Coupling of Cardiac Electrical Activity Over Extended Distances by Fibroblasts of Cardiac Origin

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Abstract—Roughly half of the cells of the heart consist of nonmyocardial cells, with fibroblasts representing the predominant cell type. It is well established that individual cardiomyocytes and fibroblasts in culture establish gap junctional communication at the single cell level (short-range interaction). However, it is not known whether such coupling permits activation of cardiac tissue over extended distances (long-range interaction). Long-range interactions may be responsible for electrical synchronization of donor and recipient tissue after heart transplantation and may play a role in arrhythmogenesis. This question was investigated using a novel heterocellular culture model with strands of cardiomyocytes interrupted by cardiac fibroblasts over defined distances. With use of optical recording techniques, it could be shown that impulse propagation along fibroblast inserts was successful over distances up to 300 μm and was characterized by length-dependent local propagation delays ranging from 11 to 68 ms (apparent local “conduction velocities” 4.6±1.8 mm/s, n=23). Involvement of mechanical stretch in this phenomenon was excluded by showing that inserts consisting of communication-deficient HeLa cells were incapable of supporting propagation. In contrast, HeLa cells expressing connexin43 permitted impulse conduction over distances as long as 600 μm. Immunocytochemistry showed that fibroblasts and cardiomyocytes expressed connexin43 and connexin45, whereas connexin40 was absent. These results illustrate that fibroblasts of cardiac origin are capable of synchronizing electrical activity of multicellular cardiac tissue over extended distances through electrotonic interactions. This synchronization is accompanied by extremely large local conduction delays, which might contribute to the generation of arrhythmias in fibrotic hearts. (Circ Res. 2003;93:421-428.)

Key Words: electrophysiology • cell culture • cardiac fibroblasts • fibrosis • gap junctions

Whereas cardiomyocytes are the dominant cell type in the normal heart in respect to volume, more than half of the cells consist of noncardiomyocytes, among which fibroblasts constitute a significant fraction. Although the main role of fibroblasts, consisting of synthesizing and maintaining the mechanical scaffold for cardiomyocytes, is undisputed, their involvement in electrophysiological processes within the working myocardium is less clear. It is well established that fibroblasts, by producing collagenous septa in the aged and hypertrophied heart, generate electrical barriers, which form the basis of discontinuous conduction and, hence, a substrate for arrhythmias. However, it is not known whether fibroblasts populating the collagenous septa might directly affect the electrophysiological properties of adjacent myocardium by forming electrical contacts with cardiomyocytes. Electrical coupling between cardiomyocytes and fibroblasts was demonstrated many decades ago in cell cultures and, more recently, in intact tissue. In cell cultures, it has been shown that single fibroblasts are capable of synchronizing contraction among individual cardiomyocytes and that these contractions are accompanied by synchronous membrane potential fluctuations in the interconnecting fibroblasts, suggesting the presence of electrical communication. More recently, it has been shown in a cell culture model consisting of fibroblasts grafted on top of monolayer cultures of cardiomyocytes that the former were able to modify the local electrophysiological properties of cardiomyocytes, suggesting the presence of electrical coupling between the two cell types. In intact tissue, evidence has been presented that mechanosensitive fibroblasts in the sinoatrial region are electrically coupled to atrial cardiomyocytes.

All of these studies concerned electrical short-range interactions between cardiomyocytes and adjacent individual fibroblasts. The possibility that multiple fibroblasts might interact electrically over extended distances with cardiomyocytes has not previously been addressed. Such long-range interactions may explain synchronization of electrical activity across scar tissue between donor and recipient cardiac tissue after heart transplantation and may be a cause of ensuing arrhythmias. More important, in the setting of cardiac fibrosis, bridges consisting of fibroblasts might establish focal electrical connections across collagenous septa, which

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may contribute to the generation of arrhythmias by forming slow pathways of excitation.

Long-range interactions between fibroblasts and cardiomyocytes were investigated using a novel cell culture system that permitted the generation of geometrically defined heterocellular preparations consisting of cardiomyocytes and noncardiomyocytes. In these preparations, it could be shown that fibroblasts of cardiac origin are capable of relaying electrical excitation over distances as long as 300 μm, thus opening the perspective that scar tissue and collagenous septa may affect conduction in a manner more complex than previously thought.

