Microscopic Conduction in Cultured Strands of Neonatal Rat Heart Cells Measured With Voltage-Sensitive Dyes

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Microscopic discontinuities in electrical activation were assessed in synthetic strands of neonatal rat myocytes cultured on a growth-directing matrix. An optical method using voltage-sensitive dye (RH-237) and a photodiode technique was used for recordings of membrane potential changes with subcellular resolution. Spatial resolution of the method (diameter of measurement area, 5.5 μm; interidiode distance, 30 μm) allowed for simultaneous measurements of cytoplasmic conduction time within a single cell and junctional conduction time across the cell border. In one-dimensional cell chains, where cells were juxtaposed by end-to-end connections but devoid of lateral connections, propagation of the excitation wave was strongly nonuniform: cytoplasmic conduction time was 38±30 (mean±SD) microseconds (n=37), whereas junctional conduction time was 118±40 microseconds (n=27, P<.0001). A mean delay introduced by a single junction was 80 microseconds, or 51% of conduction time. In two-dimensional strands consisting of several cells in width, which exhibited lateral as well as end-to-end connections, inhomogeneity of conduction was smaller: the cytoplasmic and junctional conduction times were 57±30 (n=46) and 89±40 (n=48) microseconds, respectively (P<.0001); mean junctional conduction delay was 32 microseconds (22% of conduction time). Mathematical modeling suggested that the average effect of lateral connections is caused by lateral convergence of local excitatory current beyond and lateral divergence before end-to-end connections. Our results demonstrate that the flow through lateral cell-to-cell connections smooths the excitation wave front during longitudinal conduction in myocardial tissue. (Circ Res. 1993;73:914-925.)

KEY WORDS • ventricular myocytes • cell culture • electrical activation • gap junctions • voltage-sensitive dyes

The myocardium is composed of discrete individual cells, and propagation of activation is mediated via gap junctions that interconnect the cytoplasmic spaces. Despite the discrete cellular architecture, cardiac muscle has long been considered to function as an electrically continuous medium. Experimental evidence for functional homogeneity was first provided in isolated Purkinje fibers and ventricular trabecula by Weidmann,1-2 who demonstrated that the electrotonic spread of current extends over multiple cell lengths and is well described by continuous cable theory. Also, the dependence between dV/dt_{max} of the action potential and conduction velocity upon alteration of Na+ inward current was found to follow the square relation predicted by cable theory.3,4

A number of more recent studies, however, reported results contradicting continuous cable theory. Spach et al5 showed that the dV/dt_{max} was larger during conduction in the direction transverse to fiber orientation as compared with longitudinal conduction. Treatment with uncoupling agents heptanol and octanol revealed complex nonmonotonic behavior of dV/dt_{max} that could not be explained in terms of propagation through a continuous electrical medium.6-8 It was proposed that the microscopic discontinuities of axial resistance associated with intercellular gap junctions were responsible for the noncablelike behavior of conduction.5 Such discontinuities may provide a basis for inhomogeneous conduction and block and represent a substrate for cardiac arrhythmias.5,8

Until now, the potential effects of the discontinuous cellular structure on conduction have been tested in computer simulations.6-8,10-11 The simulation studies used one-dimensional models that represented cardiac strands as a single chain of cells interconnected via gap junctions. It was shown that propagation was an inhomogeneous process on a microscopic scale, with the gap junctions introducing significant conduction delays.8,10 On a macroscopic scale, the behavior of conduction velocity and of dV/dt_{max} deviated from the behavior predicted by the continuous cable theory when the gap junctional resistivity was increased.6,10 The one-dimensional models, however, may not be fully adequate for the representation of multidimensional cardiac tissue, and in two- or three-dimensional models, propagation may be different. Thus, simulation of electrotonic spread of current in a two-dimensional anisotropic resistive network showed that the presence of lateral...
intercellular connections reduced inhomogeneities in voltage distribution.\textsuperscript{12}

The experimental assessment of propagation at a cellular level has been limited by techniques available for determination of local activation time. The majority of these techniques are based on recordings of extracellular potentials with metal electrodes. Local activation times determined from the intrinsic deflection of extracellular electrograms have provided important information about propagation on a macroscopic scale. On a smaller scale (resolution, \(<200 \ \mu\text{m}\)) extracellular mapping revealed the presence of local inhomogeneities in propagation.\textsuperscript{13} However, whether extracellular potentials (unlike transmembrane action potentials) provide an accurate measure of local excitation in a tissue with nonuniform axial resistance has been questioned recently.\textsuperscript{11,14} Accordingly, extracellular recordings may not be fully adequate for measurement of conduction delays at the spatial and temporal resolution required for the analysis of cell-to-cell conduction.

