Synthetic Strands of Neonatal Mouse Cardiac Myocytes
Structural and Electrophysiological Properties

Stuart P. Thomas, Lilly Bircher-Lehmann, Suma A. Thomas, Jianping Zhuang, Jeffrey E. Saffitz, André G. Kléber

Abstract—The aim of the present study was to morphologically and electrically characterize synthetic strands of mouse ventricular myocytes. Linear strands of mouse ventricular myocytes with widths of 34.7±4.4 μm (W₁), 57.9±2.5 μm (W₂), and 86.4±3.6 μm (W₃) and a length of 10 mm were produced on glass coverslips with a photolithographic technique. Action potentials (APs) were measured from individual cells within the strands with cell-attached microelectrodes. Impulse propagation and AP upstrokes were measured with multisite optical mapping (RH237). Immunostaining was performed to assess cell-cell connections and myofibril arrangement with polyclonal antisera against connexin43 and N-cadherins and monoclonal antibodies against cardiac myosin. Light microscopy and myosin staining showed dense growth of well-developed elongated myocytes with lengths of 34.2±4.2 μm (W₁), 36.9±5.8 μm (W₂), and 43.7±6.9 μm (W₃), and length/width ratios of 3.9±0.2. Gap junctions were distributed around the cell borders (3 to 4 junctions/μm² cell area). Each cell was connected by gap junctions to 6.5±1.1 neighboring cells. AP duration shortened with time in culture (action potential duration at 50% repolarization: day 4, 103±34 ms; day 8, 16±3 ms; P<0.01). Minimum diastolic potential and AP amplitude were 71±5 and 97.2±7.6 mV, respectively. Conduction velocity and the maximum dV/dt of the AP upstroke were 43.9±13.6 cm/s and 196±67 V/s, respectively. Thus, neonatal ventricular mouse myocytes can be grown in continuous synthetic strands. Gap junction distribution is similar to the neonatal pattern observed in the hearts of larger mammals. Conduction velocity is in the range observed in adult mice and in the higher range for mammalian species probably due to the higher dV/dtmax. This technique will permit the study of propagation, AP, and structure-function relations at cellular resolution in genetically modified mice. (Circ Res. 2000;87:467-473.)

Key Words: strands ■ mice ■ cardiomyocytes ■ connexin43 ■ conduction

Propagation of the cardiac impulse is a complex process that depends on the active electrical properties of the cardiac cells and the passive properties of the cellular network. Thus far, analysis of impulse propagation at a cellular level has been possible only through computer simulations1,2 or in experiments in which high-resolution optical mapping of transmembrane potential has been performed under high magnification in cultured myocytes grown in patterned arrays.3 Results of these experiments have provided insight into the role of discontinuous tissue structure, membrane ion channels, and cell-to-cell connections in propagation.4–6

Experimental elucidation of the functional role in impulse propagation of specific proteins that form ion channels, ion exchangers, ion pumps, and gap junction channels requires interventions that selectively and specifically inhibit or activate cellular functions. Steady-state conditions are often difficult to achieve with drugs. An alternative approach is to study genetically engineered animals to define the functional consequences of deletion or overexpression of specific genes. Until further advances occur in the development of methods and reagents for large animal transgenic technology, genetic engineering of mammals will remain practical only in mice. A number of transgenic mice with defined alterations in the expression of genes critical in depolarization, repolarization, and cell-to-cell communication have been reported.7

In the present report, we describe the production of synthetic strands of neonatal mouse ventricular myocytes and the characterization of structural and electrophysiological features pertinent to impulse propagation in this synthetic preparation. Mouse myocyte strands were produced with a technique similar to that previously described for neonatal rat myocytes.8 To relate structural features of these artificial strands to electrical function and to calibrate measurements made with voltage-sensitive dyes, optical measurements were combined with direct microelectrode recordings, and the preparations were subsequently analyzed morphometrically and immunohistochemically to assess the 2-dimensional...
structure of the multicellular strand and the expression of intercellular junction proteins. This experimental system has far-reaching potential for elucidation of the determinants of impulse propagation and arrhythmogenesis with cells from mice with defined molecular pathology.

