Rapid Communications

Anisotropic Conduction in Monolayers of Neonatal Rat Heart Cells Cultured on Collagen Substrate

Vladimir G. Fast, André G. Kléber

Abstract Anisotropic impulse conduction was studied in neonatal rat heart cell monolayers produced by culturing cells on a growth-directing substrate of collagen. Monolayers consisting of parallel-oriented cells without visible intercellular clefts were selected for experiments; cell lengths and widths were 65.8±12.5 and 12.2±3.2 μm (n=49), respectively. Action potential upstrokes were measured by using 12 photodiodes selected within a 10×10 diode array and a voltage-sensitive dye (RH-237). The size of the area sensed by a single diode was 14×14 μm. High-density multiple recordings (resolution, up to 15 μm) demonstrated the variability of local activation delays and of the maximal rate of rise of the action potential upstroke (Vmax), which are presumably related to the microscopic cellular architecture. Mean macroscopic conduction velocities measured over distances of 135 μm were 34.6±4.5 and 19.0±4.3 cm/s (mean±SD, n=13, P<.0001) in longitudinal and transverse directions, respectively. The anisotropic velocity ratio was 1.89±0.38 (n=13). Mean Vmax was not significantly different in two directions (122.0±17.4 V/s longitudinally versus 125.2±15.6 V/s transversely, n=13, P=NS). In conclusion, we developed an anisotropic cell culture model suitable for studying impulse conduction with cellular resolution. The anisotropic velocity ratio was close to values measured in vivo. By contrast, Vmax was not dependent on the direction of propagation. (Circ Res. 1994;75:591-595.)

Key Words • ventricular myocytes • cell culture • action potential propagation • voltage-sensitive dyes • collagen

Anisotropy of cardiac muscle plays an important role in the propagation of excitatory waves and in the initiation and maintenance of cardiac arrhythmias. Sano et al1 presented the first evidence that tissue anisotropy caused directional differences in impulse spread with fast velocity in a direction parallel to fiber orientation and slow velocity in a direction perpendicular to the fiber axis. Later, Clerc2 demonstrated that intracellular resistance in ventricular trabecula was 10 times larger in a direction transverse to fiber orientation than in a longitudinal direction. In accordance with continuous cable theory, conduction velocity was three times larger in the longitudinal than in the transverse direction in the same experiments. More recently Spach et al3 presented results on anisotropic propagation that could not be explained by continuous cable theory. They found that the maximal rate of rise of the action potential upstroke (Vmax) was larger in the transverse than in the longitudinal direction. Moreover, premature impulses propagating in the longitudinal direction were blocked, while they continued to propagate in the transverse direction. Both the higher transverse Vmax and the dependence of block on fiber orientation were interpreted as reflecting a higher safety factor for transverse than for longitudinal propagation. The subject of directional differences in Vmax and the occurrence of unidirectional conduction block was addressed in a large number of studies.4-12

It has been suggested that the effects of anisotropy on Vmax and the safety factor are attributed to the presence of recurrent spatial discontinuities of axial resistance related to cellular borders.3,13 Experimental verification of this hypothesis was hampered until now by the complex three-dimensional geometry of cardiac tissue and by the lack of techniques available for high-resolution mapping of activation spread. In the present study, we developed a technique that allowed growth of anisotropic two-dimensional monolayers of heart cells on a mechanically treated substrate of collagen. An optical method that allows for the recording of membrane potential with cellular resolution14 was used to assess the directional differences in conduction velocity and Vmax.