In the present study, we developed a method for the optical recording of transmembrane action potential upstrokes from neonatal rat myocytes in cultured monolayers. The resolution of the method (sensed cell membrane area, 5.5 \(\mu\text{m}\) in diameter) allowed measurement of conduction times within the cytoplasm and across intercellular junctions. This approach involves voltage-sensitive dyes and photodiode technology.\textsuperscript{15-18} The previously developed technique of patterned growth\textsuperscript{19} was used to compare cytoplasmic and junctional propagation in one-dimensional strands (one cell in width) with propagation in two-dimensional strands (four to six cells in width). Propagation along two-dimensional cell strands (consisting of end-to-end and laterally apposed cells) was found to be more homogeneous than along one-dimensional strands, where cells had no lateral connections. A computer model of cardiac propagation was used to provide a theoretical understanding of the experimental results.

**Materials and Methods**

*Preparation of Patterned Cell Cultures and Staining With Voltage-Sensitive Dye*

**Cell cultures.** The method used to prepare patterned monolayers of heart cells has been reported elsewhere in detail.\textsuperscript{19} In brief, cells were cultured on glass coverslips (22-mm diameter, 0.14-mm thickness, Haska, Bern, Switzerland) coated with photoresist KTFR (Kodak, Lausanne, Switzerland), which prevented the adhesion of myocytes. The specific growth pattern was etched in the photoresist using a photolithographic technique. Fig 1, left, shows the growth pattern used for our experiments. Regions free of photoresist formed parallel channels connecting large areas at the center and at the periphery of the coverslip. Two types of channels were used, allowing myocyte growth in one-dimensional cell chains (Fig 1, top right) and in two-dimensional cell strands (Fig 1, bottom right). The width of the channels was 20 and 80 \(\mu\text{m}\), respectively, and the length was 3.5 mm.

Cell cultures were prepared from neonatal Wistar rats (2 days old). Hearts from six to eight rats were collected, minced, and subjected to five or six cycles of serial dissociation steps in a Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free
Hanks’ balanced salt solution containing 0.1% trypsin, 60 μg/mL pancreatin, 20 U/mL penicillin, and 20 μg/mL streptomycin at 35°C. The solutions obtained after each step (except the first one) were sedimented by centrifugation, and the resulting cell pellets were resuspended in new medium M199 (GIBCO, Basel, Switzerland) having 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 μM penicillin, 20 μg/mL vitamin B12, and 10% neonatal calf serum. The fibroblast content of the cell suspension was reduced by preplating (see Reference 19 for details). After preplating, the myocytes remaining in suspension were counted with a hemocytometer and diluted to 3 × 105 cells/mL. Two milliliters of cell suspension was placed into a well containing the coverslip; 12 to 18 patterned coverslips were obtained with each culture. The cultures were incubated in cell medium M199 containing 5% serum at 37°C in a humidified atmosphere containing 5% CO2. Medium changes were performed every second day. Measurements were made between the 3rd and 11th day.

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 Tyrode’s solution composed of (mmol/L) NaCl, 150; KCl, 5; CaCl2, 1.2; MgCl2, 1; NaHCO3, 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, Eugene, Ore) was used to measure transmembrane potential changes. The dye was stored in a 2-mmol/L stock solution of dimethyl sulfoxide and diluted to yield a final dye concentration of 2 μmol/L in Tyrode’s solution (final dimethyl sulfoxide concentration, 0.1%). Cells were superfused with the dye solution for 6 to 8 minutes.

Optical and Electrical Recording of Transmembrane Potential

Optical recording. An optical system (Fig 2) was built around an inverted microscope (Axiovert 35M, Zeiss, Zürich, Switzerland) equipped for epifluorescence with a 100-W arc mercury lamp as a light source. A heat filter and a Zeiss filter set (Green H546) were used for fluorescence measurements. The filter set had a band-pass excitation filter (546±6 nm), a dichroic mirror (580 nm), and a low-pass emitting filter (>590 nm). Cells were exposed to excitation light for a single period of time (30 to 50 milliseconds) to avoid bleaching and phototoxicity. An electromechanical shutter and shutter driver (UniBlitz VS25 and UniBlitz T132, Vincent Associates, Rochester, NY) were used to turn illumination on and off with opening and closing times of <6 milliseconds. To achieve maximum optical efficiency, objectives with high numerical aperture (NA) were used (×100 Neofluar, NA 1.3 oil; ×40 Plan-Neofluar, NA 1.3 oil; Zeiss).

