Electrical Propagation in Synthetic Ventricular Myocyte Strands From Germline Connexin43 Knockout Mice

Philippe Beauchamp, Cécile Choby, Thomas Desplantez, Karin de Peyer, Karen Green, Kathryn A. Yamada, Robert Weingart, Jeffrey E. Saffitz, André G. Kléber

Abstract—To characterize the role of connexin43 (Cx43) as a determinant of cardiac propagation, we synthesized strands and pairs of ventricular myocytes from germline Cx43−/− mice. The amount of Cx43, Cx45, and Cx40 in gap junctions was analyzed by immunohistochemistry and confocal microscopy. Intracellular electrical conductance, gj, was measured by the dual-voltage clamp technique (DVC), and electrical propagation was assessed by multisite optical mapping of transmembrane potential using a voltage-sensitive dye. Compared with wild-type (Cx43+/+) strands, immunoreactive signal for Cx43 was reduced by 46% in Cx43+/− strands and was absent in Cx43−/− strands. Cx45 signal was reduced by 46% in Cx43−/− and to the limit of detection in Cx43+/− strands, but total Cx45 protein levels measured in immunoblots of whole cell homogenates were equivalent in all genotypes. Cx40 was detected in ≈ 2% of myocytes. Intercellular conductance, gj, was reduced by 32% in Cx43+/− cell pairs and by 96% in Cx43−/− cell pairs. The symmetrical dependence of gj on transjunctional voltage and properties of single-channel recordings indicated that Cx45 was the only remaining connexin in Cx43−/− cells. Propagation in Cx43−/− strands was very slow (2.1 cm/s versus 52 cm/s in Cx43+/+) and highly discontinuous, with simultaneous excitation within and long conduction delays (2 to 3 ms) between individual cells. Propagation was abolished by 1 mmol/L heptanol, indicating residual junctional coupling. In summary, knockout of Cx43 in ventricular myocytes leads to very slow conduction dependent on the presence of Cx45. Electrical field effect transmission does not contribute to propagation in synthetic strands. (Circ Res. 2004;95:170-178.)

Key Words: Cx43 • Cx45 • very slow electrical propagation • discontinuous propagation • heptanol
of murine cardiac myocytes in culture that exhibit action potentials and propagation properties similar to the intact animal.24 This experimental tool has been used to analyze propagation of electrical impulses at high spatial resolution in strands of cells with heterozygous deletion of Cx43.25 We observed that cardiac impulse propagation is relatively insensitive to a 50% reduction in Cx43 expression, in accordance with previous results from computer modeling.26,27

In the present studies, we created strands of ventricular myocytes from germline Cx43−/− mice. The goals were to assess conduction in ventricular tissue devoid of Cx43 and to characterize the nature and the role of other ventricular connexins.

Materials and Methods

Synthesis of Myocyte Strands of Specific Genotypes

The technique used to produce patterned growth of neonatal mouse ventricular myocytes has been described previously.26 Hearts were obtained from mice maintained in an inbred colony (C57BL/6J, Jackson Laboratory; Bar Harbor, Me). Cx43−/−, Cx43+/−, and Cx43−/− embryos were obtained at embryonic day 20 (E20). The genotype of each embryo was determined by polymerase chain reaction protocols modified from those of Reaume et al.28 Ventricular tissue from individual hearts was minced and 1 cell suspension was seeded on coverslips to produce patterned growth.29 Myocytes were cultured on collagen-coated coverslips as described previously.7 Cultures were fixed in paraformaldehyde and immunostained with monospecific antibodies to quantify the amounts of Cx43, Cx45, and Cx40 at intercellular junctions as previously described.26,30 Immunohistochemical analysis of Cx43 was performed using an anti-Cx45 antibody shown in previous studies to be monospecific.2 The amount of immunoreactive signal in discrete spots located at intercellular junctions was quantified using confocal microscopy and digital image processing algorithms validated in previous studies.31 When sufficient specific signal was present, it was measured and expressed as a percent of total tissue area. Cx45 protein expression was also measured by immunoblotting of whole cell homogenates prepared from Cx43+/−, Cx43−/−, and Cx43+/− cultures as described previously.2

