Ultrasound-induced modulation of cardiac rhythm in neonatal rat ventricular cardiomyocytes

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Submitted 4 November 2014; accepted in final form 2 April 2015

Fleischman A, Vecchio C, Sunny Y, Bawiec CR, Lewin PA, Kresh JY, Kohut AR. Ultrasound-induced modulation of cardiac rhythm in neonatal rat ventricular cardiomyocytes. J Appl Physiol 118: 1423–1428, 2015. First published April 9, 2015; doi:10.1152/japplphysiol.00980.2014.—Isolated neonatal rat ventricular cardiomyocytes were used to study the influence of ultrasound on the chronotropic response in a tissue culture model. The heart frequency of the cells, varying from 40 to 90 beats/min, was measured based upon the translocation of the nuclear membrane captured by a high-speed camera. Ultrasound pulses (frequency = 2.5 MHz) were delivered at 300-ms intervals [3.33 Hz pulse repetition frequency (PRF)], in turn corresponding to 200 pulses/min. The intensity of acoustic energy and pulse duration were made variable, 0.02-0.87 W/cm2 and 1–5 ms, respectively. In 57 of 99 trials, there was a noted average increase in heart frequency of 25% with 8% exposures to ultrasonic pulses. Applied ultrasound energy with a spatial peak time average acoustic intensity (Istpt) of 0.02 W/cm2 and pulse duration of 1 ms effectively increased the contraction rate of cardiomyocytes. The intensity of acoustic power tested, the lowest level of acoustic intensity and shortest pulse duration proved most effective at increasing the electrophysiological responsiveness and beat frequency of cardiomyocytes. Determining the optimal conditions for delivery of ultrasound will be essential to developing new models for understanding mechanoelectrical coupling (MEC) and understanding novel nonelectrical pacing modalities for clinical applications.

mechanoelectrical coupling; mechanosensitivity; cardiomyocytes; ultrasound modulation; electrophysiology

THE PURPOSE OF THIS STUDY was to determine whether pulsed ultrasound energy can alter the electrical rhythm of cardiomyocytes. We postulate that ultrasound produces a mechanical force on plated cardiomyocytes, eliciting a change in electrical activity. Mechanosensitivity in cardiac tissue has been well documented, involving both cell membrane electrophysiology and calcium handling (40). Mechanosensitive ion channels have been described in a wide variety of cell types including skeletal muscle, neurons, fibroblasts, epithelium, or oocytes (33–35). Stretch-activated channels have been implicated in responses to mechanical perturbation of cardiac tissue (4, 14, 29, 35, 47). In cardiomyocytes, cellular depolarization can be activated via mechanosensitive pathways in the mechanoelectric feedback circuit (1, 15, 16, 19, 37, 38, 44). Several voltage-gated channels (VGCs) have been identified in the heart as mechanosensitive, including voltage-gated sodium channels (Nav) from the human heart (35), pacemaker or hyperpolarization-activated cyclic nucleotide-gated (HCN2) channels (14, 29), voltage-gated calcium channels (Cav) (4), and potassium (Kav) channels (18). Two distinct channels are considered central to mechanoelectric coupling (MEC) in cardiac cells: a cation nonselective channel (SACNS) and a potassium-selective channel (SACK), with reversal potentials of approximately −10 to −30 mV and −90 mV, respectively (8, 20, 47). Such channels have a greater propensity to open in response to mechanical force (5, 24). Thus the physiological response to mechanical stretch is mediated by VGCs, such as SACNS (24, 47), triggering premature excitation (25, 26).

Mechanosensitive responses can be provoked in the intact heart or isolated cardiac tissues. Such responses have been elicited by augmentation of diastolic ventricular volume, increasing afterload (sudden or prolonged), indentation with a microcantilever, and axially stretching sections of cardiac tissue (2, 17, 19). Mechanotransduction manifests electrophysiologically as premature extrasystolic beats or arrhythmias (15, 16, 19). Additionally, anecdotal evidence can be found during routine procedures in the cardiac catheterization laboratory, because tactile force from a catheter on the endocardial surface frequently incites premature beats (27, 44).

