Cell-to-cell coupling in engineered pairs of rat ventricular cardiomyocytes: relation between Cx43 immunofluorescence and intercellular electrical conductance

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Connexins (Cx) are proteins that assure cell-to-cell transfer of ions and small regulatory molecules and are important determinants of cardiac electrical function. Cardiac tissue expresses three major Cx, Cx43, Cx40, and Cx45, which show specific distributions in various cardiac regions (9, 19, 32). In ventricular myocardium, Cx43 is the dominant Cx in ventricular myocardium, and Cx45 is present in trace amounts. Cx43 immunosignal has been associated with cell-to-cell coupling and electrical propagation, but no studies have directly correlated Cx43 immunosignal to electrical cell-to-cell conductance, $g_{ij}$, in ventricular cardiomyocyte pairs. To assess the correlation between Cx43 immunosignal and $g_{ij}$, we developed a method to determine both parameters from the same cell pair. Neonatal rat ventricular cardiomyocytes were seeded on micropatterned islands of fibronectin. This allowed formation of cell pairs with reproducible shapes and facilitated tracking of cell pair locations. Moreover, cell spreading was limited by the fibronectin pattern, which allowed us to increase cell height by reducing the surface area of the pattern. Whole cell dual voltage clamp was used to record $g_{ij}$ of cell pairs after 3–5 days in culture. Fixation of cell pairs before removal of patch electrodes enabled preservation of cell morphology and offline identification of patched pairs. Subsequently, pairs were immunostained, and the volume of junctional Cx43 was quantified using confocal microscopy, image deconvolution, and three-dimensional reconstruction. Our results show a linear correlation between $g_{ij}$ and Cx43 immunosignal within a range of 8–50 nS.

Electrical cell-to-cell coupling; cell engineering; microcontact printing; dual voltage clamp.

Connexins (Cx) are proteins that assure cell-to-cell transfer of ions and small regulatory molecules and are important determinants of cardiac electrical function. Cardiac tissue expresses three major Cx, Cx43, Cx40, and Cx45, which show specific distributions in various cardiac regions (9, 19, 32). In ventricular myocardium, Cx43 is the dominant Cx, and Cx45 is present in small amounts. In certain species, Cx45 modifies average gap junction channel conductance (3). Cx43 and Cx45 are remodeled in pathophysiological states; in the case of cardiac failure, downregulation of Cx43 and upregulation of Cx45 are observed (1, 12, 35).

The amount of Cx expressed by cardiomyocytes is known to play an important role in cardiac electrical function. Measurement of Cx by Western blot provides information about total cellular Cx, but may not necessarily be related to the Cx contributing to gap junction channels (4). Comparison of Cx43 immunosignals in gap junctions with gap junction plaque dimensions assessed by electron microscopy suggests that Cx43 immunosignal is a reliable measure of the gap junction area in which Cx43 is distributed (24). Relating altered levels of Cx43 in gap junctions, induced either by remodeling or genetic ablation, to electrical propagation suggests that there is a quantitative relationship between the Cx43 immunosignal measured over an area comprising >100 cells and propagation velocity (4, 8, 27, 28). However, experimental and theoretical work indicate that propagation velocity is not linearly related to cell-to-cell coupling and is relatively insensitive to moderate changes of normal cell-to-cell coupling (20).

The goal of the present study was to directly compare the Cx43 immunosignal in a pair of cultured ventricular cardiomyocytes to the electrical properties of its cell-to-cell junction. To this aim, we developed a method for generating cell pairs of controlled dimensions using microcontact printing. This also enabled us to consistently generate cell pairs with specific dimensions, allowing us to understand how cell shape can modify gap junction size. We used a method for preserving and immunostaining cell pairs after patch clamp by applying fixative while the patch clamp pipettes were still attached to the cell membranes. This allowed us to obtain electrical measurements and Cx43 immunostaining data from the same cell pair. Our results suggest a direct correlation between Cx43 immunosignal and electrical intercellular conductance, $g_{ij}$, within a range of $g_{ij}$ values from 8 to 50 nS.

Material and Methods

Photolithography, micropatterning, and cell culture. A photolithographic mask was designed in AutoCAD (Autodesk, San Rafael, CA) to produce cell pairs with control of cell shape. The mask design consisted of arrays of two rectangles (dimensions: $40 \times 12 \mu\text{m}^2$, $60 \times 12 \mu\text{m}^2$, or $80 \times 12 \mu\text{m}^2$) paired along their short axis with a $2\mu\text{m}$ separation (Fig. 1, A and B). A given group of 8–12 pairs was outlined with a 100-µm-thick rectangular cell adhesion region serving as a conditional cell layer and labeled with a number and letter (Fig. 1A). This label facilitated offline identification of cell pairs.