Materials and Methods

Cell Cultures

Hearts were rapidly excised from neonatal (1 to 2 days old) ddY mice (Japan SLC, Shizuoka Laboratory Animal Center). The ventricles were minced and placed in a dissociating solution (Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ balanced salt solution [GIBCO] containing 0.5 mg/L trypsin [Boehringer-Mannheim], 10 mg/L pancreatic [Sigma Chemical Co], and 10 mmol/L HEPES). Harvested cells were centrifuged, and the sediment was resuspended in 6 mL M199 (GIBCO) containing 20 U/mL penicillin, 20 mg/L streptomycin, 0.1 mmol/L bromodeoxyuridine, and 10% newborn calf serum (Bio Concept). The fibroblast content of the cell suspension was reduced with a differential attachment technique. Cell-rich medium (28±7×10^6 cells/mL) was placed in a multwell plate (2 mL/well) containing glass coverslips that had been prepared for patterned cell growth with a photolithographic technique described in detail previously. In the present study, cells were cultured in parallel strands 1 cm in length. Culture medium was changed after 24 hours and every 2 days thereafter with M199 containing 5% neonatal calf serum, bromodeoxyuridine (0.1 mmol/L), and epinephrine (10 μmol/L for the first 3 days). A total of 114 mice were used for 14 cell culture preparations. The study complies with the ethical principles described by the Swiss Academy of Medical Science and institutional guidelines were observed.

Optical Mapping and Analysis of Propagation

The technique of multiple-site optical recording of transmembrane potential and the staining of cell cultures with the voltage-sensitive dye RH237 have been described in detail elsewhere. The cultures were stimulated >1 mm from the recording site (cycle length 500 ms), and isochronal maps were calculated as previously described.

Microelectrode measurements were performed on days 4 (n=5), 6 (n=7), 7 (n=4), and 8 (n=4) in culture. Glass microelectrodes with a tip diameter of 1 μm were filled with a solution containing (in mmol/L) K gluconate 120, KCl 20, HEPES 20, and Mg$^{2+}$-ATP 5, with pH adjusted to 7.2. Signals were recorded with an Axoprobe 1A amplifier (Axon Instruments) in the current clamp mode and MacLab AD conversion and analysis (sampling rate 20 kHz). Junction potentials were measured and computed (correction factor = 11.8 mV) with the method described by Neher. Recordings stable over >60 seconds were used for analysis.

Immunohistochemistry

Myocytes on coverslips were fixed in 4% paraformaldehyde in PBS for 15 minutes and rinsed 3 times in PBS. Immunostaining was performed with an affinity-purified polyclonal rabbit anti-cardiac myosin (kindly provided by Dr Stacy Smith) to determine whether significant contamination by nonmyocyte cells was present. All immunostaining procedures, including the use of controls for nonspecific binding, have been described in detail in a previous report. Immunostained cells were mounted onto glass slides and examined with a Sarastro model 2000 laser scanning confocal microscope (Molecular Dynamics).

Confocal Microscopy

Three to five high-power fields of strands of different widths in 6 separate cultures were examined at a magnification of ×400 as previously described. The proportion of total cell area occupied by Cx43 immunoreactive signal was defined as the number of high-intensity pixels divided by the total number of pixels occupied by cells. The total number and mean size of individual spots of high intensity signal, operationally defined as individual gap junctions, were measured according to methods previously validated. Confocal microscopy was also used to visualize the distribution of N-cadherin immunoreactive signal to delineate the locations of fascia adherens junctions. Cultures stained with antibodies against cardiac-specific myosin were examined to define the relative proportions of myocytes (identified by intense staining in a sarcomeric pattern) and nonmyocytic cells (fibroblasts and endothelial cells) that do not express cardiac myosin. Confocal microscopy was also used to determine cell thickness in cultures stained with Fluoro-3.

Measurement of Cell Size, Cell Shape, and Connectivity

The outlines of individual cells were readily delineated in cultures stained with antibodies against Cx43 or N-cadherins. Accordingly, these preparations were used for morphometric studies to measure the length and width of cells in strands, the number and relative end-to-end or side-to-side orientation of cells interconnected by gap junctions to an individual cell within strands, the width of the strands, and the number of cells across strands of different widths. The extent of intercellular connectivity was determined by selecting index cells within the center of strands and counting the total number of neighboring cells connected to each index cell by immunohistochemically identified gap junctions. The spatial orientation of the interconnected cells was defined according to system used in previous studies.

Statistical Analysis

Trends in morphology in relation to strand width were calculated with ANOVA. Multiple regression was used to determine the effect of strand width and age in culture on conduction velocity (θ) and maximum dV/dt of the AP upstroke. Continuous variables are expressed as mean±SD. The cell orientation analysis was performed using the Mann-Whitney U test.