Microelectrodes and the patch clamp technique have led to considerable insight in the characterization of stretch-activated channels at the cellular level. Such experiments have employed a variety of mechanical stimuli to perturb isolated cells, via osmotic swelling, mechanical indentation, suction with a pipette, and imposed axial stretch (2, 41). Negative pressures and longitudinal stretch of ventricular myocytes increased the open probability of nonselective cation channels, depolarizing cardiac cells, while also prolonging the action potential duration (8, 41, 49). Additionally, in fibroblast patch clamp experiments, mechanical compression caused depolarization by way of a nonspecific inward cation current, while mechanical stretch led to hyperpolarization (21–23).

Although many techniques have been used to apply direct contact force to cardiac cells and elicit a response, ultrasound energy may be unique. Ultrasound is widely recognized for its diagnostic capabilities and therapeutic applications, such as lithotripsy and wound care (9, 43). Ultrasonic waves have a variety of effects on biological tissues, both thermal and nonthermal. Diagnostic applications seek to minimize the interaction between the acoustic field and tissues, whereas its therapeutic use exploits direct bioeffects, such as acoustic cavitation, radiation force, radiation torque, and acoustic streaming (9). The cardiac effects during the clinical use of ultrasound became apparent through observation of a high incidence of arrhythmic events during lithotripsy treatments,

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The cardiomyocytes were cultured at a density of 7,000 cells/cm² in incubator for 24 h at 5% CO₂ and 37°C. The medium was changed to high serum medium (10% fetal bovine serum) (Cellutron NS medium) and low serum (2% fetal bovine serum) (Cellutron NW medium) and allowed the cardiomyocytes to attach and spread to full confluence before the isolation procedure. For maintenance of cardiomyocytes in culture for greater than 5–7 days, the media were replaced with fresh media at a concentration of 200 μM to prevent fibroblast proliferation. This protocol was approved by the Institutional Animal Care and Use Committee at Drexel University.

Before experimentation, the medium was removed and the cardiomyocytes were washed with Tyrode’s solution, a balanced salt solution supporting cell metabolism. The cells were stained for 10 min with aniline-dpus dye (Sensitive Dyes GBR, Munich, Germany) with strong membrane binding affinity. This dye became concentrated at the nuclear membrane of the cardiomyocytes and helped to visualize and characterize the beating cells. After staining, the culture was washed three times with Tyrode’s solution. Finally, the cardiomyocytes were equilibrated in Tyrode’s solution for 1 to 2 h inside an incubator. This time period proved sufficient to restore the automaticity of the cardiomyocytes culture without compromising the viability of the cells in the culture due to limited nutrition. The cardiomyocytes were kept in the incubator at 37°C until just before the experiment.

In vitro mechanical deformation protocol using ultrasound energy. A custom 2.5 MHz, 11 mm diameter, unfocused planar ultrasound transducer was built by one of our coauthors (Chris Vecchio). A 2.5-MHz probe was chosen, considering the trade-off of a coherence of the beam and the penetration depth, to ensure the sufficient energy would have been delivered to the heart in the clinical environment (3.0–3.5 cm from the surface of the chest wall to heart). The transducer was coupled to the medium in the petri dish using a modified syringe as a mold filled with a 3% agarose and water solution (as shown in Fig. 1). This spacer was used to facilitate the experiment, allowing ultrasound exposure with concurrent microscope video imaging. The transducer was later aimed onto various confluent areas within the well plate using the microscope stage adjustments. The well plates contained high volumes of Tyrode’s solution to act as a conducting medium for the ultrasound without risk of a large acoustic impedance mismatch.

The ultrasound field distribution (checked for uniformity, not reproduced here) and the key relevant parameter, pressure amplitude, were determined using a calibrated Marconi PVDF hydrophone in a water tank. The transducer was activated by tone bursts (compromising of 25–125 cycles of 2.5-MHz frequency) and controlled via a function generator (Agilent 33220A). Tonal burst sinusoidal waveforms at a frequency of 2.5 MHz were generated by the function generator and amplified by 50 dB (or ~316 times) before reaching the transducer. The function generator was set to deliver acoustic energy at variable spatial peak time averaged intensities (I_{p,10}) in watts per square centimeter at pulse durations of 1, 2, 3, or 5 ms, which were delivered at the PRF of 3.3 Hz (300 ms). This PRF suggests delivery of 200 ultrasound bursts/min.