Standard soft lithography techniques were used to create polydimethylsiloxane (PDMS) (Dow Corning, Midland, MI) stamps for microcontact printing, as previously reported (5, 15). Briefly, silicon wafers (Wafer World, West Palm Beach, FL) were spin-coated with SU-8 2002 photoresist (MicroChem, Newton, MA) to form a 2-µm layer on the surface. The wafer was aligned under the photolithographic mask described above using a mask aligner (ABM, Scotts Valley, CA) and then exposed to UV light and submersed in propylene glycol methyl ether acetate to dissolve the regions not blocked by SU-8.
the mask. PDMS was poured over the wafer and allowed to polymerize, at which point it was peeled off the wafer and cut into smaller sizes to be used as stamps. PDMS stamps (26) were coated with 25 μg/ml fibronectin (Sigma-Aldrich, St. Louis, MO), and glass coverslips spin-coated with PDMS were treated in a UV-ozone cleaner (Jelight, Irvine, CA) before stamping. The fibronectin-coated stamps were placed on the coverslips to transfer fibronectin in the desired arrangement showing Cx43 immunofluorescence (red), nuclei [4,6-diamidino-2-phenylindole (DAPI), blue], and actin (phalloidin, green). D: cell pair in a nonstaggered arrangement showing connexin (Cx) 45 fluorescence (red).

Fig. 1. Fabrication of engineered cell pairs. A: the photolithographic mask consists of an array of blocks, with each block containing patterns for 8–12 cell pairs. Blocks are labeled with letters and numbers to serve for offline identification of cell pairs. B: each block contains cells growing on a rectangular frame. Within each frame, 8–12 cell pairs are patterned with a staggered or nonstaggered interface. C: cell pair in a nonstaggered arrangement showing Cx43 immunofluorescence (red), nuclei [4,6-diamidino-2-phenylindole (DAPI), blue], and actin (phalloidin, green). D: cell pair in a nonstaggered arrangement showing connexin (Cx) 45 fluorescence (red).

For further details on the experimental procedures and results, please refer to the original research paper. The figures and diagrams are included to illustrate the methods and findings discussed in the text. The data and images are presented to support the scientific claims made in the research.
Fig. 2. A–C: three-dimensional (3D) morphology of cell pairs. Left: maximum z-projections of wheat germ agglutinin (WGA) stain (green), Cx43 (red), and DAPI (blue). Right: rotated view of 3D reconstruction through the center of the cell-to-cell junction. A: cell pair with a length-to-width ratio (PLW) of 3.5. The height of the junction is 5.9 μm. B: cell pair with a PLW of 5.2. The height of the junction is 4.5 μm. C: cell pair with a PLW of 7.1. The height of the junction is 3.6 μm. D: junction length increases with increasing PLW. E: junction height decreases with increasing PLW. F: Cx43 immunosignal increases with increasing PLW from 3.5 to 5.2. G: junctional conductance ($g_j$) increases with increasing PLW from 3.5 to 5.2. Values are means ± SD. *P < 0.05, ANOVA.
stacks were collected with 300-nm separation between slices (z-stacks). Z-stacks were median filtered and deconvolved using blind deconvolution in Huygens Essential (Scientific Volume Imaging, Hilversum, The Netherlands) and imported into Imaris (Bitplane, Zuerich, Switzerland). For each z-stack, the same threshold was chosen to separate positive Cx43 staining from background staining. The cell-to-cell junction was determined by referencing the bright-field images. The volume of junctional Cx43 was determined by creating isosurfaces in Imaris from the thresholded z-stack of the Cx43 immunosignal and outputting the volume of the isosurfaces in micrometers cubed. The Cx43 isosurfaces not located in the cell-to-cell junction were not used for the correlation between the electrophysiological measurements and the immunosignal.

To determine parameters characterizing cell shape, cell pairs were fixed and incubated with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Invitrogen) for 10 min at room temperature. The actual width and length of each cell pair was measured and averaged. The ratio of the length to the width was defined as “cell pair length-to-width ratio” (PLW). To determine the junction height, WGA z-stacks were imaged using the same protocol mentioned above for Cx43, reconstructed in Imaris (NIH, Bethesda, MD) and resliced in the y-plane to facilitate viewing of the junction from the side. Manual measurements of the height of the junction were taken midway through the junction. The length of the cell-to-cell junction was manually measured in ImageJ using maximum projections of the WGA stain.