Dual-Voltage Clamp in Cell Pairs and Action Potential Recordings

Methods used to assess gJ in cell pairs have been described previously.28 In brief, freshly dispersed myocytes were plated onto glass coverslips coated with collagen. After 2 to 5 days in culture, spontaneously formed cell pairs were selected for measurement of transjunctional current, Ij, and junctional conductance, gJ = Vj/Ij, using dual-voltage clamp recordings at room temperature. Initially, the membrane potentials of both cells were clamped to the same voltage (Vj = Vl = −100 mV). Thereafter, voltage pulses of different amplitudes (up to 130 mV) and of either polarity were administered to cell 1 and Ij was measured in cell 2 (Ij = −Ij). Analyses that yielded maximal conductance, gJ max, and the function of normalized conductance at steady state, gJ = f(Vj), were performed as previously described.32 Weakly coupled pairs and normally coupled pairs treated with heptanol (1 mmol/L) were used to determine single-channel events. Transmembrane action potentials were obtained in current clamp mode. Subthreshold voltage responses were used to calculate input resistance.33

Optical Action Potential Measurement and Analysis of Propagation in Synthetic Strands

Staining of cell cultures with the voltage-sensitive dye RH237, multiple site optical recording of transmembrane potential with a light-sensitive diode array (10×10 diodes), and determination of conduction velocity, θ, have been described in detail.24,34 Cell strands were stimulated at a site >1 mm from the recording site (cycle length 400 to 500 ms, rectangular pulse of 5 ms duration, 1.5-fold threshold strength). The spatial resolution of the system was 15 μm with a 4× objective, and 6 μm with a 100× objective. Time resolution between data points was 80 μs. Conduction velocity was calculated along a strand segment of 105 μm. In Cx43−/− strands in which conduction delays were confined to cell borders, an additional estimate of mean conduction velocity was obtained by dividing the average cell length (44 μm)24 by the average conduction delay.

Statistical Analysis

Student t test was used for pairwise comparisons. Analysis of variance was used for multiple comparisons between genotypes. Variables are expressed as means±SD.

Results

Immunohistochemistry of Cx Distribution in Synthetic Strands of Cx43−/− Myocytes

Immunohistochemical analysis of Cx43 expression was performed in 3 Cx43+/− cultures, 3 Cx43−/− cultures, and 6 Cx43+/− cultures. All strands of each genotype showed a normal appearance and regular contractions when viewed by light microscopy. Immunohistochemical staining showed a reduction in the area occupied by Cx43 immunoreactive signal of 46% in Cx43+/− preparations, caused mainly by a reduction in number of gap junctions (Table 1) as previously reported.25 Cx43 immunoreactive signal was undetectable in all Cx43−/− preparations, consistent with the complete absence of Cx43 in these cells (Figure 1A through 1D).

Cx43 expression has been demonstrated previously in Cx43+/−, Cx43−/−, and Cx43+/− neonatal mouse ventricular
myocyte cultures. To confirm these observations, we performed Cx45 immunohistochemical staining in 2 Cx43/+/+ strands, 4 Cx43/+/- cultures, and 6 Cx43/-/- cultures. Compared with Cx43/+/+ strands, the area occupied by Cx45 signal was decreased by 49% in Cx43/+/- strands (Table 1). This was caused by a decrease in the number of individual gap junctions containing Cx45. Cx45 signal at gap junctions was apparent in all Cx43/-/- cultures as tiny regular spots around the cell perimeter in amounts too low to reliably quantify (Figure 1D). Despite the marked differences in Cx45 signal in gap junctions, total Cx45 protein levels, determined by immunoblotting whole cell homogenates, were equivalent in Cx43/+/+, Cx43/+/-, and Cx43/-/- cultures (Figure 1E and Table 1). This observation confirmed results obtained in neonatal Cx43/-/- hearts.

Although working ventricular myocytes do not express Cx40, immunostaining with specific anti-Cx40 antibodies was performed in 4 Cx43/-/- and 2 Cx43/+/- cultures to determine whether cultures from minced ventricles included conduction system cells, which are known to be coupled by Cx40. In 26 microscopic fields from 4 Cx43/-/- cultures, 2.4±0.0% of all cells expressed Cx40. These occurred as individual cells interspersed among myocytes showing no Cx40 signal. In Cx43/+/- cultures, 3.1±4.4% of all cells were
determined by dual-voltage clamp recordings. Voltage pulses of ±10mV amplitude were administered to cell 1 to produce transjunctional gradients, $V_j$, and associated non-inactivating gap junction currents, $I_j$, to determine the maximal gap junction conductance, $g_{j,max}$. As shown in Figure 2 and Table 2, $g_j$ in Cx43$^{+/+}$ and Cx43$^{-/-}$ cell pairs was reduced to 68% and 4% of the Cx43$^{+/+}$ level, respectively.

