182.1±13.8</td>
<td>165.9±15.6*</td>
<td>182.5±14.4‡</td>
</tr>
</tbody>
</table>

Values are mean±SD measured at a basic cycle length of 500 msec. APD_{50}, action potential duration measured at 50% full repolarization; APD_{90}, action potential duration measured at 90% full repolarization; ERP, effective refractory period. Amplitudes of phases 1 and 2 were measured from resting membrane potential to the end of phase 1 and peak plateau, respectively.

* p<0.01; analysis of variance, vs. control.
‡ p<0.01; analysis of variance, vs. isoproterenol.
§ p<0.05; analysis of variance, vs. control.
¶ p<0.05; analysis of variance, vs. isoproterenol.

produced no change in the action potential recorded from endocardium. The changes in ERP paralleled those of APD.

Table 3 summarizes the results of 12 experiments in which the interaction of ACh and isoproterenol was evaluated at a concentration of 8 x 10^{-7} M. In epicardium, isoproterenol produced a 50% decrease in the magnitude of phase 1 (22% increase in phase 1 amplitude), which was almost completely reversed by the addition of an equimolar concentration of ACh. The isoproterenol-induced abbreviation of APD_{50}, APD_{90}, and ERP (12.4%, 10.9%, and 12%, respectively) in epicardium was largely reversed by ACh. In endocardium, isoproterenol produced a lesser abbreviation of APD_{50}, APD_{90}, and ERP (8.3%, 7.6%, and 8.9%, respectively); ACh completely antagonized the isoproterenol-induced changes.

**Direct Versus Indirect Effects of Acetylcholine in Epicardium**

Although exogenous catecholamines were not present in the experiments illustrated in Figures 1–5 and summarized in Tables 1 and 2, it is possible that the effects of ACh are in part due to ACh antagonism of the effects of residual catecholamines (adrenergic influence exerted by tonic release of catecholamines from nerve endings). To test this hypothesis, we examined the effect of ACh in the absence and presence of a β-blocker (propranolol). The concentration of propranolol selected was shown to be capable of completely antagonizing the effects of the residual catecholamines released in response to tyramine (1.5 mM). Figure 7 shows two representative examples. The addition of propranolol alone caused a slight accentuation of the spike and dome with little change in the other action potential parameters. The action potential returned to control values after 30 minutes of washout. The addition of tyramine caused an abbreviation of APD_{50} (panel A: 184 to 153 msec; panel B: 183 to 158 msec) secondary to a diminution of the spike and dome and a decrease in time to peak plateau. The reintroduction of propranolol in the presence of tyramine resulted in an action potential morphology identical to that seen with propranolol alone. The addition of ACh to the Tyrode’s solution containing tyramine and propranolol produced a further accentuation of phase 1, which led to either a prolongation (Figure 7B) or a marked abbreviation (Figure 7A) of the epicardial action potential. ACh accentuated the spike and dome and prolonged or abbreviated APD in all six preparations tested. In four of these, propranolol alone produced little or no change in APD, whereas in two preparations, propranolol alone caused a
prolongation of APD$_{90}$ (presumably because of blockade of residual catecholamines). In the latter, the ACh-induced prolongation of APD (in the presence of propranolol) was always as large or larger than that produced by propranolol alone.

**Discussion**

Only recently have data been advanced suggesting the existence of a marked heterogeneity of active membrane properties in ventricular myocardium. In the present study, we demonstrate a prominent heterogeneity in the pharmacological responsiveness of tissues spanning the ventricular wall to ACh and isoproterenol.

Our results provide the first demonstration of a muscarinic effect of relatively low concentrations of ACh on the APD and ERP of isolated canine ventricular myocardium. The lack of a significant effect of ACh in endocardium, although consistent with the classical paper of Hoffman and Suckling, contrasts sharply with the distinct effects of ACh seen in epicardium (Figures 1 and 2, Tables 1 and 2).

Under certain conditions, ACh also produced a marked abbreviation of APD and refractoriness in epicardium (Figures 1–4). This action of ACh was usually observed only at fast stimulation rates with ACh concentrations of 2 to 5×10$^{-6}$ M but was rate independent with higher ACh concentrations. When rate dependent, an abrupt acceleration led to a triphasic time course of APD changes characterized by an initial period of abbreviation followed by a period of slight prolongation and a second period of marked abbreviation (Figure 3). A steady state was not achieved for many (10–170) beats after the change in rate.