Statistics. All data are reported as mean values ± SD. Data were statistically analyzed using ANOVA for comparison between groups, with $P < 0.05$ considered significant (MATLAB, MathWorks, Natick, MA). Regression lines with $R^2$ values were determined using the curve-fitting tool of MATLAB.

RESULTS

Interface of cell pairs and Cx43 immunosignal. After 3–5 days in culture, pairs of cells were most commonly observed on a single island or grown across the engineered border (Fig. 2). Pairs spanning two islands with the cell border anchored at the engineered gap were rare (10%) and, therefore, not used. The pairs were subdivided into three groups, with PLW of 3.5 ± 0.2 (n = 9, Fig. 2A), 5.2 ± 0.2 (n = 12, Fig. 2B), and 7.1 ± 0.4 (n = 10, Fig. 2C). As illustrated in Fig. 2, the length of the cell-to-cell junction increased with increasing PLW. Cell pairs with PLW of 3.5, 5.2, and 7.1 had junction lengths of 24.9 ± 2.7 μm (n = 9), 35.9 ± 6.4 μm (n = 11), and 48.9 ± 6.2 μm (n = 10), respectively (Fig. 2D). Conversely, the height of the junction was inversely related to PLW, with junction heights of 5.7 ± 1.2 μm (n = 9), 4.5 ± 1.1 μm (n = 10), and 4.0 ± 0.6 μm (n = 11) for PLW of 3.5, 5.2, and 7.1, respectively (Fig. 2E). Cx43 immunosignal volumes in the PLW of 3.5, 5.2, and 7.1 groups were 4.4 ± 3.1 μm$^3$ (n = 6), 15.2 ± 9.5 μm$^3$ (n = 7), and 12.7 ± 7.1 μm$^3$ (n = 6), respectively (Fig. 2F). Cell pairs with PLW of 3.5, 5.2, and 7.1 had an average $g_j$ of 24.7 ± 15.9 nS (n = 8), 66.6 ± 37.6 nS (n = 11), and 39.2 ± 22.7 nS (n = 7), respectively (Fig. 2G). Both the decreases of Cx43 immunofluorescence and $g_j$ between PLW 3.5 and 5.2 were statistically significant ($P < 0.05$ and $P < 0.01$, respectively). Increasing PLW beyond 5.2 did not further increase Cx43 immunofluorescence or $g_j$ (Fig. 2, F and G).

Electrical cell-to-cell coupling. To assess the functional consequences of the mixed Cx43/Cx45 composition of rat ventricular gap junctions, we analyzed single gap junction channel currents, as shown in Fig. 3. Figure 3A illustrates four individual recordings of openings of single gap junction channels. To assess the change in $g_j$ upon the change in $V_j$, recordings were selected that showed only one single-channel opening. In the depicted traces, the difference between $g_j$ of the last opening before the change in polarity of $V_j$, and $g_j$ of the first opening after the change in $V_j$, ranged from 1 to 15 pS (17). For the nine recordings in which one channel opening was observed during the clamp at both polarities of $V_j$, the difference in $g_j$ upon the change in polarity amounted to 12.9 ± 2.6 pS. This was significantly different from the level of noise in each trace ($P < 0.05$), which amounted to 1.1 ± 0.1 pS. As shown in Fig. 3B, the distribution of single-channel conductances yielded a close fit to a Gaussian with a peak at 45 pS ($\pm 0.8$ pS; $R^2 = 0.96; n = 745$). This frequency distribution of $g_j$ values was closely similar to the distribution in wild-type murine ventricular myocyte pairs (3). The single-channel recordings favor the hypothesis that rat ventricular gap junction channels are composed of mixed heteromeric Cx43/Cx45 connexons (see DISCUSSION).

Correlation between $g_j$ and Cx43 immunosignal. The major goal of this study was to correlate the junctional Cx43 immunosignal to the electrical properties of its cell-to-cell junction. To do this, we patched cell pairs and then fixed and immuno-
stained against Cx43 to directly correlate the conductance $g_j$ with the presence of Cx43 at the cell-cell junction, as illustrated schematically in Fig. 4A. Figure 4B illustrates a cell pair used to correlate $g_j$ to the Cx43 immunosignal and shows the suction marks of the patch-clamp pipettes in a confocal slice near the top of the z-stack. This suction mark, together with the label of the patterned square (Fig. 1), allowed for the direct correlation between the Cx43 immunosignal and $g_j$ in each individual pair. This relationship is illustrated for four cell pairs in Fig. 4, C and D, E and F, G and H, and I and J, ordered according to increasing value of $g_j$. The plaque shape of the junctions in the Y/Z projection is demonstrated in the 3D animation showing Cx43 immunofluorescence in the pair shown in Fig. 4, I and J (Supplemental Movie S1; the online version of this article contains supplemental data). Of note, the pair with the largest PLW ratio (Fig. 4, G and H) had a smaller $g_j$ and Cx43 immunofluorescence signal than the pair with the smaller PLW ratio shown in Fig. 4, I and J. This was due to the formation of a cleft in the center of the interface between the pair with the largest PLW (Fig. 4G).