Materials and Methods

Cell Cultures

Cardiomyocytes

Primary cultures of neonatal rat ventricular cardiomyocytes were obtained using previously published procedures.10 Experiments were approved by the State Veterinary Department. In brief, hearts from 4 to 6 neonatal rats (Wistar, 1 to 2 days old) were excised, the ventricles were minced with scissors, and the resulting small tissue pieces were dissociated in Hanks’ balanced salt solution (HBSS, without Ca2+ and Mg2+, Bioconcept) containing trypsin (0.1%, Boehringer) and pancreatin (60 μg/mL, Sigma). The dispersed cells were centrifuged and resuspended in medium 199 (M199) with Hanks’ salts (Sigma). The medium was supplemented with penicillin (20 U/mL, Fakola), streptomycin (20 μg/mL, Fakola), vitamin B12 (20 μg/mL, Sigma), 100 μmol/L bromodeoxyuridine (Sigma), l-glutamine (100 μg/mL), and 10% neonatal calf serum (Boehringer). The cell suspension was preplated in a large culture flask and incubated for 2 hours at 35°C in a humidified atmosphere containing 1.2% CO2. This procedure served to enrich the cardiomyocyte fraction and provided the source for cardiac fibroblasts. The cardiomyocytes remaining in suspension were collected and seeded at a density of 1.4×106 cells/mm2 on patterned substrates (see below). Medium exchanges were performed on the first day after seeding and every other day thereafter with supplemented M199 containing a reduced concentration of calf serum (5%) and lacking bromodeoxyuridine.

Fibroblasts

Fibroblasts obtained during the preplating step described above were kept under conditions identical to those used for cardiomyocytes, with the exception that the M199 did not contain bromodeoxyuridine. After 1 week in culture, fibroblasts were harvested with trypsin17 and used for establishing heterocellular cultures.

HeLa Cells

Wild-type HeLa cells (HeLaC3T3) and connexin43 (Cx43)-transfected HeLa cells (HeLaC41) (both kindly provided by Prof R. Weingart, Department of Physiology, University of Bern) were kept in culture using standard procedures.10 They were harvested using trypsin and, after the establishment of heterocellular cultures, were incubated under conditions identical to those used for cardiomyocytes.

Preparation of Heterocellular Cultures With Defined Geometry

In a first step, cell culture substrates were patterned using a previously described method.10 In short, glass coverslips (22 mm in diameter, Haska) were spin-coated with agar, followed by coating with a photoresist (KTFK, Kodak). These substrates were exposed to the desired growth patterns (80-μm-wide, 10-mm-long strands) and were, after etching, coated with collagen type VI (type IV, human placenta, Sigma). Thereafter, the photoresist was lifted off, resulting in substrates consisting of agar-coated regions (no cell adhesion) and in bands of collagen, permitting cell adhesion (Figure 1A, a). These substrates were then subjected to the following procedures:

1. Spatially defined masking of collagen strands (Figure 1A, b): Commercially available clear adhesive tape (B/N 436135, Conrad Elektronik) was cut into strips of defined width (range ~50 to 800 μm). After complete drying of the substrates with nitrogen gas, three of these strips were firmly attached perpendicular to the collagen-coated lines at equal intervals (2.5 mm). Thereafter, the substrates were submersed in HBSS and placed in a custom-built UV illuminator for sterilization for at least 2 hours.

2. Cell seeding and washing: After sterilization, HBSS was withdrawn, and freshly dissociated cardiomyocytes were seeded (Figure 1A, c). After 20 to 24 hours, the preparations were washed twice with HBSS. This step revealed the growth pattern by
removing all unattached cells, i.e., the cells in the regions covered by agar and adhesive strips (Figure 1A, d).

3. Unmasking and seeding of fibroblasts or HeLa cells: As final step, the adhesive strips were removed mechanically using small forceps (Figure 1A, e), and fibroblasts or HeLa cells were seeded to a density of $1.4 \times 10^4$ cells/mm². In the case of fibroblasts, preparations were washed after 30 minutes with HBSS to minimize contamination by the few cardiomyocytes still present in the fibroblast suspension. In the case of HeLa cells, washing was performed after 24 hours because of the slow adhesion process of this cell type. After they were washed, the heterocellular preparations (Figure 1A, f) were kept in the incubator, and experiments were performed 2 to 3 days later.