Results

Structural Features of Synthetic Mouse Cardiac Myocyte Strands

The arrangement of cells in strands was clearly demonstrated in cultures stained with an antibody against cardiac myosin (Figure 1). These preparations showed closely packed, elongated cardiac myocytes aligned with their long axes parallel to the long axis of the strand. Alignment of cells was almost complete in the narrow strands. Thus, the absolute value of the angle between the long axes of the cells and the strand axis was 3±1° in the small strands and 15±4° in the large strands (P<0.001). The cells exhibited robust staining in a sarcomeric pattern, indicating well developed contractile organelles. An examination of both strands and peripheral areas composed of confluent monolayers of cells revealed no cells that failed to demonstrate myosin staining (Figure 1). Thus, contamination with nonmyocytic cells was not detectable.

Table 1 summarizes the results of morphometric analyses. Cells were grown in strands of 3 widths: 34±4.4 (W1), 57.9±2.5 (W2), and 86.4±3.6 (W3) μm. The average dimensions of individual cells within strands varied as a function of strand width (Table 1). Both cell length and width increased with increasing strand width. In contrast, there was a trend...
toward an inverse relationship between cell thickness and stand width that did not achieve statistical significance. The number of cells across each strand fell within a narrow range of 3 or 4 cells across small strands to 8 or 9 cells across the largest strands (Table 1). Cell dimensions were independent of duration in culture between days 4 and 8.

The number of cells interconnected by gap junctions to an individual cell located within the center of a strand was 6.5 ± 1.1. Of the neighbors interconnected to an average index cell, 2.5 or 38% were connected in a purely or predominantly side-to-side orientation, whereas 4.0 or 62% were connected purely or mainly in an end-to-end juxtaposition. No differences were observed in the number or spatial orientation of interconnected neighbors in strands of different widths or with duration of cells in culture between 4 and 8 days.

### Immunohistochemistry and Confocal Microscopy of Intercellular Junction Proteins

The Cx43 immunoreactive signal in mouse myocytes in strands was analyzed with quantitative confocal microscopy. Figure 2 shows representative confocal images of immuno-stained strands of different widths. In all cases, the Cx43 signal appeared as discrete spots of high-intensity signal against a dark background. Table 2 shows the results of quantitative analysis of the amount of Cx43 immunoreactive signal as well as the number of individual spots of high-intensity signal (operationally defined as individual gap junctions) and the mean size of individual gap junctions. These parameters were independent of strand width.

Figure 3 shows cultured neonatal mouse ventricular myocytes incubated simultaneously with anti-Cx43 antibodies and with an antibody against a conserved sequence in the N-cadherins that stains fascia adherens junctions in the intercalated disk. Patches or linear arrays of discrete spots of high-intensity signal were seen at the edges of neighboring cells where junctional membranes had formed. Simultaneous visualization of fascia adherens junctions and gap junctions in double-label preparations revealed an intimate spatial relationship between these 2 types of junctions. There was minimal overlap in the distribution of the 2 signals, however, indicating that each of these organelles occupied separate discrete regions of the junctional membrane (Figure 3).

### Electrical Properties of Synthetic Cardiac Myocyte Strands

A major goal of the present study was to characterize APs and propagation with multisite optical recordings. Because the fluorescence change of the voltage-sensitive dye does not indicate the absolute value of the voltage change, it was necessary to calibrate the optical signals with microelectrode measurements. As illustrated in Figure 4, the AP duration and shape of the plateau phase were dependent on the age of cells in culture. At day 4, the plateau phase was present and repolarization was relatively delayed. By day 8, the AP had a short spiked appearance, similar to that seen in adult mice. The amplitude of the AP and the minimum diastolic

### Table 1. Morphometric Features of Mouse Myocytes Strands

<table>
<thead>
<tr>
<th>Strand Width Group</th>
<th>W₁</th>
<th>W₂</th>
<th>W₃</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>22</td>
<td>23</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Strand width, μm</td>
<td>34.7±4.4</td>
<td>57.9±2.5</td>
<td>86.4±3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length, μm</td>
<td>34.2±4.2</td>
<td>36.9±5.8</td>
<td>43.7±6.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Width, μm</td>
<td>9.3±1.0</td>
<td>9.4±1.2</td>
<td>11.2±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Depth, μm</td>
<td>8.1±3.1</td>
<td>7.2±1.1</td>
<td>6.1±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>L/W</td>
<td>3.7</td>
<td>4.0</td>
<td>3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Cells across strand, n</td>
<td>3.5±0.5</td>
<td>5.7±0.5</td>
<td>8.4±0.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*ANOVA.