Materials and Methods

Cell Culture

Collagen Coating

Extracellular matrix protein collagen type IV from human placenta (Sigma) was dissolved in phosphate buffer at a concentration of 20 to 50 μg/mL. Two milliliters of the solution was applied to one side of the glass coverslips (22-mm diameter, 0.14-mm thickness, Haska) for 1 hour at room temperature. Afterward, the coverslips were rinsed with distilled water and air-dried. A growth-directing substrate was obtained by gently rubbing the collagen coat with a fine brush. This mechanical treatment produced an adhesion matrix leading to a parallel alignment of the cultured cells. Myocytes were isolated from neonatal Wistar rats (2 days old) by using a procedure reported in detail previously.15 In brief, the ventricles of the excised hearts were dissociated with trypsin (0.1%). The dispersed cells were suspended in medium M199 (GIBCO) with an ionic composition of (mmol/L) NaCl 137, KCl 5.4, CaCl2 1.3, MgSO4 0.8, NaHCO3 4.2, KH2PO4 0.5, and NaH2PO4 0.3 and containing 20 U/mL penicillin, 20 μg/mL vitamin B12, and 10% neonatal calf serum. The cell suspension

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was plated to reduce the fibroblast content and then diluted to $3 \times 10^{10}$ cells per milliliter. Two milliliters of cell suspension was placed into a well containing the collagen-coated coverslip. The cultures were incubated in cell medium M199 containing 5% serum at 37°C in a humidified atmosphere containing 1.5% $\text{CO}_2$. Medium changes were performed every second day. Measurements were made between the third and eighth day.

**Voltage-Sensitive Dye Staining**

Coverslips were transferred into the experimental chamber mounted on a vibration-free table. Cells were superfused at a rate of 2 mL/min with a solution composed of (mmol/L) NaCl 150, KCl 5, CaCl$_2$ 1.2, MgCl$_2$ 1, NaHCO$_3$ 5.8, HEPES 5, and glucose 5. pH was 7.4, and temperature was 34°C to 35°C. The voltage-sensitive styryl dye RH-237 (Molecular Probes) was used to measure transmembrane potential changes. The dye was stored in a 2-mmol/L stock solution of dimethyl sulfoxide (DMSO) and diluted to yield a final dye concentration of 2 mmol/L in Tyrode’s solution (final DMSO concentration, 0.1%). Cells were superfused with the dye solution for 4 to 6 minutes.

**Optical Recording of Impulse Conduction**

**Optical Setup**

An optical system to record the fluorescence change of voltage-sensitive dye was used as described previously with several modifications. The system included an inverted microscope (Axiovert 35M) with a Plan-Neofluar objective (×40; numerical aperture, 1.3 Oi, Zeiss) and a filter set with a band-pass exciting filter (530 to 585 nm), dichroic mirror (600 nm), and a low-pass emitting filter (>615 nm). Cells were exposed to excitation light for 50 milliseconds. The fluorescence emitted by the dye was measured using a 10×10 photodiode array (Centronic) located in the image plane of the microscope. An area of $14 \times 14 \mu\text{m}^2$ of cell culture corresponded to each photodiode. The photocurrents from 96 diodes were converted to voltage, and 12 channels selected out of 96 were directed into second-stage amplifiers with a sample-and-hold circuit for subtraction of DC signals. Signals were sampled at a rate of 100 kHz per channel and 12 bits resolution and stored on a personal computer (Macintosh IIIfx, Apple Computer). To eliminate high-frequency noise, signals were digitally filtered using a gaussian low-pass filter with a cut-off frequency of 2 kHz. The activation times were determined at the level of 50% change of optical upstream.

**Electrical Stimulation**

Electrical stimulation was performed via a bipolar electrode composed of the conducting core of a glass pipette (tip diameter, 50 to 70 $\mu\text{m}$) filled with the superfusion solution and a silver wire coiled around the pipette tip. Cells were stimulated with rectangular pulses (duration, 1 millisecond; double threshold strength). To ensure that electrical stimulation did not interfere with propagation measurements, the stimulation electrode was placed >1 mm from the measurement site.

**Measurements of Activation Map and $V_{\text{max}}$**

Activation maps were constructed by using linear interpolation between diodes. At a given measuring site and with a given position of the diode array, impulse conduction was measured in the direction parallel to the long cell axes and in the transverse direction by changing the location of the stimulation electrode. To calculate $V_{\text{max}}$, signals were scaled to 100-mV amplitude, filtered with a cutoff frequency of 2 kHz, and digitally differentiated. Repetitive exposures of cells to excitation light resulted in the reduction of $V_{\text{max}}$ by 5% (phototoxic effect). To compensate for this effect in the comparison of longitudinal with transverse velocity, the order of measurements was alternated from experiment to experiment.