Although the ACh concentrations observed to prolong APD and refractoriness in epicardium (10$^{-7}$–10$^{-8}$) are thought to represent physiologically meaningful levels (see Levy and Zieske), it is not clear whether the same holds for the ACh concentrations (≥2×10$^{-8}$ M) needed to produce the marked abbreviation of the epicardial action potential. Our observation in a parallel study that hypoxia sensitizes epicardium to the ACh-induced depression of the action potential plateau is relevant to this issue. Abbreviation of the epicardial action potential was observed with an ACh concentration of 10$^{-6}$ M after brief exposure of the preparation to hypoxia. Whether lower concentrations produce a similar effect under these conditions or with longer hypoxic periods remains to be investigated.

When the ACh-induced abbreviation was rate independent, premature beats elicited in early diastole displayed action potentials whose amplitude and duration were greater than those of the basic beat. The restitution of APD showed a relation opposite to that of control (Figure 4).

In addition to the clear differences in the responsiveness of epicardium and endocardium to ACh, our results indicate that epicardium is also more responsive to isoproterenol (Figure 6, Table 3). Moreover, the data show that the effect of ACh to prolong APD

**FIGURE 6.** Acetylcholine (ACh) antagonism of the effects of isoproterenol (Iso) in epicardium and endocardium. In epicardium (panel A), Iso 5×10$^{-7}$ M attenuated the spike and dome morphology and abbreviated the action potential. The addition of ACh (10$^{-8}$ M) completely reversed the effects of Iso on the spike and dome and action potential duration. An increase in the concentration of ACh to 5×10$^{-6}$ resulted in a further accentuation of the spike and dome and a prolongation of action potential duration beyond control. In endocardium (panel B), Iso (5×10$^{-7}$ M) elevated the plateau and slightly abbreviated the action potential. ACh (1 to 5×10$^{-6}$ M) completely antagonized the Iso-induced effects in endocardium. Action potential traces were reconstructed with illustration tape from enlarged projection of 35-mm film.
and ERP in the presence of a β-adrenergic agonist is more pronounced in epicardium than in endocardium. These findings may provide an explanation for the observation of Martins and Zipes\(^\text{10}\) that the effect of vagal stimulation to prolong ERP may be greater in epicardium than in endocardium during augmented sympathetic activity. These findings also provide support for the concept of accentuated antagonism\(^\text{17}\) as a mechanism by which the effects of parasympathetic influences on ERP may be amplified in the presence of sympathetic tone.

**Ionic Mechanisms**

Differences in the electrophysiological characteristics of epicardium and endocardium have been shown to be due to the presence of a prominent \(I_{\text{Ca}}\) in epicardium but a relatively weak \(I_{\text{Ca}}\) in endocardium\(^\text{19}\). \(I_{\text{Ca}}\) is believed to be predominantly carried by \(K^+\) ions and shows voltage-dependent activation, inactivation, and reactivation. A calcium-activated component has been reported in a number of studies\(^\text{19,26}\).

Two exponential processes describe the reactivation of \(I_{\text{Ca}}\) in canine ventricular epicardium: 1) a slow component that recovers with a time constant of approximately 400 msec and is largely abolished by the \(I_{\text{Ca}}\) blocker 4-AP (1–5 mM), and 2) a fast component with a time constant of about 50 msec that is diminished by 4-AP but is also inhibited by ryanodine or \(Sr^{2+}\) replacement of \(Ca^{2+}\). Interventions known to inhibit the \(Ca^{2+}\)-activated component of \(I_{\text{Ca}}\)\(^\text{19}\).

The electrophysiological differences between epicardium and endocardium have been shown to contribute importantly to differences in the responsiveness of these two tissues to a variety of pharmacological agents.\(^\text{8}\) Agents that exert an effect on either \(I_{\text{Ca}}\) or the slow inward current (\(I_{\text{Ca}}\)) produce different electrophysiological changes in epicardium and endocardium.

\(ACh\) has been reported to diminish \(I_{\text{Ca}}\) in mammalian ventricular tissues.\(^\text{5,6,27}\) Using whole-cell patch-clamp techniques, G.-N. Tseng (personal communication) has found that \(ACh\) (10 \(\mu\)M) depresses the L-type calcium current in canine ventricular myocytes isolated from the midmyocardial region. \(ACh\) produces a 20–30% decrease in peak \(I_{\text{Ca}}\) in the absence of catecholamines (direct effect) and an 80% decrease in the presence of isoproterenol (accentuated antagonism). \(ACh\) also produced a positive shift in the voltage dependence of activation and inactivation of the L-type calcium current.