Figure 1. Mouse myocytes stained with antibody to myosin to demonstrate well-developed contractile unit structure. A, Myocytes in a narrow strand were aligned with the long axis of the strand. B, Myocytes in an area of confluent monolayer showing absence of nonmyocytic cells. Scale bar in μm.
potential, averaged over all recording days, were 97.2±7.6 and 71±5 mV, respectively. The AP upstroke velocity was 196±67 V/s; higher than that previously observed in synthetic strands of rat ventricular myocytes. The propagation velocity recorded with optical mapping was 43.9±13.6 cm/s and was independent of strand width. Figure 5 illustrates propagation, measured optically, in a medium-sized (W2) street.

There was an increase in maximum dV/dt of the AP upstroke with increasing age of the culture. This trend was clearly apparent in the microelectrode recordings but did not achieve statistical significance. However, the optical measurements of upstroke velocity confirmed this trend (P<0.01). The lower recorded amplitude of dV/dt max in the optical signals is due to low-pass filtering at 1.5 kHz. The change in maximum velocity of the AP upstroke with increasing age of the culture was not associated with a significant increase in \( \mu \) despite an apparent slight trend in the mean values (Table 3).

**Discussion**

**Gap Junction Distribution and Connectivity**

Cell shape, orientation, and connections between adjacent myocytes are major determinants of the passive properties of conduction within multicellular networks. Therefore, the description of cell morphology, connectivity, and Cx43 distribution are key components of this work. In normal adult humans, ventricular myocyte gap junctions are distributed anisotropically. In these structures, there is colocalization of gap junctions and fascia adherens junctions predominantly at the cell poles.\(^1\)\(^8\)\(^1\)\(^9\) This is not the case in human neonates, in whom Cx43 in myocytes has a uniform punctate distribution throughout the appositional cell membrane. Initially, gap junctions and adhering junctions are not usually closely associated. The adult pattern develops progressively during the first 6 years of life.\(^2\)\(^0\) Previous studies have shown that Cx43 in neonatal rat ventricular myocyte cultures exhibit a uniform distribution pattern within the cell membrane similar to that of the human neonate.\(^2\)\(^1\) In the present study, we showed that neonatal mouse ventricular myocytes have a similar distribution of Cx43. Changes in connexin distribution are also part of the remodeling process observed in several disease states. In the border zone between necrotic and healthy tissue after myocardial infarction, marked alter-

**Figure 2.** Pattern of distribution of Cx43 in neonatal mouse cultures. Cx43 has a uniform punctate distribution throughout the appositional cell membrane. The pattern is independent of strand width. A, 30-\( \mu \)m strand (W1). B, 50-\( \mu \)m strand (W2). C, 80-\( \mu \)m strand (W3). D, Monolayer. Scale bar in \( \mu \)m.

<table>
<thead>
<tr>
<th>Strand Width ( \mu )m</th>
<th>n</th>
<th>Area, %</th>
<th>Strands/100 ( \mu )m²</th>
<th>Size, ( \mu )m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small (W1)</td>
<td>9</td>
<td>1.69±0.94</td>
<td>3.85±1.31</td>
<td>0.43±0.17</td>
</tr>
<tr>
<td>Medium (W2)</td>
<td>12</td>
<td>1.67±0.75</td>
<td>4.04±1.01</td>
<td>0.41±0.13</td>
</tr>
<tr>
<td>Large (W3)</td>
<td>10</td>
<td>1.56±0.41</td>
<td>3.00±0.62</td>
<td>0.53±0.11</td>
</tr>
</tbody>
</table>

\( * \)ANOVA.

*\( P^*\)N S N S N S N S*
ations of Cx43 distribution have been observed.\textsuperscript{22,23} A similar pattern of gap junction distribution was recently observed in the hypertrophied right ventricles of rats with pulmonary hypertension\textsuperscript{24} and in hypertrophic cardiomyopathy.\textsuperscript{25}

The length/width ratio and connectivity of cardiac myocytes are important determinants of anisotropic conduction. The number of cells connected to an individual cell was 6.5 ± 1.1. This value is comparable to the connectivity in adult canine ventricular tissue when the 2-dimensional structure is taken into consideration.\textsuperscript{26} As in the intact human ventricle, end-to-end connections dominate. In the adult canine ventricle, purely or predominantly end-to-end connections form 53% of connections compared with 62% in our preparations.\textsuperscript{15} By contrast, the length/width ratio was lower than that seen in adult dogs and neonatal rat cultures.\textsuperscript{11} These factors need to be taken into account if these cell cultures are to be used to investigate aspects of anisotropic conduction (see later).