The summarized data of the correlation between the Cx43 immunosignal and $g_j$ is presented in Fig. 5, in which each point represents a cell pair ($n = 15$). The total Cx43 signal showed a positive correlation to $g_j$, with exception of one outlier value (79.8 nS vs. 10.7 $\mu$m$^3$), which deviated from this correlation. Between 0 and 25 $\mu$m$^3$ Cx43 immunosignal, the dependence of $g_j$ was highly linear ($R^2 = 0.83$). However, the two cell pairs with Cx43 immunosignals > 25 $\mu$m$^3$ showed a very high value of $g_j$ (exponential fit: $R^2 = 0.70$). This deviation from linearity may be related to the very large interference of the electrode access resistances at this level of coupling (see DISCUSSION) (31, 34). Interestingly, the correlation line does not pass through the origin. The observation that the “zero intersect” of the correlation corresponds to an average $g_j$ level of ~10 nS (corresponding to ~100 channels in the open state) suggests a lower limit for immunofluorescence detection of Cx43 in gap junctions in our experiments (see DISCUSSION).

**DISCUSSION**

As a main finding of this study, our experiments show a linear correlation between the $g_j$ of patterned cell pairs and the Cx43 immunosignal within a range of electrical conductances of 8–50 nS in rat ventricular cardiomyocytes. Moreover, the measurements of single-channel conductances suggest the presence of mixed Cx43/Cx45 channels in the engineered neonatal ventricular rat myocytes.

Soft lithography and microcontact printing techniques were used to control the dimensions of cell pairs used for DVC experiments. Because isolated cardiomyocytes generally conserve their volume (15), we were able to increase the height of the cells by decreasing the surface area of the pattern. The ability to modulate cell height was especially advantageous for DVC experiments because increased cell height reduces the amount of strain on the cell membrane during seal formation with the pipette, thereby minimizing damage to the cell. Micropatterning also increased the probability of cell pair formation, compared with plating cells seeded at random onto an
7.1 did not show further increases of either fluorescence showed a significant increase with increasing PLW. Between 0- and 25-μm3 Cx43 immunosignal, the dependence of one outlier value, there is direct relationship between the two parameters. With exception of one outlier value, there is direct relationship between the two parameters. Between 0- and 25-μm3 Cx43 immunosignal, the dependence of gj is highly linear (R² = 0.83). However, the two cell pairs with Cx43 immunosignals > 25 μm³ showed a very high value of gj (exponential fit to the whole data set showed an R² = 0.70; see DISCUSSION).

A goal of this study was to correlate the immunofluorescence signals of junctional Cx43 to the intercellular electrical conductance gj. It has been shown previously that reduction of Cx43 expression is associated with a decrease in average cumulated gap junction length and number of gap junctions, whereas the average size and size distribution of gap junctions remain the same (24). Our finding of a linear relationship between the Cx43 immunosignal and gj within a given range of junctional conductances from ~8 to 50 nS supports the notion that the Cx43 immunosignal is a measure of integrated gap junction size or area at the cell interface rather than gap junction protein. Moreover, the present work suggests that there is a lower threshold for immunodetection of gap junction size, below which significant electrical coupling can still be detected. In our work, the electrical conductance corresponding to this threshold amounted to ~8 nS (intersect of linear and exponential fits with ordinate in Fig. 5B). Theoretical computations of the relationship between electrical propagation velocity and cell-to-cell resistance have shown that, independent of the model (continuous vs. discontinuous), propagation velocity in linear structures is relatively insensitive to changes in the level of cell-to-cell coupling (25). These arguments altogether suggest that electrical ventricular propagation, albeit at a significantly lower level, can be preserved in the absence of detectable immunosignal for Cx43. The suggestion that very low levels of Cx43 are not detectable by immunofluorescence is also supported from the comparison of gj measurements in cultured rat (our study) and murine cell pairs (3) with genetic ablation of Cx43. Full Cx43 knock out in murine cell pairs produced a decrease of average gj to 1.4 nS (3), i.e., to a level amounting to ~15% of the gj level at the Cx43 immunodetection threshold. The observation of an immunodetection threshold of cell-to-cell coupling may, at least partially, explain several previously obtained observations in experimental and pathological settings. In a mouse model of conditional knock-out of Cx43, which increased with age, ventricular arrhythmias and inducible ventricular tachycardias started to occur only at markedly reduced levels of Cx43 immunosignals in gap junctions (7). In arrhythmogenic right ventricular cardiomyopathy in humans, a condition most frequently associated with desmosomal mutations, Cx43 in gap junctions is downregulated and shows a faint immunosignal. Although arrhythmogenic right ventricular cardiomyopathy patients frequently die from ventricular tachycardia, their hemodynamic function before the arrhythmic event is not markedly impaired, which implicates significant cell-to-cell communication in the working myocardium, despite postmortem evidence of markedly reduced Cx43 in right and left ventricular myocardium (2).