**Time-Lapse Videomicroscopy**

To follow the structural and functional development of the preparations, they were mounted on the stage of an inverted microscope (TMS, Nikon), which was placed in an incubator. The microscope was equipped with a videocamera (AVC-D7CE, Sony), and a custom-built motorized x-y table served to move the microscope without opening the incubator. Immediately after the seeding of fibroblasts or HeLa cells, cell insert of interest were selected, and time-lapse videomicroscopy (AG-6720A recorder, Panasonic) was started. Intermittent real-time recording served to document synchronization of contractile activity across inserts.

**Optical Measurements of Impulse Propagation**

The characteristics of impulse propagation in heterocellular preparations were determined with a system for multiple-site optical recording of transmembrane voltage, described in detail elsewhere. In short, the preparations were mounted in a temperature-controlled chamber and transferred to the stage of a microscope equipped for epifluorescence (Axiovert 135 M, Zeiss). They were stained with the voltage-sensitive dye di-8-ANEPPS (135 μmol/L, Molecular Probes). After the staining procedure, the preparations were constantly superfused at 36°C with HBSS containing (mmol/L) NaCl 137, KCl 5.4, CaCl₂ 1.3, MgSO₄ 0.8, NaHCO₃ 4.2, KH₂PO₄ 0.5, and NaH₂PO₄ 0.3, and HEPES 10. The solution was supplemented with 1% calf serum and titrated to pH 7.4. Individual strands were stimulated with a bipolar electrode consisting of a glass micropipette filled with HBSS and a silver wire coiled around the shank. The electrode was placed ±1 mm from measurement site to permit propagation to reach steady state. The preparations were prestimulated for 10 seconds by a stimulator (SD9, Grass Instruments) at a basic cycle length of 500 ms before recording a single propagating action potential. Recordings were made at ×20 magnification (Fluar 20×, 0.75 numerical aperture, Zeiss), resulting in a spatial resolution of 50 μm. The amplitudes of optically recorded action potential upstrokes were scaled to 100% (%APA). Maximal upstroke velocities (dV/dtₘₚ) were calculated from %APA values and are given as %APA/ms. With an assumed action potential amplitude of 100 mV, %APA/ms values correspond to V/s. Activation profiles of the preparations were assessed from the time when depolarization reached 50% of the entire action potential amplitude.

**Immunocytochemistry**

After the measurements, the preparations were washed with PBS, followed by fixation with 2% paraformaldehyde for 5 minutes at room temperature. Thereafter, they were incubated at room temperature for 1 hour with blocking buffer (PBS containing 3% goat serum) before being exposed for 1 hour to the primary antibodies dissolved in PBS containing 1% goat serum and 0.15% Triton X-100. Finally, the preparations were washed and incubated for 2 hours at 37°C with secondary antibodies (Alexa Fluor 488, goat anti-mouse, Molecular Probes; Alexa Fluor 546, goat anti-rabbit, Molecular Probes; Cy3-conjugated goat anti-guinea pig, Chemicon). The preparations were imaged on an inverted microscope equipped for epifluorescence (Axiovert 35 M, Zeiss) using a slow-scan camera (Spot RT, Diagnostic Instruments). Primary antibodies used included anti-myomesin (mouse monoclonal, kindly provided by Prof Perriard, ETH Zürich), anti-connexin40 (anti-Cx40, S15C/R85, rabbit polyclonal) and anti-connexin45 (anti-Cx45, Q14E/GP42B, guinea pig polyclonal) (both kindly provided by Drs Severs and Coppen, Imperial College London), and anti-Cx43 (mouse monoclonal, Zymed). Anti-myomesin stainings as shown in Figure 1B permitted the exact determination of insert lengths and the exclusion of preparations containing aberrant cardiomyocytes within the insert.

**Results**

**Synchronization of Contractile Activity of Cardiomyocytes by Fibroblasts**

To investigate whether multiple fibroblasts in series are capable of synchronizing contractile activity of cardiac tissue, linear strands of cardiomyocytes interrupted over variable distances by fibroblasts of cardiac origin were constructed in cell culture. Fibroblast inserts between ~50 and 800 μm in length were studied. Before the seeding of fibroblasts, individual cardiomyocyte strands showed spontaneous contractile activity. Because the frequency of spontaneous contractions varied among the strands, functional linking of these strands by fibroblasts could be detected on the basis of the establishment of synchronization. The development of fibroblasts in the inserts and the establishment of synchronized contractions were followed by time-lapse videomicroscopy performed under stable incubating conditions. A typical example of such a recording is shown in the data supplement (available online at http://www.circresaha.org). It illustrates that fibroblasts spread rapidly after attaching to the substrate and formed contacts with the cardiomyocytes. Synchronization of contractile activity between linked cardiomyocyte strands occurred as soon as ~8 hours after seeding of the fibroblasts in short- to middle-sized inserts (~200 μm), whereas it took up to 24 hours until synchronized activity developed across longer inserts.