Further evidence for the ability of \(ACh\) to diminish \(I_{\text{Ca}}\) derives from its negative inotropic effects\(^\text{20,22}\) as well as from its ability to depress \(Ca^{2+}\)-dependent "slow" responses.\(^\text{27,30}\) In a recent study, we have shown that \(ACh\) rapidly suppresses slow responses (14 mM KCl plus 1 \(\mu\)M isoproterenol) in both epicardial and endocardial tissues (S. Litovsky and C. Antzelevich, unpublished observation, 1989). The available data suggest that the principal action of \(ACh\) in epicardium is to diminish \(I_{\text{Ca}}\), but one cannot rule out the possibility that other currents may be affected as well (e.g., \(I_{\text{Na}}\)). In this context, it is noteworthy that calcium channel blockers (manganes-, cobalt, verapamil, nisoldipine) produce effects on canine ventricular epicardium and endocardium similar to those produced by \(ACh\) (A. Lukas and C. Antzelevich, unpublished observation, 1989).

Although it was beyond the scope of this study to quantitate the extent to which \(ACh\) inhibition of \(I_{\text{Ca}}\) was direct versus indirect (antagonism of residual catecholamines), the data argue well for the presence of a direct action of \(ACh\) on APD and ERP (Figure 7).

The greater sensitivity of epicardium to \(ACh\) observed in the present study may be explained on the basis of a shift in the delicate balance of currents that exist at the end of phase 1 of the epicardial action potential. Under normal conditions, the early repolarization phase in epicardium brings the mem-

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**Figure 7.** Effects of acetylcholine (\(ACh\)) in the presence of \(\beta\)-blockade. Each panel shows transmembrane responses recorded from an epicardial preparation under control conditions and after 1) 20 minutes of exposure to propranolol (Prop, 0.2 \(\mu\)g/ml), 2) 30 minutes of washout of propranolol (same as Control), 3) 20 minutes of exposure to tyramine (Tyr, 1.5 \(\mu\)M), 4) 20 minutes of exposure to tyramine plus propranolol, and 5) 20 minutes of exposure to tyramine, propranolol, and \(ACh\) (8 \(\mu\)M). \(ACh\) accentuated the spike and dome and produced either a prolongation or marked abbreviation of the action potential in the presence of complete \(\beta\)-blockade. These results indicate that the effects of \(ACh\) in epicardium are in large part direct and not mediated through antagonism of residual catecholamines. Action potential traces were reconstructed with illustration tape from enlarged projection of 35-mm film.
brane potential close to the threshold voltage for $I_{Ca}$ (approximately $-20$ to $-30$ mV). At these potentials, $I_{Ca}$ is relatively weak and slow to activate and is opposed by any $I_{Na}$ not yet inactivated. The balance of these two currents in large part determines the rate of rise of the second upstroke or, alternatively, the failure of a second upstroke to emerge. A relatively small decrease of $I_{Ca}$ by ACh (direct plus indirect effects) would shift the balance of currents so that the net inward current at the end of phase 1 is weaker. A further decrease of $I_{Ca}$ may result in a net outward current and an “all-or-none repolarization” at the end of phase 1. Based on this scheme, a slight inhibition of $I_{Ca}$ by ACh would slow and/or delay the second action potential upstroke in epicardium, thus giving rise to an increase in APD. A greater ACh inhibition of $I_{Ca}$ would lead to a progressive depression of the plateau, resulting in a marked abbreviation of the action potential. This hypothesis is supported by the lack of a significant effect of ACh in epicardial preparations in which the spike and dome is eliminated by pretreatment with the $I_{Na}$ blocker 4-AP. Also noteworthy is the fact that the effect of ACh is less pronounced in preparations displaying a less accentuated spike and dome.

Because of the lack of a spike and dome in endocardium or in epicardium pretreated with 4-AP, a similar inhibition of $I_{Ca}$ by ACh would be expected to produce only a slight depression of the action potential plateau (Figures 2 and 5) with little change in APD. The end of phase 1 determines the voltage at which activation of the calcium current will begin. The availability of $I_{Ca}$ would be expected to be much greater in endocardium, and a slight decrease in the current may be of little import. Furthermore, if ACh inhibition of $I_{Ca}$ is in large part due to a positive shift in the activation and inactivation curve for the L-type calcium current (as discussed above), the effects of ACh on epicardium and endocardium would be expected to be different. ACh-induced decrease of $I_{Ca}$ would be most prominent when the end of phase 1 is relatively negative (steep portion of the activation curve) and much diminished when phase 1 is small (endocardium or epicardium pretreated with 4-AP) and the plateau currents are activated from much more positive potentials (top of activation curve for $I_{Ca}$).