The determination of gj at high levels of cell-to-cell coupling is hampered by the fact that the sum of the access resistances of the patch electrodes largely determines the flow of current in the measuring circuit. A small change of these values during the experiment may, therefore, introduce an error in the measurements of gj (31, 34). The correlation between Cx43 immunofluorescence signals and gj at high levels of coupling has, therefore, to be interpreted with caution.

Immunofluorescence signals for Cx45 have been shown to be present in small amounts in rat, mouse, and human ventricles (3, 18, 33, 35). The low level of Cx45 in ventricular myocardium was confirmed in our study, and interference from background fluorescence precluded a quantitative 3D analysis of this immunosignal. Despite its small quantitative contribution, Cx45 may play a modulatory role in ventricular cell-to-cell coupling. Cx45 has been implicated in electrical remodeling in left ventricular hypertrophy and may play a role in limiting gap junction size (16, 35). Moreover, knock out of the coxsackie-adenovirus receptor protein both specifically decreases Cx45 expression in gap junctions and concomitantly increases intercellular dye diffusion between ventricular myocytes (21). In our study, two observations indicate a functional role for Cx45 in rat ventricle. First, the frequency distribution of γj showed a peak at 45 pS for the main channel states. These data, which are almost superimposable to the findings in wildtype murine ventricular cells pairs (3), indicate that Cx45 may decrease average γj, compared with a population of pure,

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Fig. 5. Relationship between Cx43 fluorescence and gj in individual cell pairs. Each point on the graph illustrates the relationship between gj and the Cx43 immunosignal in an individual pair for a total of 15 cell pairs. With exception of one outlier value, there is direct relationship between the two parameters. Between 0- and 25-μm³ Cx43 immunosignal, the dependence of gj is highly linear (R² = 0.83). However, the two cell pairs with Cx43 immunosignals > 25 μm³ showed a very high value of gj (exponential fit to the whole data set showed an R² = 0.70; see DISCUSSION).
homomeric-homotypic Cx43 channels (23, 30). Second, we observed a significant change of gap junction channel conductance upon a switch in the polarity of $V_{j}$. This suggests the presence of channels composed of different heteromeric Cx43/Cx45 mixtures. Since the presence of consecutive single-channel openings, as shown in Fig. 3A, during a voltage clamp does not assert that the openings can be attributed to the same channel, these measurements are not fully conclusive. However, the immunofluorescence data in this and earlier studies, the single-channel measurements, and the earlier observations of heteromeric Cx43/Cx45 formation in heterologous expression systems make it likely that Cx43 and Cx45 form heteromeric connexons and mixed gap junction channels in rat ventricle (6, 11).

A potential limitation of our study may be due to the fact that we assessed cell junctions that have been reformed from association of dissociated cells. However, none of the multiple studies involving neoformation of cell junctions in cultured cardiac or heterologous cell systems has shown basic differences in gap junction composition compared with gap junctions in vivo.

In summary, our study demonstrates a direct correlation between the volume occupied by the Cx43 immunosignal and intercellular electrical conductance. Although we did not associate levels of electrical coupling <$7$ nS with immunofluorescence, this observation raises the question of whether immunofluorescence can detect very small, but still electrically conducting, gap junctions.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

5. Darrow BJ, Fast VG, Kléber AG, Beyer EC, Saffitz JE. Functional and structural assessment of intercellular communication: increased conduc-
6. Danik SB, Liu F, Zhang J, Suk HJ, Morley GE, Fishman GI, Gutstein DE. Modulation of cardiac gap junction expression and arrhythmic sus-

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H449

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H449