**Impulse Conduction Across Fibroblast Inserts**

To assess the mechanism underlying the synchronization of contraction, the characteristics of impulse propagation along preparations were assessed using multiple-site optical recording of transmembrane voltage. A typical example of such a measurement is shown in Figure 2. The length of the fibroblast insert as determined by anti-myomesin staining was 134 μm (Figure 2B). Impulses elicited at 2 Hz on the left propagated successfully across the insert at a coupling ratio of 1:1. As shown in Figure 2C, action potential upstrokes in the regions of the fibroblasts typically showed a biphasic shape, with the first rapid component being due to electrotonic current flow from the proximal cardiomyocyte strand and the second rapid component being evoked by electrotonic current flow from the distal strand, which was activated with a delay. Upstroke velocities recorded along the preparation (Figure 2D) showed a decline of dV/dtₘₚ in the region of the fibroblasts, consistent with passive charging of their membranes. In the distal cardiomyocyte strand, dV/dtₘₚ stayed depressed, suggesting partial inactivation of Na⁺ channels due to slow subthreshold charging, as evident from the prominent feet potentials in Figure 2C. As shown in Figure 2E, the fibroblast insert introduced a substantial local activation delay of 30 ms. This result illustrates that multiple
fibroblasts in series (about two or three in this example) are capable of bridging discontinuities in cardiac tissue and that, as suggested by the transmembrane voltage fluctuations in the region of the fibroblasts, electrotonic coupling might play an essential role in this process.

To characterize the dependence of activation delays on fibroblast insert lengths, the latter were varied between $50 \mu m$ and $800 \mu m$. As shown in Figure 3, increasing the insert length led to an increase in activation delays that became as long as $68 \text{ ms}$. With increasing length, the percentage of conducting fibroblast inserts gradually declined until, at lengths $>302 \mu m$, propagation invariably failed. Relating impulse propagation delays to insert lengths resulted in apparent local "conduction velocities" along fibroblasts ranging from $2.2$ to $10.5 \text{ mm/s}$ ($4.6 \pm 1.8 \text{ mm/s, mean} \pm \text{SD, } n=23$).

**Impulse Conduction and HeLa<sub>wt</sub> Cell Inserts**

Whereas the presence of bidirectional electrotonic interactions between cardiomyocytes and fibroblasts suggested an involvement of gap junctions in successful impulse conduction, it did not rule out the possibility that mechanical stretch relayed passively by fibroblasts may have elicited electrical activity in the distal cardiomyocyte strand by activation of stretch-sensitive channels. To investigate this possibility, we produced heterocellular constructs with communication-deficient HeLa<sub>wt</sub> cells instead of fibroblasts (Figure 4).

![Figure 2](image_url)

**Figure 2.** Characteristics of impulse propagation across a fibroblast insert. A, Phase-contrast image of the preparation consisting of an 80-µm-wide strand of cardiomyocytes with a central fibroblast insert. Circles indicate the position of individual photodetectors. B, Anti-myomesin staining shows that the detectors flanking the central two detectors (black and white circles) received a mixed input from both cardiomyocytes and fibroblasts and that the overall insert length was 134 µm. C, Propagating action potentials recorded along the preparation after stimulation on the left show biphasic upstrokes in the region of the fibroblasts that are typical of bidirectional electrotonic interaction with the proximal and distal cardiomyocyte strands. D, Upstroke velocities exhibit double peaks in the region of the fibroblasts, and $dV/dt_{max}$ is diminished in the distal compared with the proximal cardiomyocyte strand. E, Plot of activation times along the preparation indicates the presence of a delay in the region of the fibroblasts. Its size was calculated as the difference between linear fits of activation times along proximal and distal cardiomyocyte strands.

![Figure 3](image_url)

**Figure 3.** Activation delays as a function of insert length. Delays across fibroblast inserts were positively correlated with the length of the inserts. At insert lengths $>302 \mu m$, impulse propagation failed. The data point with a circle corresponds to the experiment shown in Figure 2.