**Transmembrane APs**

In the present study, APs from dense cultured monolayer networks of mouse ventricular myocytes were recorded for

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**Figure 3.** Cultured neonatal mouse ventricular myocytes incubated simultaneously with antibodies against Cx43 and a conserved sequence in the N-cadherins. In double-label preparations, fascia adherens junctions are green and gap junctions are red. Although both of these structures are distributed evenly around the perimeter of the cell, there was little overlap in the distribution of the 2 signals. Scale bar in \( \mu \text{m} \).

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**Figure 4.** Shortening of AP duration with increasing time in culture. Examples of microelectrode action potentials recordings taken at 4, 6, 7, and 8 days. The graph contains all of the action potential duration (APD) data and clearly shows the rapid decrease in AP duration between 4 and 8 days.
the first time. Minimum diastolic membrane potentials and AP amplitudes were comparable to previous recordings of resting and diastolic ventricular APs from isolated cells or intact tissue fragments. In intact ventricular tissue fragments from neonatal and adult mice, the resting membrane potentials were $-76$ to $-81$ mV. The AP amplitude was similar to that observed in the present study and slightly lower than that of isolated cells. The maximum upstroke velocity of the AP in tissue fragments was similar to that observed in the present study. Wang et al. found no difference in the resting membrane potential or the maximum upstroke velocity of phase 0 of the AP between neonatal and adult mice.

In contrast to the absence of depolarization changes during postnatal development, Wang and Duff and Wang et al. demonstrated a progressive reduction in ventricular AP duration in the mouse right ventricle with normal postnatal development. The action potential duration at 50% repolarization (APD$_{50}$) was 43 ms in hearts from 1-day-old neonates, 18 ms after 3 days, and 10 ms in adult hearts. Studies in isolated cells indicated that changes in the density and inactivation kinetics of $I_{\text{Na}}$ contribute to this development. Babij et al. showed that $I_{\text{Na}}$ did not increase during postnatal development. Another study that involved the targeted disruption of the gene minK, which encodes a subunit of $I_{\text{Kr}}$, showed no QT prolongation in neonatal or adult mice. In the present study, a similar dramatic reduction in AP duration was observed for the first time in cultured networks. This finding suggests that developmental changes in the cultured network mimic those seen in vivo. We demonstrated an 85% reduction in APD$_{50}$ and a 52% reduction in action potential duration at 90% repolarization (APD$_{90}$) between days 4 and 8. If AP durations of myocytes freshly prepared from neonatal mice of varying postnatal ages are compared with myocytes after a similar number of days in culture, we observe that the APs are longer in our preparations and that the reduction in duration develops more slowly.

**Propagation Velocity**

Several differences between the cultures described here and adult myocytes in vivo would be expected to affect $\theta$. Conduction velocity is determined not only by depolarizing ion channels and cell-to-cell connections but, as demonstrated in a recent study by Spach et al., also by cell size. The small cell size and distribution of gap junctions may suggest that $\theta$ in these preparations would be low. Instead, we found that $\theta$ was in the upper range for ventricular conduction in larger mammals. Clearly, other factors may have affected $\theta$ in this model. The rapid AP upstroke velocity that suggests a high density of Na$^+$ channels may at least in part compensate for the relatively small cell size.

Cell morphology was not uniform across the different strand widths assessed in this study. We observed that the cell length and width were greater in the large strands. By contrast, there was a trend toward an increase in cell thickness in small strands. This suggests that the difference in thickness may have compensated for the tendency of the larger cell length to increase $\theta$. Moreover, the greater alignment of cells in the smaller strands with the direction of propagation also may have attenuated the effect of a decrease in cell length on $\theta$. Our failure to detect any significant difference in $\theta$ between the strand widths suggests that within the range of strand widths studied, these opposing effects are small or that they cancel each other out.

**Implications**

This model allows the combination of several powerful tools for the investigation of impulse propagation in cardiac tis-

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**TABLE 3. Change in Electrical Parameters With Increasing Duration in Culture**

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>Microelectrode Data</th>
<th>Optical Data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APA, mV</td>
<td>APD$_{50}$, ms</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>95.4±2.3</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>94.3±10.6</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>97.8±4.7</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>104.0±5.0</td>
</tr>
<tr>
<td>$P^*$</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

APA indicates action potential amplitude; MDP, minimum diastolic membrane potential.

*ANOVA.
sues: genetic engineering in mice, patterned growth of synthetic strands of neonatal ventricular myocytes, and high-resolution optical mapping of transmembrane potentials. These methods will contribute to the investigation of the basic mechanisms of propagation and repolarization at a cellular level.

Acknowledgments
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References
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