ACh inhibition of $I_{Ca}$ in the ventricle may be due to an elevation of cyclic GMP through cyclic GMP-mediated phosphorylation of a calcium channel protein.31 This mechanism may also in part account for the ACh antagonism of the effects of isoproterenol. Another mechanism that likely contributes importantly to the latter effect is the well-known action of ACh to inhibit adenylate cyclase via the $N_{i}$ (inhibitory) coupling protein and thus to diminish cyclic AMP levels, especially after activation of this system by a $\beta$-adrenergic agonist.31-33

The only other current reported to be inhibited by ACh in ventricular tissues (guinea pig papillary muscles) is the delayed rectifier ($I_{K}, I_{Na}$).5,24 Our data are not consistent with an effect of ACh on this current in the absence of $\beta$-adrenergic stimulation, because the repolarization phase (phase 3) was not conspicuously altered by ACh in either tissue. This effect, however, may contribute to ACh antagonism of the effects of isoproterenol (Figure 6), whose actions are in part mediated by an increase in $I_{K}$.34,35

The rate dependence of the effects of ACh on epicardium (Figures 2 and 3) is comparable to the use-dependent inhibition of contractility by ACh demonstrated by others.36,37 The specific mechanism underlying this action of ACh remains to be elucidated.

The unique restitution characteristics observed after ACh-induced suppression of the action potential plateau (Figure 4) may be explained on the basis of the slow reactivation kinetics of $I_{Na}$.19,35 A decreased availability of $I_{Na}$ is expected for responses elicited in early diastole, because $I_{Na}$ has not had sufficient time to reactivate. As a consequence, less outward current is available to oppose $I_{Ca}$ during phase 1. The plateau is thus restored and the action potential prolonged. With progressive recovery of $I_{Na}$ later in diastole, the net current at the end of phase 1 would become outward, and the plateau is once more suppressed.

Isoproterenol, among its many actions, augments $I_{Ca}$ by increasing cyclic AMP levels secondary to stimulation of adenylate cyclase via the $N_{c}$ coupling protein.31 As expected, its effects on the epicardial response are opposite to those of ACh. Isoproterenol also augments $I_{K}$ in ventricular myocardium.34,35 The differences in the responses of epicardium and endocardium to isoproterenol appear to be due to the fact that augmentation of $I_{Ca}$ can produce a major change in the early phases of the action potential in epicardium but a relatively minor change in endocardium (Figure 6) for reasons discussed above. Through its effects to diminish the spike and dome configuration of the action potential in epicardium, isoproterenol permits an earlier attainment of the peak plateau, thus causing an abbreviation of the action potential over and above that produced by augmentation of $I_{K}$. In endocardium, the early phases of the action potential are less affected and the isoproterenol-induced abbreviation of APD may be in large part attributed to an increase in the level of $I_{K}$. ACh antagonism of the effects of isoproterenol are likely due to its effect to modulate the levels of cyclic AMP and cyclic GMP and thus to diminish $I_{Ca}$ and $I_{K}$. Evidence for the involvement of a G protein ($G_{i}$) has also been documented.34

**Physiological Implications**

The physiological implications of the findings presented may be far reaching. First, the results provide a basis to better understand the effects of autonomic influences on the electrophysiological characteristics of the ventricle. The demonstration of an effect of ACh to prolong APD and refractoriness in epicardium provides for the first time a potential mechanism by which to explain the findings of in vivo studies that argue for a direct parasympathetic influ-
ence on ventricular refractoriness\textsuperscript{14,16} as well as hemodynamics.\textsuperscript{28,29} The differential responsiveness of epicardium and endocardium to ACh and isoproterenol and the different degrees of accentuated antagonism observed in the two types of tissue also provide a framework by which differences in responsiveness of epicardium and endocardium to parasympathetic and sympathetic stimulation\textsuperscript{10} may be better understood. Our demonstration of a 6–13-msec average prolongation of epicardial APD and ERP in response to $10^{-7}$–$10^{-6}$ M ACh can be compared with a 3–8-msec prolongation of ERP observed by others in response to vagal stimulation in vivo in anesthetized dogs\textsuperscript{9–11} (ERP measured using either endocardial or epicardial electrodes in either right or left ventricle). Comparison of these values is difficult because of the many variables that are likely to influence the effects of vagal stimulation in vivo. These include the effects of anesthesia, heart rate, stimulation site for ERP determinations, and the level of adrenergic tone. An indirect measure of the influence of resting vagal tone on ERP in the unanesthetized human was obtained by Prystowsky and coworkers\textsuperscript{16} by measuring ERP (endocardial site) before and after the administration of atropine in the presence of $\beta$-adrenergic blockade. Resting vagal tone was found to account for a 20-msec average prolongation of ERP.