Data recording and analysis of cell membrane translocation. A Nikon Diaphot 300 inverted phase contrast microscope was connected to a high-speed Dalsa CA-D1-0128T camera with NI-1422 frame-grabber. Timing and image capture from the CCD were controlled via custom NI Labview VI. This setup recorded 12 bit, 128 × 127 pixel arrays at a frame rate of 155 frames/s for 8 s. Chroma 86000 series filters were used with a mercury lamp to excite the aniline-dplus dye at ~490 nm, and emitted light was directed through a 617/70 filter. For each trial, frames were captured for 8 s without ultrasound exposure to establish a control. Immediately after this control, the same sample was exposed to ultrasound while capturing an additional 8 s of frames. These frames were further analyzed using software from ImageJ (National Institutes of Health).

The contraction rate of the cardiomyocytes was measured based upon the translocation of the nuclear membrane captured by this high-speed camera. A pattern detection algorithm (Image J and a custom Matlab and C software package) translated captured images into beat frequency, measured as beats per minute.

An average of the duration of the cardiac cycle from all recorded beats was calculated for both the control and ultrasound exposure groups. Statistical comparisons were made between controls before ultrasound exposure and after exposure for each trial using a two-way ANOVA test with a post hoc Dunnett’s test. The results were considered significant at P < 0.05.
sample unequal variance $t$-test. Results from individual trials were pooled for each set of conditions and analyzed with a Wilcoxon Signed Rank Test. $P$ values $<0.05$ were considered statistically significant.

RESULTS

The cardiomyocyte contraction rate and beat frequency were recorded for 11 ultrasound settings summarized in Table 1. Specifically, Table 1 summarizes the results of all 99 trials and the influence of ultrasound exposure on beat frequency. Individual trials using a single set of ultrasound parameters were repeated between 3 and 13 times, and the resulting beat rate of the cardiomyocytes was compared with its own control.

Of the 99 trials, the beat frequency increased with respect to controls in 57 trials, contrasting with a decreased beat frequency in 42. Only 19 trials showed a statistically significant rate response change ($P < 0.05$) compared with control: 11 trials with a significant increase and 8 with a significant decrease in beat frequency. Of the 11 trials showing a significant increase in beat frequency, 10 of these trials employed ultrasound intensities of 0.2 W/cm$^2$ or less. Of the 8 trials showing a significant decrease in beat frequency, 4 utilized an intensity of 0.2 W/cm$^2$ or greater. A ratio of the beat frequency during ultrasound exposure to its baseline (control) was determined for each trial. The pooled data showed an increase in