![Figure 4](image_url)

**Figure 4.** Impulse propagation failure across HeLa<sub>wt</sub> cell inserts. A, Shown is a phase-contrast image of the preparation consisting of an 80-µm-wide strand of cardiomyocytes with a central insert of HeLa<sub>wt</sub> cells (length $\sim 100 \mu m$). B, Optical signals (raw, nonscaled) recorded along the preparation show, on the left of the insert, a rapid initial positive deflection (action potential upstroke) followed by large random excursions due to contraction (motion artifact). A motion artifact signal was also recorded from the HeLa<sub>wt</sub> cells (arrows), whereas the remainder of the preparation showed no signals. C, Action potential upstrokes indicate the presence of conduction block at the left cardiomyocyte-HeLa<sub>wt</sub> cell border. Corresponding $dV/dt_{max}$ values are given below the traces. D, Propagation is characterized by a very high local conduction velocity in front of the insert. This is typical of a sealed-end situation as represented by the border between proximal cardiomyocyte strand and communication-deficient HeLa<sub>wt</sub> cells.
Similar to fibroblasts, HeLa\(_{wt}\) cells rapidly filled the inserts and established physical contacts with the cardiomyocytes. Those HeLa\(_{wt}\) cells in close vicinity to the proximal cardiomyocyte strand began to move with each contraction. Indications of passive movement of HeLa\(_{wt}\) cells for a 100-μm-wide insert are illustrated in Figure 4B. This panel shows optical compound signals consisting of action potentials and motion artifacts. Fast initial positive deflections (action potential upstrokes, shown in expanded form in Figure 4C) could be recorded only from the proximal cardiomyocyte strand, but subsequent excursions of random amplitude and polarity, which were due to motion (and, in the case of cardiomyocytes, were overlaid on the repolarization signal),\(^{11}\) were also manifest in the region of the HeLa\(_{wt}\) cells. Despite this movement, no synchronized activity could be recorded from the distal cardiomyocyte strand, indicating the presence of conduction block. As expected for an insert consisting of communication-deficient cells, propagation along the proximal cardiomyocyte strand showed the electrophysiological signature of a sealed end, ie, a local increase in \(\frac{dV}{dt_{\text{max}}}\) (Figure 4C) and a steep increase of conduction velocities to more than twice the values of steady-state conduction (1 versus \(0.4\) m/s, Figure 4D).

In a total of 17 preparations with HeLa\(_{wt}\) cell insert lengths ranging from 48 to 274 μm (168±72 μm, mean±SD), conduction invariably failed at the proximal border between cardiomyocytes and HeLa\(_{wt}\) cell inserts. Therefore, even though HeLa\(_{wt}\) cells passively relayed motion, this motion was not sufficient to elicit action potentials in the distal cardiomyocyte strand, thus excluding mechanical coupling as a mechanism of impulse conduction across nonexcitable cells.

**Impulse Conduction Along HeLa\(_{Cx43}\) Cells**

To confirm that indeed connexin deficiency and not the cell type as such was the cause of failure of conduction across the HeLa\(_{wt}\) cell inserts, an additional series of experiments was performed in which the inserts were formed by HeLa\(_{Cx43}\) cells. In the example shown in Figure 5, the HeLa\(_{Cx43}\) cell inserts span a distance of 480 μm. Similar to fibroblasts, HeLa\(_{Cx43}\) cell inserts led to a functional coupling of the cardiomyocyte strands. Transmembrane voltage changes recorded from HeLa\(_{Cx43}\) cells showed biphasic positive deflections, indicating bidirectional electrotonic interaction with the cardiomyocyte strands (Figure 5C). The plot of activation times along the preparation shown in Figure 5D reveals a local activation delay of 41 ms. The “overshoot” of activation times in the distal part of the HeLa\(_{Cx43}\) cell insert is the result of the slow passive charging of these cells after activation of the distal cardiomyocyte strand. Interestingly, activation of the distal cardiomyocyte strand close to the insert occurred in a virtually simultaneous manner. This behavior was routinely observed in the case of long activation delays and can be explained by the slow subthreshold charging of the cardiomyocytes in the distal strand, which caused cardiomyocytes to reach threshold almost simultaneously.\(^{14}\) Slow subthreshold charging is evident not only from the pronounced feet of action potentials recorded in the distal cardiomyocyte strand close to the insert (Figure 5C) but also from the slow action potential upstrokes at the same locations (Figure 5E), which are likely due to partial inactivation of Na\(^+\) channels.