An area not addressed in the present study is to what extent a direct parasympathetic influence on epicardial APD may affect the measurement of ERP in endocardium. The degree to which this direct effect on epicardium may be electrotonically or otherwise transmitted to endocardium remains to be investigated.

The ERP-prolonging effects of ACh, whether direct or secondary to modulation of sympathetic tone or its influence, may exert an antirhythmic action that could contribute to the well-known protective effect of enhanced vagal activity.\textsuperscript{9,38} The accentuation of the effect of ACh to prolong APD and ERP in epicardium at rapid rates (Figure 2) may act to effectively abort any reentrant rhythms that develop and thus to limit tachyarrhythmias to paroxysmal episodes.

Ischemia and hypoxia have been shown to produce a marked abbreviation of APD and ERP in epicardium but not endocardium.\textsuperscript{8,22} Our data suggest the possibility that vagal influence in a setting of hypoxia or ischemia may contribute to the development of a marked dispersion of refractoriness across the ventricular wall, which may in turn set the stage for a variety of reentrant ventricular arrhythmias, transmural or epicardial reentry in particular. A high level of vagal activity attending ischemia, as with an inferior myocardial infarction,\textsuperscript{39} might provide an appropriate setting for this phenomenon to manifest.

In addition to creating a marked dispersion of refractoriness between epicardium and endocardium, the ACh-induced suppression of the epicardial action potential plateau may contribute to deterioration of conduction across regions of impaired conductivity or regions displaying marked anisotropy. The decrease in the intensity of the local circuit current resulting from the depression of the plateau could influence the success or failure of impulse conduction across regions of the heart where the impedance to the flow of local circuit current is high.\textsuperscript{8,40}

Because of the unique restitution characteristics encountered under these conditions (Figure 4), it may also be possible for premature beats to conduct under conditions in which the basic beats are blocked (supernormal conduction). The possibility of supernormal conduction in ventricular muscle has long been a matter of debate. In vivo studies\textsuperscript{41,42} using extracellular stimulating electrodes (in many cases applied to the ventricular epicardium) have demonstrated a supernormal period, whereas studies of ventricular muscle tissues (endocardium) in vitro have failed to uncover a period of supernormal conduction or excitability.\textsuperscript{43} In a recent study, we have demonstrated supernormal conduction in epicardial strips of tissue whose central segment was depressed by superfusion with an “ion-free” isotonic sucrose solution.\textsuperscript{8} Under conditions in which conduction of the basic beats was totally blocked, early premature beats were shown to conduct successfully across the inexcitable zone (sucrose gap). Successful conduction of the premature beats was due to the greater amplitudes of the early phases of these action potentials (see Figure 4, control). Differences between the amplitudes of the early phases of the basic beat and those of early premature beats are even more exaggerated in the presence of ACh (Figure 4, ACh). These findings suggest that parasympathetic influence may contribute to a greater margin of safety for conduction of premature beats than of basic beats, thus permitting the manifestation of supernormal conduction in ventricular epicardium.

Finally, suppression of the dome portion of the epicardial action potential as seen with ACh would be expected to give rise to an ST segment elevation in the electrocardiogram similar to that observed in patients with the “early repolarization syndrome”\textsuperscript{44} or acute pericarditis,\textsuperscript{45} as well as those with acute myocardial infarction. To what extent, if any, these phenomena are interrelated remains to be established.

Acknowledgments

We dedicate this paper to the memory of Gordon K. Moe, MD, PhD, whose teachings and friendship we will treasure always. We also thank Gea-Ny Tseng, PhD, for her valuable input and acknowledge the expert technical assistance of Judith Hefferon and Robert Goodrow.

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Key Words: ventricle, epicardium, acetylcholine, isoproterenol, electrophysiology
Differences in the electrophysiological response of canine ventricular subendocardium and subepicardium to acetylcholine and isoproterenol. A direct effect of acetylcholine in ventricular myocardium.
S H Litovsky and C Antzelevitch

Circ Res. 1990;67:615-627
doi: 10.1161/01.RES.67.3.615

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