**Morphology**

Before each recording, cell morphology was observed in red light (filter, >630 nm), and the photodiodes were positioned over a chosen area. Pictures showing the bright-field illumination image of the cells and of the photodiodes and the phase-contrast picture of cells (magnifications of ×20 and ×40) were made by a video camera (WV-CD50, Panasonic) and a videographic printer (UP-811, Sony). This procedure avoided phototoxic damage of cells. Morphological parameters of cells were measured from photographs. Cell length was taken as the longest straight line within a cell contour. The longest line within the contour perpendicular to the long axis was taken as the cell width. Statistical data are expressed as mean±SD. Two-tailed nonpaired Student’s $t$ test was used for comparison, and a value of $P<.05$ was considered statistically significant.

**Results**

**Morphology of Cell Monolayers**

Fig 1 demonstrates partial overviews of isotropic (panel A) and anisotropic (panel B) cell monolayers. The culture depicted in panel A was grown on a coverslip coated with collagen; the culture shown in panel B was grown on a coverslip that was mechanically treated after collagen coating to obtain anisotropic cell growth. In isotropic monolayers, the cell shapes were polymorphic, ranging from almost polygonal or round to elongated. Altogether, cells formed a dense network showing no preferential direction of cell orientation. By contrast, the cells grown on directed collagen substrate (panel B) were typically of elongated shape and oriented in one direction. The aver-
age cell length and width were 65.8±12.5 and 12.2±3.2 μm (n=49), respectively.

Conduction in Isotropic Monolayers

In isotropic cultures, macroscopic conduction was not dependent on the direction of the propagation wave. The mean value for conduction velocity was 24.8±3.1 cm/s (n=14). Vmax in these measurements was 121.4±8.8 V/s, when assuming a value of 100 mV for action potential amplitude.

Conduction in Anisotropic Monolayers

Activation Maps During Longitudinal and Transverse Conduction

Fig 2 demonstrates an example of impulse propagation in an anisotropic cell culture. In this experiment, 12 recording points were distributed over the whole area covered by the 10×10 diode array (150×150 μm²) to assess the general pattern of excitation spread. Panel A shows the anisotropic cell monolayer with the localization of photodiodes; panels B and C show the activation maps during longitudinal and transverse conduction, respectively. No clefts were visible on the culture image. The interdiode distance varied between 40 and 60 μm. Propagation in panel B was obtained by stimulation of the monolayer at a point located =1 mm to the right of the measurement area. The map of longitudinal propagation demonstrates that the general direction of propagation coincided with the cell orientation. The shape of the isochrones revealed small nonuniformities in longitudinal activation. Overall longitudinal conduction velocity measured between peripheral diodes (activation times at three proximal and three distal diodes were averaged) amounted to 38.6 cm/s. Transverse propagation in the same culture is shown on panel C. In this case, the culture was stimulated by the electrode located 1 mm below the measurement area. The general direction of propagation slightly deviated from the transverse axis of the monolayer. The overall conduction velocity was 23.3 cm/s, which was 1.7 times smaller than longitudinal velocity. Measurements of Vmax revealed significant variability during both longitudinal and transverse conduction. Vmax varied in the range of 123 to 177 and 94 to 169 V/s, respectively. However, there was no significant difference between mean Vmax values measured during longitudinal (149.1±15.4 V/s) and transverse (135.9±25.4 V/s, n=12 recording points, P=NS) propagation.