As shown in Figure 6, HeLa\(_{Cx43}\) cell inserts successfully
relayed impulses between strands of cardiomyocytes over distances as long as 600 μm. Activation delays introduced by HeLa cells varied with increasing insert lengths from 1 to 55 ms, and were, at any given insert length, considerably shorter than those seen in fibroblast bridge preparations. Accordingly, apparent local “conduction velocities” were faster and ranged from 8.8 to 89 mm/s (21.4±15.2 mm/s, mean±SD, n=47).

Connexin Isoforms Involved in Impulse Propagation Across Fibroblast Inserts

To identify the isoforms of connexins responsible for impulse conduction across cardiac fibroblasts, mixed monolayer cultures of cardiomyocytes and fibroblasts of cardiac origin were subjected to immunocytochemistry for Cx40, Cx43, and Cx45. As shown in Figure 7A, distinct labeling for Cx43 was found both at fibroblast-fibroblast cell abutments and at the contact sites between cardiomyocytes and fibroblasts. The labeling was mostly of the punctate type as opposed to the plaquelike staining normally observed at cardiomyocyte-cardiomyocyte junctions. A similar staining pattern was found for Cx45 (Figure 7B). Staining for Cx40 was negative in all instances. Figure 7C illustrates that contact sites between HeLa cells and cardiomyocytes displayed a plaquelike Cx43 signal, which fits the observation that HeLa cells were functionally better coupled than fibroblasts. These results indicate that gap junctions involved in electrotonic interactions between fibroblasts and cardiomyocytes were composed of both Cx43 and Cx45 but not of Cx40.

Discussion

Impulse Propagation Along Multiple Fibroblasts

The major finding of the present study was that electrical interactions between cardiomyocytes and fibroblasts can occur over distances much longer than the short-range interactions previously described. Furthermore, the present study showed that propagation supported by fibroblasts is characterized by long local conduction delays. Optical mapping of impulse propagation along geometrically defined heterocellular constructs paced at 2 Hz showed that fibroblast inserts from 54 to 302 μm in length were capable of relaying electrical activity between strands of cardiomyocytes. This process was characterized by local activation delays in the regions of the fibroblasts, which increased from 11 ms up to 68 ms with increasing insert length. These large delays translate into apparent “conduction velocities” in the regions of the fibroblasts ranging from 2.2 to 10.5 mm/s. Interestingly, similarly slow conduction velocities in cardiac tissue were previously observed in highly nonuniform anisotropic tissue from aged human subjects exhibiting extensive microfibrosis. In that study, large propagation delays during transverse conduction supported the establishment of reen-
transient excitation on a very small scale. Whereas slow transverse propagation was suggested to be based on the zigzag nature of the spread of excitation, the present study opens the possibility that sporadic lateral coupling of otherwise electrically isolated bundles of myofibers by fibroblasts might additionally contribute to very slow conduction. Moreover, the observed extremely long activation delays could set the stage for reflection of activation and, hence, initiate reflected reentry. Finally, the present finding of substantial conduction delays across fibroblast inserts might also contribute to the explanation of the large step delays (~40 ms) observed previously at the input of the atrioventricular node.

Electrical Coupling, Electromechanical Feedback, or Active Response?

In the case of transplanted hearts, it has been speculated that electrical synchronization between donor and recipient tissue might be based on either electrical coupling or electromechanical feedback. Because both of these mechanisms might underlie impulse propagation in our heterocellular constructs, fibroblasts were replaced by communication-deficient HeLa wt cells, which were (similar to fibroblasts) stretched passively by adjacent cardiomyocytes during contraction. The finding that HeLa wt cells invariably blocked conduction even at very small insert lengths suggests that mechanical stretch relayed passively along the cells in the insert was not responsible for activating distal cardiomyocytes by electromechanical feedback mechanisms. This failure of relaying activation was not caused by the specific cell type (ie, HeLa wt cells) per se because using HeLa Cx43 cells permitted the synchronization of cardiomyocyte strands over distances up to 600 μm. This confirms that electronic current flow, as visualized by membrane potential fluctuations synchronous to activation of the cardiomyocyte strands, was the main mechanism underlying successful impulse transmission along fibroblasts.