The fluorescence emitted by the dye was measured by a linear array of three circular photodiodes (OSDI-5T, Centronic, Craydon, England) located in the image plane of the microscope. With the ×100 objective, each measurement spot had a diameter of 5.5 μm, and the distance between spots was 30 μm; with the ×40 objective, the corresponding values were 11 and 60 μm. The generated photocurrent was converted to voltage by current-voltage converters built with operational amplifiers (OPA 128, Burr-Brown, Tucson, Ariz) and 100-MΩ feedback resistors (Eltec Instruments Inc, Daytona Beach, Fla). Signals were displayed on a four-channel digital storage oscilloscope to measure the background fluorescence. Simultaneously, they were fed into custom-built second-stage amplifiers (gain ×60, ×110, or ×220) with a sample-and-hold circuit for subtraction of DC signals. The signal rising time (0% to 63%) upon application of a square light pulse was 94±5 microseconds for all three channels. Signals were sampled using a data acquisition card with four channels, 1-MHz throughput, and 12-bit resolution (NB-A2000, National Instruments, Wettingen, Switzerland) and a personal computer (Macintosh IIfx, Apple Computer, Wallisellen, Switzerland). The sampling rate was either 50 or 100 kHz per channel (see “Results”). To eliminate high-frequency noise, signals were digitally filtered using a gaussian low-pass filter.20 A cutoff frequency of 2 or 1.5 kHz was chosen for filtering. Filtering at these frequencies did not change the shape of the action potential upstream. The filtered signal (F) was then differentiated digitally to determine the activation time, corresponding to dF/dtmax. Software for the data acquisition and data processing was written in THINK PASCAL using the NI-DAQ driver’s library (National Instruments).

Electrical recordings. To validate the optical method for measurement of activation times at microscopic resolution (≤10 μm), optical signals were compared with action potential upstreams simultaneously recorded with glass micropipettes at closely adjacent sites on a cellular membrane. Preliminary experiments showed that capacitance compensation of the slow time response of conventional high-resistance microelectrodes (tip resistance, 30 to 40 MΩ) could introduce an error in determination of dV/dtmax. To avoid this error, we recorded action potentials with low-resistance tight-seal suction pipettes in combination with a conventional microelectrode amplifier (World Precision Instruments, New Haven, Conn). Pipettes were filled with the following solution (mmol/L): potassium aspartate, 120; NaCl, 10; MgCl2, 1; CaCl2, 1; EGTA, 10; ATP, 3; and HEPES, 5 (pH 7.2) (filtered through 0.22-μm pores). The patch pipettes (tip opening, 1.5 to 2 μm) had resistances of 1...
to 3 MΩ. Upon application of a square pulse, the rise time of the signal was <40 microseconds.

**Electrical stimulation.** Electrical stimulation was performed via a bipolar electrode composed of a glass pipette (tip diameter, 50 to 70 μm) filled with Tyrode’s solution and a silver wire coiled around the pipette tip. Cells were stimulated with rectangular pulses (duration, 1 millisecond; double threshold strength) at 1 Hz. Stimuli were delivered from an eight-channel digital impulse generator (Master-8, A.M.P.I., Jerusalem, Israel). This generator was also used to synchronize the shutter opening and the data acquisition. To ensure that electrical stimulation did not interfere with propagation measurements, the stimulation electrode was placed >1 mm from the measurement site.

**Conduction velocity measurements.** Microscopic velocity was calculated from the ratio of the total interdiode distance (60 μm) and the corresponding conduction time. This distance is close to the average length of a cell (55 μm) in a synthetic strand. To compare the microscopic measurements to the average propagation in the entire strand, macroscopic velocity was also determined. It was calculated from the difference in activation times determined consecutively between sites distal and proximal to the stimulation electrode (separated by 0.4 to 1 mm).

**Morphology**

Before each recording, cell morphology was observed in red light (filter, >610 nm), and the photodiodes were positioned over a chosen area. A picture showing the bright-field illumination image of the cells and of the photodiodes was made by a video camera (model WV-CD50, Panasonic, Wettingen, Switzerland) and a videographic printer (model UP-811, Sony, Baden, Switzerland). This procedure avoided phototoxic damage. After optical recordings, cells were observed once more in fluorescent light to verify the position of the photodiodes with respect to the cell borders, which were clearly delineated by fluorescent images. In some experiments, fluorescent pictures were taken by a Nikon 35-mm camera. In a control culture, patterned strands were fixed, permeabilized, and immunohistochemically stained with a specific mouse monoclonal antibody against connexin 43. This experiment revealed the presence of abundant aggregates of connexin 43 at cell borders (J.E. Saffitz, Department of Pathology, Washington University, St Louis, Mo; V.G. Fast and A.G. Kléber, unpublished observation).