Fig 3 shows an example of another type of measurement, in which recording points were aligned in a row to record activation sequences at a higher spatial resolution (interdiode distance, 15 μm) and to compare transverse with longitudinal Vmax, along a single axis. Action potential upstrokes were smooth in both transverse (panel B) and longitudinal (panel C) directions showing no discontinuities or "humps." At the same time, the activation sequence measured along the transverse axis was rather nonuniform. The local conduction times between neighboring diodes varied from 60 to 160 microseconds. In most cases, a single diode collected light from two cells; therefore, it was not possible to

Fig 2. Impulse conduction in an anisotropic cell culture. A, Phase-contrast image of the cell culture. The white rectangles correspond to the localization of the 12 photodiodes. An individual diode covers a cell membrane area of 14×14 μm². B, Isochrone map during longitudinal impulse spread. Numbers within rectangles indicate local activation time (in microseconds). Isochrones are drawn at an interval of 100 microseconds. C, Isochrone map recorded during transverse conduction.

Fig 3. Activation sequences of transverse and longitudinal conduction. A, Phase-contrast image of the cell culture and photodiodes. Numbers within rectangles indicate photodiode numbers. B, Normalized optical upstrokes recorded by the photodiodes shown in panel A during transverse conduction. C, Normalized optical upstrokes recorded from the same localization during longitudinal conduction.
attribute the delay between two diodes to a given cell border with certainty. The measurements, however, are consistent with the variability of conduction delays introduced by intercellular junctions. The average transverse conduction velocity measured between diodes 1 and 10 was 18.0 cm/s, and the average longitudinal conduction velocity between diodes 11 and 12 was 37.5 cm/s, i.e., 2.1 times larger than transverse velocity. The 10 diodes located along the transverse axis were activated within 80 microseconds during longitudinal spread. This was consistent with the wave front nonlinearity shown in Fig 2. As in the experiment presented in Fig 2, there was substantial variability of $V_{\text{max}}$; it varied from 116 to 158 V/s (longitudinal conduction) and from 114 to 143 V/s (transverse conduction). However, the mean values of longitudinal and transverse $V_{\text{max}}$ were only slightly different: 142.6±10.9 and 130.7±9.5 V/s (n=12), respectively.

From all the measurements in anisotropic cultures, the average conduction velocity amounted to 34.6±4.5 and 19.0±4.3 cm/s in longitudinal and transverse directions, respectively (n=13 experiments, P<.0001). The mean anisotropic velocity ratio was 1.89±0.38. In contrast to anisotropic conduction velocity, $V_{\text{max}}$ was not different in two directions. From all the experiments, the average of mean $V_{\text{max}}$ values was 122.0±17.4 V/s during longitudinal conduction and 125.2±15.6 V/s (n=13, P=.64 [P=NS]) during transverse conduction.

**Discussion**

Anisotropy of cardiac tissue plays an important role in impulse propagation and generation of cardiac arrhythmias. So far, the effects of anisotropy have been studied in isolated tissue preparations including atrial tissue,2 papillary muscle,3,15 and sheets of ventricular myocardium.2,5,10-17 Variations in the whole heart, anisotropy was assessed on the epicardial surface of the intact heart.22-24 In a thin rim of subepicardial muscle surviving after the intramural infarction,25 or after cryodestruction of intramural layers,12,25,26 Typically, a sheet of viable tissue in such experiments consists of many cell layers, which complicates the assessment of impulse conduction at the cellular level. A possible solution to avoid the complexity of three-dimensional heart tissue is to use cultured cell monolayers.14 Thus, we developed a technique allowing the production of two-dimensional anisotropic monolayers of heart cells. This was achieved by coating glass coverslips with collagen, which is known for its ability to promote cell adhesion, and by scratching the collagen substrate with a brush. This mechanical treatment produced cell adhesion patterns in which cells formed an anisotropic structure. Mean cell length (66 μm) and cell width (12 μm) in anisotropic monolayers were slightly smaller than values found in adult rat myocytes (cell length, 73 to 98 μm; cell width, 15 to 18 μm27) and similar to the values found in cultured strands of neonatal rat myocytes grown in channels formed by a photosensitive polymer.14,15 The degree of the structural anisotropy given by the ratio of cell length to width as well as by cell alignment was similar to the myocardial structure in vivo. The method of multisite optical recording of action potential upstrokes, which was validated in our previous work,14 was used for the first time to characterize the directional differences in conduction velocity and $V_{\text{max}}$.