### Table 1. Summary of results from 99 trials using 11 ultrasound parameters

<table>
<thead>
<tr>
<th>$I_{\text{spa}}$ (W/cm$^2$)</th>
<th>Pulse Duration</th>
<th># of Trials</th>
<th># Trials Increase in beats/min</th>
<th># Significant Increase*</th>
<th>Ratio</th>
<th># Trials Decrease in beats/min</th>
<th># Significant Decrease*</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>1 ms</td>
<td>13</td>
<td>10 (77%)</td>
<td>3</td>
<td>1.39</td>
<td>3 (23%)</td>
<td>0</td>
<td>0.92</td>
</tr>
<tr>
<td>0.04</td>
<td>1 ms</td>
<td>12</td>
<td>8 (67%)</td>
<td>1</td>
<td>1.18</td>
<td>4 (33%)</td>
<td>0</td>
<td>0.87</td>
</tr>
<tr>
<td>0.05</td>
<td>3 ms</td>
<td>10</td>
<td>3 (30%)</td>
<td>1</td>
<td>1.15</td>
<td>7 (70%)</td>
<td>1</td>
<td>0.95</td>
</tr>
<tr>
<td>0.07</td>
<td>1 ms</td>
<td>9</td>
<td>3 (33%)</td>
<td>0</td>
<td>1.25</td>
<td>6 (67%)</td>
<td>2</td>
<td>0.94</td>
</tr>
<tr>
<td>0.08</td>
<td>2 ms</td>
<td>3</td>
<td>3 (100%)</td>
<td>1</td>
<td>1.36</td>
<td>0 (0.0%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.09</td>
<td>5 ms</td>
<td>12</td>
<td>9 (75%)</td>
<td>2</td>
<td>1.17</td>
<td>3 (25%)</td>
<td>1</td>
<td>0.88</td>
</tr>
<tr>
<td>0.12</td>
<td>3 ms</td>
<td>12</td>
<td>7 (58%)</td>
<td>1</td>
<td>1.35</td>
<td>5 (42%)</td>
<td>0</td>
<td>0.93</td>
</tr>
<tr>
<td>0.20</td>
<td>5 ms</td>
<td>8</td>
<td>5 (63%)</td>
<td>1</td>
<td>1.25</td>
<td>3 (38%)</td>
<td>1</td>
<td>0.79</td>
</tr>
<tr>
<td>0.21</td>
<td>3 ms</td>
<td>3</td>
<td>1 (33%)</td>
<td>0</td>
<td>1.16</td>
<td>2 (67%)</td>
<td>0</td>
<td>0.79</td>
</tr>
<tr>
<td>0.35</td>
<td>5 ms</td>
<td>13</td>
<td>5 (39%)</td>
<td>0</td>
<td>1.06</td>
<td>8 (62%)</td>
<td>3</td>
<td>0.91</td>
</tr>
<tr>
<td>0.87</td>
<td>2 ms</td>
<td>4</td>
<td>3 (75%)</td>
<td>1</td>
<td>1.50</td>
<td>1 (25%)</td>
<td>0</td>
<td>0.94</td>
</tr>
<tr>
<td>99</td>
<td></td>
<td>57 (58%)</td>
<td>11</td>
<td>1.25</td>
<td>42 (42%)</td>
<td>8</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant ($P < 0.05$) - 2 sample $t$-test with unequal variance. $I_{\text{spa}}$, spatial peak time average acoustic intensity; Ratio, ratio of beat frequency during ultrasound exposure to its baseline.
beat frequency and a mean ratio of 1.25 (range of 1.06 to 1.50), consistent with a net increase in beat frequency of 25%. This response was asymmetric in trials showing a decrease in beat frequency, with a mean ratio of 0.91 (range of 0.79 to 0.95), representing a net decline of 9.2% in beat frequency response.

Table 2 summarizes the pooled data from all 99 trials that include the results of all 11 conditions and the average beat frequency from all pooled trials before and after exposure to ultrasound. The beat frequency increased relative to the control in 8 of the 11 conditions of $I_{\text{spatial}}$ and pulse duration. Of these conditions, the ratio of beat frequency after ultrasound exposure relative to the control ranged from 1.007 to 1.292, with three conditions showing an increase over 20%. However, only one condition (0.02 W/cm², 1-ms pulse duration) was determined to be statistically significant ($P < 0.05$) in producing an increase in beat frequency. Similar to the results from individual trials, all three conditions with a decline in beat rate employed acoustic intensities greater than 0.2 W/cm²; however, none reached statistical significance. The remainder of ultrasound intensities showed an increase in beat frequency, without achieving statistical significance.

**DISCUSSION**

Previously, other investigators employed pulses of intense ultrasound above a threshold energy and pulse duration to successfully pace rat and porcine hearts (30, 32, 42, 46). In similarly designed frog studies, premature ventricular contractions resulted from intermittent 5-ms pulses of ultrasound at acoustically generated pressures between 5 and 10 MPa (11, 12). Evidence for the effectiveness of ultrasound in eliciting a mechanoelectric response also exists at the cellular level. However, these studies have not identified the acoustic intensity and pulse duration that can induce pacing without myocardial tissue injury. It remains unclear whether demonstrated induction of a premature ventricular contraction (PVC) is the result of acute injury or the result of a mechanical electrically excitable “healthy” cell. It is also possible that the observed ultrasound-mediated effect occurs through production of microbubbles and inertial cavitation causing cell membrane injury resulting in PVC (32, 36).