**Computer Simulations of Propagation**

Computer simulations were performed on one- and two-dimensional models of cardiac strands that incorporated gap junctional structures. The one-dimensional model shown in Fig 3A was similar to models introduced by others. It represented a chain of 50 cells arranged in an end-to-end fashion and connected by a junctional resistance. No-flow boundary conditions were imposed at the ends of the strand (sealed ends). Geometrical cell parameters approximated morphometric measurements of cells grown in narrow channels, as reported previously. An individual cell had a length of 64 μm and was divided into eight isopotential patches each 8 μm long. The cell surface-to-volume ratio was 6700 cm⁻¹. This corresponds to a flat cell with an average thickness of 3 μm. Ionic currents were described by the Beeler-Reuter model with Ebihara-Johnson modification of the fast sodium current. The maximum sodium conductance was set to 35 millisiemens/cm² to give the value of ≈190 V/s for dV/dtmax, which was close to values measured in our experiments.

The model of the two-dimensional cell strand was composed of five identical one-dimensional cell chains coupled in transverse direction and aligned in a fashion shown in Fig 3C. Every cell had four lateral neighbors interconnected via a variable resistance. The excitation wave was initiated by a current stimulus applied at the end of the strand, and the action potentials were recorded at several selected sites in the middle portion of the strand. Action potentials were differentiated, and the activation times were determined at dV/dtmax.
Both one- and two-dimensional models were computed using an alternating-direction implicit algorithm of Peaceman and Rachford\textsuperscript{24} and the simplifying method of Rush and Larsen\textsuperscript{25} and Victorri et al\textsuperscript{26} with the time integration step of 2 microseconds. The Peaceman-Rachford algorithm is designed for two-dimensional calculations. To use it for a one-dimensional strand, the strand was extended to two dimensions. Computer-generated lookup tables with voltage steps of 0.1 mV were used to calculate ionic rate constants. Simulations were programmed in FORTRAN and carried out on VAX-6410 with \textsc{vector processor}.

Statistical data are expressed as mean±SD. A two-tailed nonpaired Student’s \textit{t} test was used for comparison.

**Results**

**Validation of Optical recordings of Action Potential Upstroke and Activation Time**

Optical recording of action potential upstroke. An optical recording of the upstroke of a transmembrane potential from an area (5.5 μm in diameter) of a cultured myocyte is shown in Fig 4A. In this single recording, the fluorescence change (ΔF/F, corresponding to action potential amplitude) was 8% of total fluorescence. The following parameters for optical signals were measured (mean±SD, \textit{n}=27): ΔF/F, 5.5±1.6%, signal-to-noise ratio \([S/N_{\text{rms}}]\), 63±13. The fluorescent upstroke was not disturbed by a mechanical contraction artifact. The bleaching of the dye was insignificant during recording of the action potential upstroke.

In a series of experiments, optical signals were compared with electrical measurements obtained simultaneously from closely adjacent membrane sites within the same cell. This spatial correspondence was achieved by positioning the circumference of the circular membrane area (radius, 11 μm) sensed by the light-sensitive diode in contact with the micropipette tip. The sampling rate in these experiments was 50 kHz per channel. The parameters of the electrically recorded upstrokes were close to those previously reported for rat myocytes\textsuperscript{19,27} (mean±SD): action potential amplitude, 108±9 mV; maximal upstroke rate of rise, 214±49 V/s. Fig 4B shows a superimposition of the optical (ΔF/F) with the electrical (V) signal. It demonstrates that the time courses of the two signals were nearly identical. The superimposed first time derivatives (Fig 4C) show that the local activation time was equal with both methods. From all the measurements, the value of the difference in activation times between the two methods was 5±30 microseconds (\textit{n}=15). Thus, the systematic difference between electrical and optical recordings was very small (5 microseconds), whereas the optical signal showed significant random variation (±30 microseconds).

**Sampling rate, noise, and filtering as sources of error.**

An additional test was performed to determine the potential contributions of sampling rate, noise, and digital filtering to the error in the optical determination of local activation time from a single measurement.