**Conduction Velocity Anisotropy**

Longitudinal conduction velocity reported for adult ventricular myocardium is 43 to 63 cm/s, transverse velocity is 16 to 29 cm/s, and the anisotropic velocity ratio is 1.7 to 3.5.2,3,12,18,20,24-26 It is generally believed that the directional differences in conduction velocity are due to the increased axial resistivity in transverse direction. Our mean values for longitudinal and transverse conduction velocities in anisotropic dense cell monolayers are somewhat lower (34 and 19 cm/s, respectively), but the mean anisotropic ratio (1.9) falls within the range known for the adult tissue. The potential reasons for the lower velocity in neonatal rat heart cell cultures with respect to adult myocardial tissue include a larger surface-to-volume cell ratio and a lower degree of electrical coupling in cultured monolayers. They have been discussed elsewhere in detail.14

**Local Nonuniformity of Conduction**

Two types of anisotropic conduction were reported previously,17 characterized either by smooth shapes of extracellular potentials in both longitudinal and transverse directions (uniform anisotropy) or by smooth longitudinal conduction and fractionated electrograms in transverse direction (nonuniform anisotropy). The intracellular recordings of transmembrane potential in papillary muscle demonstrated the presence of notches on the upstroke when propagation occurred in the transverse direction but not in the longitudinal direction.18 A similar observation was made in ventricular muscle treated with the uncoupling agent heptanol.19 Correlating structure to function revealed that the presence of connective tissue separating bundles of myocytes was associated with fractionated extracellular electrograms.20 More recently, another type of nonuniformity was described, which was characterized by small fluctuations in extracellular potentials that became apparent only in the second time derivative of the signals and were observed on a small time scale.21 This indicates that nonuniformities of propagation may occur at several scales. Our results support this idea: even in dense cell monolayers, conduction was nonuniform on a microscopic scale. This was most obvious in transverse direction, where the activation time varied from 60 to 160 microseconds, i.e., threefold between diodes located 15 μm apart. Such a variability is consistent with the variability of conduction delays at intercellular junctions.14 However, discontinuities or “notches” in the initial or terminal portions of the action potential upstrokes were not apparent with the inhomogeneity introduced by cell borders.

**Tissue Anisotropy and $V_{\text{max}}$**

It has been shown in most studies that $V_{\text{max}}$ is larger in the transverse than in the longitudinal direction. The values for $V_{\text{max}}$ reported in the literature were 94 to 140 V/s for longitudinal and 123 to 180 V/s for transverse conduction, resulting in a transverse-to-longitudinal ratio of 1.3 to 1.4.3,6,10,19,21 These directional differences in $V_{\text{max}}$ have been attributed to the presence of the recurrent spatial discontinuities of axial resistivity.3 It was postulated that in the transverse direction, cardiac tissue behaves as loosely coupled, relatively isolated membrane patches, each patch being nearly isopotential.
and therefore having larger $V_{max}$ than during continuous longitudinal conduction. The anatomic substrate explaining such recurrent discontinuities is not precisely known yet. The present study demonstrates that it is possible to grow networks of cardiac cells with structural anisotropy and anisotropic conduction velocity but no directional-dependent differences in $V_{max}$. Therefore, the discontinuities introduced by cell borders alone do not explain the directional differences of $V_{max}$ observed in vivo. This is consistent with the simulation study showing that the effects of discrete cell length on $V_{max}$ are negligible in homogeneous one-dimensional cable models.

Other type of discontinuity caused by arrangement of cardiac cells into unit bundles and arrangement of these bundles into fascicles (which both are separated by connective and vascular tissue) may be responsible for the directional differences of $V_{max}$ in vivo. This would be consistent with the observation that the higher margin of safety for transverse longitudinal conduction is only observed in “nonuniform” anisotropic tissue, i.e., in the presence of connective tissue sheets separating the fiber bundles.

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