Because it can be expected that fibroblasts close to the cardiomyocytes underwent membrane potential excursions comparable in magnitude to those of the cardiomyocytes, the question arises whether activation of Ca²⁺ inward currents might have supported active propagation along fibroblasts, as reported previously for monolayer cultures of normal rat kidney fibroblasts. In the present experiments, an active involvement of fibroblasts in impulse propagation was unlikely because (1) the initial fast depolarizing signal shown by the fibroblasts after activation of the cardiomyocytes was decremental and, hence, not active, and (2) measurements with the Ca²⁺-indicator fluo 3 showed no signals in the region of the fibroblasts (data not shown).

Which Type of Connexin Is Involved?

Probing the types of connexins underlying successful impulse propagation across fibroblast inserts with immunocytochemistry showed that both Cx43 and Cx45 were expressed, whereas Cx40 could not be detected. Stainings for Cx43 and Cx45 between fibroblasts and between cardiomyocytes and fibroblasts showed a fine punctate pattern, whereas stainings for both Cx43 and Cx45 between cardiomyocytes were more prominent and plaquelike. Plaquelike staining patterns indicating a high density of connexins were also found at cardiomyocyte-HeLa Cx43 cell interfaces. This might explain the finding that propagation delays at given insert lengths were substantially smaller in HeLa Cx43 cells and that they were able to support propagation over distances twice as long as was possible with fibroblasts.

Phenotype of Fibroblasts

Previous studies of contacts between fibroblasts and cardiomyocytes in intact and healthy hearts have shown an absence of robust gap junctional coupling. This finding is in clear contrast to the data of the present study and previous investigations of short-range electrical interactions between individual cardiomyocytes and fibroblasts in culture. This raises the question of whether fibroblasts in culture assume a phenotype different from that in intact tissue. In vivo, fibroblasts are known to retain pluripotentiality, as exemplified by their conversion into myofibroblasts after a local loss of cardiomyocytes. Myofibroblasts express α-smooth muscle actin and, on the basis of their capability to form cell-cell and cell-stroma contacts, are responsible for scar retraction (reparative fibrosis). They have been found as early as 4 to 6 days after infarction in human hearts and seem to persist many years thereafter. According to a recent report, such a transformation of cardiac fibroblasts into myofibroblasts occurs regularly during primary culture because of hyperoxia. Whereas gap junctional coupling of myofibroblasts among themselves and to cardiomyocytes has not been studied yet, intestinal myofibroblasts were shown to be linked by gap junctions. Moreover, myofibroblasts of breast cancer stroma and myofibroblasts derived from corneal fibroblasts were found to express Cx43. Thus, the observation of absence of gap junctional coupling between fibroblasts and cardiomyocytes in healthy intact myocardium might possibly be reconciled with the findings of electrical coupling in primary cell culture by assuming that fibroblasts are converted into myofibroblasts and establish gap junctional communication only thereafter. Such a de novo coupling of nonexcitable cells to cardiomyocytes under pathological conditions, such as myocardial infarction, might constitute an important new arrhythmogenic mechanism, and the signaling pathways responsible for this conversion may become targets for new antiarrhythmic drugs.

Limitations of the Study

The main limitations of the present study in terms of extrapolating the results to intact tissue concern (1) the use of a cell culture model and (2) the question of whether the linking of multiple fibroblasts might occur in vivo. Whereas cardiomyocyte strands display an electrophysiological phenotype that is close to that of intact tissue in terms of action potential configuration and conduction velocities, little is known about how fibroblasts of cardiac origin in culture compare with those in intact tissue. Also, there is no information available regarding the microscopic spatial distribution of fibroblasts or myofibroblasts in the heart. Answers to these question will require a detailed analysis of gap junctional coupling and of the 3D organization of fibroblasts and myofibroblasts in intact tissue.
In conclusion, the finding that fibroblasts of cardiac origin can interact electrotonically over extended distances with cardiomyocytes opens the perspective that these cells might influence the network of electrically coupled cardiomyocytes in intact tissue not only by forming insulating collagenous septa but also by modulating active and passive electrical properties of the network directly by gap junctional communication. Such a modulation might become especially important under conditions of fibrosis, in which, in addition to zigzag conduction, fibroblast bridges might form slow conducting pathways, thus contributing to the generation of arrhythmias.

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References
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