A noise-free signal generated from the computer model was compared with the same signal mixed with the experimental noise (Fig 5A). Experimental noise was recorded from myocytes in the absence of activation. Both signal and noise were sampled at a rate of 100 kHz per channel—the same rate as was used subsequently in conduction time measurements. The noise was scaled with respect to the action potential to give a specified \(S/N_{\text{rms}}\). Subsequently, the mixed signal was filtered and differentiated to determine the activation time. This measurement was repeated 14 times for each \(S/N_{\text{rms}}\), and the standard deviation of activation times was taken as the random error. The whole procedure was repeated for different values of \(S/N_{\text{rms}}\). Fig 5B shows the calculated error and its dependence on the \(S/N_{\text{rms}}\) for two different cutoff frequencies of the gaussian filter (\(f_c\)). At \(S/N_{\text{rms}}\) of 63 (typical for our measurements), the random error (SD) was 25 microseconds (\(f_c\), 2 kHz) and 16 microseconds (\(f_c\), 1.5 kHz). Thus, in average, the random error was ±20 microseconds. This error includes contributions from both the noise and from the finite sampling interval. It is comparable to ±30 microseconds obtained from the comparison of optical and electrical recordings.
The effect of electrical filtering on activation times was evaluated using the electrical (smooth) recordings. The filtering procedure resulted in shift of activation times to longer values. For the familiar used in the present study, this shift was 7 microseconds (2 kHz) and 12 microseconds (1.5 kHz). For the signals with similar shapes, the value of the shift in activation time is expected to be the same. Therefore, this error will be canceled when conduction time is calculated. In the worst case, when this shift is taken into account as an independent random error, the total estimated error (quadratic average of the filtering and noise errors) would amount to 26 microseconds (fc, 2 kHz) and 20 microseconds (fc, 1.5 kHz).

S/Nrms could be improved by the averaging of several successive recordings from the same point. However, the deterioration of cells due to the phototoxicity of the dye rendered the averaging impossible. Even a single exposure to excitation light resulted in a significant decrease of the maximum upstroke rate of rise (not shown) and changed conduction time. Therefore, only the first recording at each site was taken for conduction time measurements in subsequent experiments.

**Electrical Propagation in One- and Two-Dimensional Cell Strands**

**One-dimensional strands.** A fluorescent picture of a one-dimensional cell strand is shown on Fig 1, top right. Those channels were chosen for measurements where cell growth was limited at most sites to a chain of single cells. The result of a single measurement is illustrated in Fig 6A and 6B. Three light-sensitive diodes equally spaced on a straight line were positioned over two cells (Fig 6A). Two diodes collected the fluorescent light from circular areas of an individual cell. The third diode was located across the cell junction in the neighboring cell. In the majority of measurements, the sampling rate was 100 kHz per channel. Fig 6B shows the upstrokes of action potentials (ΔF/F) measured at three sites and their first time derivatives in a representative measurement. All signals are normalized with respect to amplitude. Conduction time within the cytoplasm (between points 1 and 2) was significantly shorter than conduction time across the cell border (between points 2 and 3).

The collected data from all measurements are represented by the histogram in Fig 6C. The cytoplasmic conduction time (mean±SD) was 38±30 microseconds (n=37); the junctional conduction time was 118±40 microseconds (n=27, P<.0001). The average conduction delay (difference between junctional and cytoplasmic conduction times) introduced by a single end-to-end cell connection was 80 microseconds, which corresponded to 51% of the overall conduction time of 156 microseconds (sum of cytoplasmic and junctional conduction times). The shape of the upstroke of action potential showed no major discontinuities in these experiments. Measurements in which control of the diode position after optical recording revealed an overlap of the sensed area with cell borders were discarded. This explains the reduced n value in measurements of junctional conduction times. From these measurements, the mean microscopic conduction velocity measured over a distance of 60 μm was 0.38±0.08 m/s. It was close to the overall macroscopic conduction velocity of 0.34±0.05 m/s (n=11).

**Two-dimensional strands.** With the rationale to test the role of side-to-side connections for propagation along a strand, cytoplasmic and junctional conduction times were measured in wide strands, ie, in myocytes embedded in growth channels forming strands of four to six cells in width (Fig 1C). Analogous to the experiments in one-dimensional strands, two diodes collected the fluorescent light from two circular areas of an individual cell, and the third diode was located across the cell junction in the neighboring cell (Fig 7A). In all measurements the sampling rate was 100 kHz per channel. Fig 7B shows the upstrokes of action potentials (ΔF/F) measured at three points and their first time derivatives from a single measurement. The collected data are represented by the histogram in Fig 7C. With respect to the one-dimensional cell strands, the conduction delay at the end-to-end connection was reduced, and the cytoplasmic conduction time was increased. Cell-to-cell conduction time was 89±40 microseconds (n=48), and cytoplasmic conduction time was 57±30 microseconds (n=46, P<.0001). Therefore, the gap junctional delay (32 microseconds) decreased to 22% of the overall conduction time (from 51% in the single cell..
chains). Microscopic velocity (0.41±0.09 m/s) and macroscopic velocity (0.36±0.04 m/s, n=16) in the two-dimensional cell strands were not significantly different from the velocity values along the one-dimensional strands, indicating no major differences in electrical properties between the two types of cultured strands.

Computer Simulation

Comparison of the conduction times measured in one-dimensional cell strands with conduction times obtained from the two-dimensional cell strands suggest that lateral cell-to-cell contacts reduce inhomogeneities in longitudinal conduction. To elucidate the mechanism of this averaging effect, the spread of excitation was compared in computer models of one-dimensional and two-dimensional strands shown in Fig 3.