In this study, we examined the electrophysiologic responsiveness and resultant beat frequency of cultured neonatal rat ventricular cardiomyocytes using pulsed 2.5-MHz ultrasound source as the driving stimulus. Of the 99 individual trials, 19 trials produced a change in beat frequency that was statistically significant. Moreover, in 13 of these trials, ultrasound produced an increase in beat frequency and in 8 trials ultrasound produced a decrease in beat frequency, reflecting the complexity of the relative spatial temporal organization of our two-dimensional model. Nevertheless, it is possible that the observed negative chronotropic effect at the higher acoustic energy is a marker of injury resulting from an altered electrical network of cells. In the 57 trials that showed a positive chronotropic effect, there was a mean increase in beat frequency of 25%. This is contrasted by a decrease of 9.2% in 42 trials, demonstrating further the collective behavior of a network of cardiac cells in terms of their electrophysiological responsiveness. The ultrasound parameters that caused a decrease in beat frequency employed acoustic intensities greater than 0.2 W/cm². Specific ultrasound parameters (intensity and pulse duration) caused different changes in beat frequency, some potentiating the beat frequency, whereas other parameters inhibited the beat frequency. The lowest intensity of ultrasound exposure, 0.02 W/cm² and 1-ms pulse duration, was most effective at increasing the beat frequency.

Pulse duration was used as a variable to control the delivery of acoustic energy to modify the amount of radiation force delivered to the cells. The chosen pulse duration values of 1, 2, 3, and 5 ms were deliberately selected to operate within a duty cycle of the stimulus based on models that would be appropriate for stimulating cells with a spontaneous beat frequency of 40–100 beats/min. Our goal was to achieve the desired $I_{\text{plta}}$ values using a minimum acoustic intensity without exceeding a 5-ms burst duration. We achieved the final spatial peak time average acoustic intensity ($I_{\text{spatial}}$) based on progressive increases in the drive voltage and pulse duration. Before this series of experiments, we postulated that a narrow range of $I_{\text{plta}}$ and shorter pulse duration would provide the greatest physiological effect. Our results showed that the lowest level of acoustic intensity and shortest pulse duration proved most effective at increasing the electrophysiological responsiveness and beat frequency of cardiomyocytes.

Cardiomyocytes in 2D retain an inherent spontaneous rhythmogenicity consistent with other studies, as in vitro rate of contraction was observed to be 43 ± 21 beats/min (28). Although ultrasound pulses were delivered at the effective rate of 200 per minute, they could not be synchronized with the underlying spontaneous beat frequency as in other animal studies and thus did not capture one-to-one. The temporal distribution of HIFU insonation is likely key to beat capture, because animal studies have shown a majority of successful pacing to be achieved after the ECG T wave (30). Additionally, a recent study suggests mechanical pacing demonstrates a rate-dependent loss of 1:1 capture (39).

Although there is no inherent spatial control on a 2D culture plate as in animal studies, culture conditions were controlled to achieve the intrinsic variability in cell phenotype of ventricular cells in a confluent network. Such a network of excitable cells would exhibit a dominant hierarchical rhythm. The study design was predicated upon translation to the clinical application of extracorporeal targeted acoustic stimulation to the human heart. The large surface area of the lateral and anterior ventricular territory would be easily accessible to acoustic energy delivered transcutaneously with a skin surface-mounted ultrasonic probe, hence the use of isolated ventricular cells.