Application of $V_j$ pulses of increasing amplitude leads to progressive inactivation of $g_j$. Currents at the beginning and end of each $V_j$ pulse, $I_{j,inst}$ and $I_{j,ss}$ (instantaneous and steady-state currents, respectively), are useful to characterize the properties and connexin composition of gap junction channels. We determined the amplitudes of $I_{j,inst}$ and $I_{j,ss}$ for each $I_j$ signal. Figure 3A and Table 2 summarize normalized $g_i$ values plotted versus $V_j$ in Cx43$^{+/+}$ and Cx43$^{-/-}$ cell pairs. The fit of the data to the Boltzmann equation at negative and positive $V_j$ revealed that $V_{j,0}$, corresponding to $V_j$ at half maximal inactivation, was smaller in Cx43$^{-/-}$ than in Cx43$^{+/+}$ cell pairs (43.6mV versus 64.7mV).

Single-channel recordings from Cx43$^{+/+}$ and Cx43$^{-/-}$ cell pairs revealed marked differences between genotypes (Figure 3B). Two distinct conductance levels were observed in Cx43$^{+/+}$ cell pairs, suggesting the presence of a single gap junction channel type exhibiting a main open state at $33\pm5$ pS ($n=66$) and a residual state at $13\pm5$ pS ($n=47$). In contrast, a wide variety of conductances was observed in Cx43$^{-/-}$ cell pairs (range, 7 to 66 pS; $n=141$), rendering it difficult to attribute the values to 2 conductance states only. Moreover, changing the polarity of $V_j$ pulses produced a change in single-channel conductance, which is characteristic of heterotypic channels (see Discussion).

### Electrical Propagation in Synthetic Strands of Cx43$^{+/+}$, Cx43$^{+/+}$, and Cx43$^{-/-}$ Ventricular Myocytes

Multisite optical mapping of transmembrane potential was performed in 10 different cell cultures from Cx43$^{+/+}$ mice, and in 3 Cx43$^{+/+}$ and 8 Cx43$^{-/-}$ cultures (Table 2). Propagation in Cx43$^{+/+}$ and Cx43$^{-/-}$ strands was fast and continuous. As observed previously, there was a slight difference in propagation velocity between Cx43$^{+/+}$ and Cx43$^{-/-}$ strands (52±1 cm/s in Cx43$^{+/+}$ and 48±9 cm/s in Cx43$^{-/-}$ strands), but this was not statistically significant (Table 2). A small difference was also seen in maximal upstroke velocity of the

### Table 2. Electrophysiological Parameters of Wild-Type Cx43$^{+/+}$, Heterozygous Cx43$^{+/+}$, and Knock Out Cx43$^{-/-}$ Mice Cultures

<table>
<thead>
<tr>
<th></th>
<th>$g_{j,max}$ [nS]</th>
<th>$g_{j,min}$ [nS]</th>
<th>$V_{j,0}$ [mV]</th>
<th>$\theta$ [cm/s]</th>
<th>$dV/dt_{max}$ [V/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Cx43$^{+/+}$ (n)</td>
<td>36.8±16 (8)</td>
<td>2.82</td>
<td>64.7 (3)</td>
<td>52±1 (3)</td>
<td>115±7 (3)</td>
</tr>
<tr>
<td>HZ Cx43$^{+/+}$ (n)</td>
<td>24.8±13* (20)</td>
<td>NM</td>
<td>NM</td>
<td>48±9 (8)</td>
<td>130±9* (5)</td>
</tr>
<tr>
<td>KO Cx43$^{-/-}$ (n)</td>
<td>1.4±1.3**,*** (33)</td>
<td>0.32</td>
<td>42.3 (6)</td>
<td>6.1±0.5**,*** (10)</td>
<td>134±15 (9)</td>
</tr>
</tbody>
</table>

$g_{j,max}$ indicates maximal gap junctional conductance; $g_{j,min}$, minimal gap junctional conductance relative to $g_{j,max}$; $V_{j,0}$, mean transmembrane voltage at half-maximal inactivation; $\theta$, conduction velocity; $n$, number of measurements; NM, not measured.

Data are presented in means±SD.

*P<0.05 HZ vs WT; **P<0.001 knock out vs wild type; ***P<0.001 knock out vs heterozygous.
transmembrane potential in Cx43\(^{+/+}\) and Cx43\(^{-/-}\) strands, confirming previous measurements.\(^{25}\)