For the one-dimensional model, the parameters were chosen to approximate the excitable properties and the conduction times determined experimentally in one-dimensional chains. These approximations yielded a junctional resistance of 9.3 MΩ and a cytoplasmic specific resistivity of 180 Ωcm and corresponded to an average axial resistivity of 280 Ωcm. Fig 8A shows four action potential upstrokes recorded from two cells in the one-dimensional strand. Conduction characteristics were the following: dV/dt max, 188 V/s; average conduction velocity, 0.40 m/s; cytoplasmic conduction time, 38 microseconds; junctional conduction time, 122 microseconds (measured over a distance of 32 μm). The junctional conduction delay was 84 microseconds or 52% of overall conduction time.

In the model of a two-dimensional strand, lateral coupling was added, and conduction times were computed as a function of variable lateral-coupling resistance. The presence of lateral coupling did not change the average macroscopic conduction velocity, since the macroscopic tissue parameters (surface-to-volume ratio, longitudinal resistivity, etc) remained constant. It decreased, however, the difference between junctional and cytoplasmic conduction times. Fig 8B illustrates the conduction in two-dimensional strands with an average lateral resistivity 10-fold larger than the longitudinal resistivity (2800 versus 280 Ωcm), which is close to the physiological degree of anisotropy in ventricular myocardium.26 Similar to our experiments, the inhomogeneity of conduction was smaller than in two-dimensional strands. The cytoplasmic and junctional conduction times were 68 and 86 microseconds, respectively; the junctional conduction delay amounted to 18 microseconds, or 12% of overall conduction time. The dependence of conduction times on average lateral resistivity is shown on Fig 9. The situation observed experimentally in two-dimensional strands (junctional delay representing 22% of conduction time) is indicated by a vertical solid line. It corresponds to a ratio of transverse to longitudinal resistance of ≈20. At lateral resistances that are 60 times the longitudinal resistance, virtually no lateral averaging occurs, and propagation shows the
same degree of inhomogeneity as in one-dimensional strands.

The explanation of the averaging effect of lateral cell-to-cell connections on longitudinal conduction is given in Fig 10. Fig 10 depicts qualitatively local current flow (arrows) at the time of local activation for membrane locations beyond (open symbols) and before (filled symbols) end-to-end cell connections. The distribution of transmembrane voltage at a given time of activation determines the direction of these currents. At sites beyond end-to-end connections there is convergence of local current from the laterally apposed cells. This convergence explains why such membrane locations are activated earlier than the respective sites in the one-dimensional model. By contrast, there is divergence of local excitatory currents from membrane sites located before end-to-end cell connections. Such sites of divergence are activated later than respective sites in one-dimensional strands.

**Discussion**

In the present study, we measured cytoplasmic and intercellular conduction in synthetic strands of cardiac myocytes in order to analyze the role of electrical cell-to-cell connections for homogeneity of impulse propagation. Because this is the first systematic application of the optical technique to record the spread of myocardial propagation at high spatial and temporal resolution, an analysis of the potential sources of error in the measurements of microscopic activation times was carried out.

**Optical Recording of Electrical Activation at Subcellular Resolution**

Voltage-sensitive dyes, such as the styryl dye RH-237, are known to be fast and linear transducers of changes in transmembrane potential. In the present experiments, there was no systematic difference in the shapes of the electrical and optical upstrokes within the noise deviations of the optical signals and no statistically significant differences in activation times measured optically and electrically from closely adjacent membrane sites.

In several studies, performed in whole cardiac tissues and isolated myocytes, differences in onset of activation between optical and electrical signals were observed. In some of these studies, the optical signal was delayed in onset and slower in rate of upstroke than the electrical signal. These experiments were performed on whole cardiac tissue preparations. The delay was explained by the optical recordings collecting light from an area including a large number of conducting cells and the consequent integration of light. In other studies, the relation between electrical and optical recordings was opposite: optical signals measured either in tissue preparations or in isolated single myocytes were faster in onset than the electrical signals. In the study by Windisch et al., the spatial resolution of optical measurements was similar to ours. It was reported, however, that the optical signal measured from a small portion of a cellular membrane preceded the electrical recording (measured with a...
Fig 8. Numerical modeling of propagation in a one-dimensional cell chain (A) and a two-dimensional cell strand (B). Graphs show upstroke of simulated transmembrane action potential (E_m) measured at four sites in two neighboring cells. The distance between measurement sites was 32 μm. Arrow indicates the direction of propagation. A, The parameters for the simulation in one-dimensional chains were taken to approach the experimental results obtained in one-dimensional cell chains (Fig 6). Cytoplasmic conduction time was 38 microseconds; junctional conduction time, 122 microseconds. The junctional conduction delay was 84 microseconds (52% of overall conduction time). B, The lateral coupling resistance in the two-dimensional chain was taken as 10 times larger than the longitudinal resistance. Cytoplasmic conduction time was 68 microseconds; junctional conduction time, 86 microseconds. The junctional conduction delay was 18 microseconds (12% of overall conduction time).