**Table 2. Summary of results after combining all trials using the same conditions**

<table>
<thead>
<tr>
<th>$I_{\text{plta}}$ (W/cm²)</th>
<th>Pulse Duration</th>
<th># of Trials</th>
<th>Control beats/min</th>
<th>Ultrasound beats/min</th>
<th>Ratio</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>1 ms</td>
<td>13</td>
<td>72.3</td>
<td>87.8</td>
<td>1.22</td>
<td>0.004*</td>
</tr>
<tr>
<td>0.04</td>
<td>1 ms</td>
<td>12</td>
<td>44.8</td>
<td>45.7</td>
<td>1.02</td>
<td>0.319</td>
</tr>
<tr>
<td>0.05</td>
<td>3 ms</td>
<td>10</td>
<td>64.2</td>
<td>64.7</td>
<td>1.01</td>
<td>0.508</td>
</tr>
<tr>
<td>0.07</td>
<td>1 ms</td>
<td>9</td>
<td>67.6</td>
<td>67.5</td>
<td>1.00</td>
<td>0.767</td>
</tr>
<tr>
<td>0.08</td>
<td>2 ms</td>
<td>3</td>
<td>56.07</td>
<td>78.5</td>
<td>1.29</td>
<td>0.054</td>
</tr>
<tr>
<td>0.09</td>
<td>5 ms</td>
<td>12</td>
<td>45.5</td>
<td>48.0</td>
<td>1.06</td>
<td>0.120</td>
</tr>
<tr>
<td>0.12</td>
<td>3 ms</td>
<td>12</td>
<td>53.7</td>
<td>59.0</td>
<td>1.00</td>
<td>0.079</td>
</tr>
<tr>
<td>0.20</td>
<td>5 ms</td>
<td>8</td>
<td>49.0</td>
<td>49.7</td>
<td>1.01</td>
<td>0.337</td>
</tr>
<tr>
<td>0.21</td>
<td>3 ms</td>
<td>3</td>
<td>51.9</td>
<td>45.0</td>
<td>0.87</td>
<td>0.593</td>
</tr>
<tr>
<td>0.35</td>
<td>5 ms</td>
<td>13</td>
<td>92.4</td>
<td>89.1</td>
<td>0.97</td>
<td>0.196</td>
</tr>
<tr>
<td>0.87</td>
<td>2 ms</td>
<td>4</td>
<td>37.0</td>
<td>46.5</td>
<td>1.26</td>
<td>0.137</td>
</tr>
</tbody>
</table>

*Statistical significance ($P < 0.05$, Wilcoxon Signed Rank Test).
Targeting the sinus node region would be challenging and impractical, and additionally isolation of a specific phenotype of sinus node cells would be difficult in itself.

Isolated neonatal rat cardiomyocytes were selected for these experiments for their robustness and reproducibility in 2D culture conditions. Furthermore, studies have demonstrated that cultured neonatal cardiomyocytes form functional electro-mechanical coupling more comparable with an in situ heart (48). Adult rat cardiomyocytes are particularly sensitive to culture conditions; they lack resistance to cellular disintegration and quickly lose their contraction capability (6).

Finally, these studies were confined to conditions in which cell density was preselected to achieve full confluence, and rhythm changes were the result of a cohesive, synchronous response. A lack of confluence has been shown to have a strong influence on asynchronous rhythms of individual cell clusters. Unpublished observations of neonatal cardiomyocytes on electrode arrays have demonstrated characteristic rhythm “islands.” Although cardiac cells within these clusters beat synchronously implying cell coupling remains intact, coupling between clusters was not achieved because of desperate plating. Without such “island” clusters, as in a confluent culture, the cell population converges to a single overriding rhythm (spontaneous or paced). Cells are mechanically coupled by the deformable substrate to which they adhere, which in this case would be the culture plate itself. Mechanoelectric feedback is not easily propagated; this is especially true with increasing distance between cells and stiff plating substrates. Thus sparse culture conditions were avoided in this experiment in favor of culture confluence (45).

Although many methods have been used to mechanically perturb cardiac cells, this study provides evidence for ultrasound as a novel approach for eliciting mechanosensitive responsiveness in cardiac tissue. Specifically, it was demonstrated that ultrasound has the capacity to manipulate the spontaneous rhythm of a confluence of neonatal rat ventricular cardiomyocytes in culture. Ultrasound may offer to be a practical noninvasive means for imparting mechanical forces to capture and pace myocardial tissues.

Limitations. There were several limitations to our study. First, this study was limited to only neonatal ventricular cardiomyocytes in 2D culture conditions. The significant baseline beat rate variability could have influenced our observations in both exaggerating and dampening the effects from ultrasound. The cells were plated on glass and the adhesion to a stiff substrate such as glass could have influenced the observed effects of ultrasound.

Conclusions. The data presented suggest that low (2.5 MHz) frequency ultrasound has a modulating effect on the rate of cardiomyocyte contraction. Rate-response varied depending on the amount of acoustic power and pulse duration. The lowest level of acoustic power and shortest pulse duration tested proved most effective at increasing the responsiveness and beat frequency of cardiomyocytes. Ultrasound-induced stimulation of cardiomyocytes via stretch and compression-mediated mechanosensitive pathways appears to be a plausible mechanism of action and hence may prove to be a practical, noninvasive clinical tool for capture and pacing of myocardium in both emergent and nonemergent settings.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