Fig 9. Graph showing simulated dependence of junctional, cytoplasmic, and overall conduction times on lateral resistivity (R_i). Interrupted line denotes an anisotropic ratio (R_i/R_j, where R_j is average longitudinal resistivity) of 10, taken for the simulation of the results shown in Fig 8B. The solid line corresponds to R_i/R_j of 20. At this ratio, the experimental and simulated degree of inhomogeneity introduced by end-to-end cell connections (22% of overall conduction times) are equal. Above R_i/R_j of 60, the averaging effect of lateral connections is absent. Note that variation of lateral coupling resistance has almost no effect on overall propagation velocity.

Fig 10. Schematic diagram showing local current flow in a cell of a two-dimensional strand coupled to six neighboring cells. Gap junctions are symbolized by filled oval dots. The electrotonic currents flowing in the wave front of propagation (horizontal arrows) and between laterally apposed cells (vertical arrows) are depicted at the instants of local activation for a point distal to the end-to-end cell connection (open symbols) and a point proximal to the end-to-end connection (filled symbols). The direction of these currents is given by the computed potential distribution at the two instants of activation. At the distal point, lateral convergence of local current accelerates activation; at the proximal point, lateral divergence delays activation. Both divergence and convergence of local currents through lateral connections are responsible for the reduced inhomogeneity of propagation in two-dimensional strands.

patch pipette) by ~100 microseconds. Comparison with our results is difficult because the electrical recording technique was not specified in these experiments. One of the potential errors with electrical recording of action potentials relates to the use of patch pipettes in combination with patch-clamp amplifiers. In the current-clamp mode, such devices can produce delays of ~110 microseconds in the onset of rapid voltage recordings, as verified in preliminary experiments (not shown). Therefore, we used a standard microelectrode amplifier in combination with a tight-seal patch pipette for our electrical control measurements (Fig 4).

The spatial resolution of optical recordings in our experiments was 5.5 μm (smaller than cell width); the interdiode distance, 30 μm. The conduction measurements on a such small spatial scale require high tempo-
ral resolution for determination of activation time. In our experiments, it was limited by the experimental noise and depended mainly on the S/N_{\text{rms}} value. At S/N_{\text{rms}} of 63, typical for our measurements, the accuracy of a single measurement was ±20 microseconds. This accuracy limited the interpretation of differences of conduction times in a single measurement; however, the differences between the means of the collected data are highly significant.

**Patterned Strands of Cultured Neonatal Rat Heart Cells as a Model for Studying Propagation**

The main advantages of the technique of patterned growth consist of the possibility to restrict the spread of propagation to two dimensions, to vary the geometry of cell arrangement by adjusting the photoresist pattern, and to make the synthetic strands accessible to high-resolution optical mapping.

Since cultured strands are formed by cells that rearrange and reestablish gap junctional contacts after dissociation, their electrophysiological properties may not fully correspond to the original myocardial tissue. With respect to ionic currents responsible for action potentials, no major differences appear to exist. This was shown in a previous study and confirmed by the present recordings of action potential amplitude and upstroke rate of rise. Propagation velocity in one-dimensional and two-dimensional strands (34 to 36 cm/s) was lower than in whole adult hearts (50 to 60 cm/s\textsuperscript{5,32}). One of the reasons for this moderate reduction is likely to be the lower degree of electrical cell-to-cell coupling. The electrical resistance is \( \approx 30 \text{ M} \Omega \) in 2-day-old neonatal rat cell pairs\textsuperscript{34} and 2 \text{ M} \Omega in adult cell pairs.\textsuperscript{35} In the 3- to 11-day-old cultures taken for measurements, which show a high degree of differentiation,\textsuperscript{19} some intermediate value is likely to prevail. A close fit with the experimental data was obtained with a 9-M\Omega intercellular resistance in the computer model. Such a degree of electrical cell-to-cell coupling would still be lower than in adult cells by at least fourfold. Another possible reason for reduced conduction velocity is the larger surface-to-volume ratio of cultured myocytes as compared with adult cells in vivo. Adult cells can be roughly represented as cylinders with a diameter of 20 \( \mu \text{m} \textsuperscript{38} \) and a surface-to-volume ratio of 2000 cm\textsuperscript{-1}. This is approximately three times smaller than the value assumed for cultured neonatal myocytes, which adhere to the glass and are of flat shape.

**Inhomogeneity of Conduction in One- and Two-Dimensional Strands Caused by Cell-to-Cell Connections**

Conduction measurements in one-dimensional neonatal cell chains revealed that propagation on a microscopic scale is a discontinuous process with slowing at the intercellular junction. This is in accordance with previous simulation studies indicating that approximately half of the overall conduction time is due to delay at gap junctions, if the setting of the model parameters are taken to closely approach physiological values of adult tissue.\textsuperscript{10} At this degree of inhomogeneity, the shape of the upstroke of transmembrane action potentials exhibited no major discontinuities in both experimental measurements and simulations. The standard deviations of the optical conduction time measurements and the estimated errors were similar, suggesting that a large portion of the variability in cytoplasmic and cell-to-cell conduction times was due to random optical noise.

Computer simulations of voltage distribution in passive anisotropic resistive networks have shown that, after point application of current, the lateral connections smooth the discontinuities in longitudinal voltage distribution.\textsuperscript{32} Our experiments demonstrate that a similar process reduced the inhomogeneity during propagation in two-dimensional tissue. Consequently, there was an increase in cytoplasmic conduction time and a decrease in cell-to-cell conduction time, resulting in a reduction of the junctional delays. At a physiological degree of anisotropy\textsuperscript{28} (lateral coupling equal to 1/10 of longitudinal coupling), the simulated conduction delay of cell-to-cell transmission in two-dimensional strands was reduced to 12% of overall conduction time. According to the computer model, the experimentally obtained value (22%) would correspond to an anisotropic ratio of 20 (Fig 9). This quantitative comparison has to be made with caution, because the computer simulations depended on assumptions that could not be fully verified (e.g., cell thickness) and simplifications (geometric arrangement of cells). However, qualitatively, both procedures clearly demonstrate the averaging effect of lateral cell-to-cell connections. In three-dimensional compact ventricular tissue, nine cells were connected in mean to an individual myocyte (versus six cells in the two-dimensional model).\textsuperscript{36} This increase in electrotonic coupling is predicted to enhance the averaging effect of lateral connections and to reduce the degree of inhomogeneity even more.

The explanation of the averaging effect was provided by the computer model. In the case of lateral cell-to-cell apposition, membrane sites located before end-to-end cell contacts represent points where local electrotonic current diverges into the cells located laterally and ahead. At such sites, a relatively large amount of the ionic current generated by the cell membrane is furnished to diverging electrotonus; only the remainder current discharges membrane capacitance and changes membrane voltage. The opposite process takes place beyond longitudinal cell-to-cell connections where divergence of electrotonic current from laterally apposed cells accelerates the local change in membrane potential. Since on the lateral axis points of convergence and divergence alternate, the longitudinal wave front is smoothed.

**Possible Implication for Adult Myocardial Tissue**

A number of studies based on recordings of local extracellular electrograms reported discontinuities of conduction in cardiac tissues. In atrial tissue\textsuperscript{37} and in the Purkinje system,\textsuperscript{38} this is likely to indicate local macroscopic branching of trabecula and longitudinal dissociation in cell strands. In ventricular tissue, discontinuities in local electrograms during transverse propagation can reflect the presence of connective tissue sheets, separating whole strands of ventricular cells.\textsuperscript{39} The dense microvasculature of cardiac tissue, which separates cell bundles,\textsuperscript{40} might be a further source of microinhomogeneities in propagation. The question of whether borders of individual cells introduce significant inhomogeneities under physiological conditions in whole heart tissue has not been answered yet. The present experiments suggest
that inhomogeneities during longitudinal conduction will be at least partially canceled by current flow through lateral connections. This might explain why analysis of extracellular waves in small arterially perfused rabbit papillary muscles (diameter, 0.6 to 1 mm) has failed to show inhomogeneities when cellular excitability and cell-to-cell coupling are normal.41 Such muscle cylinders consist essentially of uniformly packed myocyte strands separated by a dense capillary network and interstitial clefts.40

With electrical uncoupling or reduction in membrane excitability in pathophysiological settings, inhomogeneities related to electrical cell-to-cell transmission might appear. In isolated cell pairs, marked delays in the cell-to-cell transfer of the electrical impulse were reported with decreased electrical cell-to-cell coupling and/or excitability.42 Similarly, discontinuities in extracellular and intracellular waveforms have been demonstrated in ischemic papillary muscle with reduced excitability during cell-to-cell uncoupling.43 In isolated Purkinje strands, local reduction of excitability was responsible for the generation of ectopic activity and arrhythmias, leading either to microreentry44 or reflection reentry.45 The techniques of patterned growth and high-resolution optical mapping offer a possibility for investigating these mechanisms at microscopic resolution.

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