Propagation in Cx43\(^{-/-}\) strands was dramatically different than in Cx43\(^{+/+}\) and Cx43\(^{+/+}\) strands, as illustrated in Figure 4. Figure 4A depicts upstrokes of transmembrane action potentials measured during propagation from left to right along a strand distance of 105 \(\mu\)m with a resolution of 15 \(\mu\)m per measuring diode. The upstrokes are clustered in 4 groups with prolonged conduction delays (1587 \(\mu\)s to 2489 \(\mu\)s) between groups. This type of highly discontinuous conduction is typical of cardiac tissue with a very low degree of intercellular coupling, in which excitation within individual cells is virtually instantaneous and delays are localized to cell borders.\(^{27,37}\) At a resolution of 15 \(\mu\)m (Figure 4A), the light-sensing diodes overlap cell borders leading to upstrokes with multiple components. To assess the topology of the conduction delay more accurately, experiments were performed in 4 cultures at the maximal magnification of 100× (resolution of 6 \(\mu\)m). Figure 4C and 4D show the progress of propagation from one single cell to the next. Action potentials occurred virtually simultaneously at multiple sites within a single cell, whereas a very large conduction delay occurred at the cell border. A histogram of intercellular delays is shown in Figure 4B. A major population of delays occurred with a peak value of 2250 \(\mu\)s. A second smaller population had a peak delay of 5250 \(\mu\)s, and a few very large delays were observed as well. Full conduction block, confined to local cell-to-cell contacts that presumably contained no gap junctions, is the most likely explanation for the presence of the second smaller peak. In this case, the delay between adjacent cells was determined by the sum of the cell-to-cell delays along the detour path of the excitation wave.

Conduction velocity, \(\theta\), calculated from the conduction time along a distance of 105 \(\mu\)m was 2.1 cm/s in Cx43\(^{-/-}\) cultures. This was more than an order of magnitude slower than in Cx43\(^{+/+}\) and Cx43\(^{+/+}\) cultures (Table 2). A second, independent estimate of conduction velocity in Cx43\(^{-/-}\) cultures, the ratio of the mean cell length and the average conduction delay (2460 \(\mu\)s, first population in Figure 4B), yielded a velocity of 1.8 cm/s.

In a recent theoretical study, it was shown that slow electrical propagation could occur under highly specialized conditions in the absence of gap junctional coupling (see Discussion). To determine whether this occurred in Cx43\(^{-/-}\) strands, we performed 3 experiments in which propagation was studied in the presence and absence of the uncoupling agent heptanol (1 mmol/L).\(^{38}\) As shown in Figure 5, application of 1 mmol/L heptanol abolished propagation within 30 seconds. Washout of heptanol restored electrical propagation within a few seconds, indicating that very slow propagation in Cx43\(^{-/-}\) strands required the presence of functional gap junction channels. Higher concentrations of heptanol (3 mmol/L) have been reported to decrease \(I_{Ca}\), \(I_{K1}\),\(^{39}\) and \(I_{Na}\).\(^{40}\) We therefore

![Figure 3](http://circres.ahajournals.org/)

Figure 3. A. Relationships between normalized gap junction conductance at steady state, \(g_{j,ss}\), and the transjunctional voltage, \(V_j\), determined in Cx43\(^{-/-}\) (○) and Cx43\(^{+/+}\) (□) cell pairs. B, Single gap junction channel currents, \(I_j\), recorded from Cx43\(^{-/-}\) and Cx43\(^{+/+}\) cell pairs during 2 pulses of alternating \(V_j\) applied across intercellular junctions. The short dashes denote the levels of the main conductance states, and the long dashes indicate the levels of subconductance states. Left panel, \(I_j\) from a channel in a Cx43\(^{-/-}\) pair at \(V_j = \pm 70\) mV (upper trace) and \(\pm 130\) mV (bottom trace). Right panel, \(I_j\) recorded from 2 channels in a Cx43\(^{+/+}\) cell pair (upper and lower traces) at \(V_j = \pm 90\) mV. The consistent unitary conductance (g value) of 30 pS is characteristic of channels formed entirely by Cx45. Note the asymmetrical behavior of \(I_j\) on reversal of \(V_j\) polarities in channels recorded in Cx43\(^{-/-}\) cell pairs.
tested the effect of 1 mmol/L heptanol on action potentials in single Cx43+/−, Cx43+/−, and Cx43−/− cells. Exposure to heptanol for 60 seconds in 6 experiments (2 Cx43+/−, 2 Cx43+/−, 2 Cx43−/−) had no significant effect on action potential amplitude (126±4 mV versus 128±8 mV control) or input resistance (2.6±0.7 GΩ, versus 3.1±0.9 GΩ control), whereas action potential duration at 50% repolarization was significantly decreased (66±76 ms versus 37±26 ms control). These observations ruled out the possibility that depression of action potential upstroke or decreased membrane resistance contributed to the propagation block observed in Cx43−/− strands 30 seconds after application of heptanol.

Discussion

The purpose of this study was to assess the effect of deletion of Cx43 in ventricular tissue on electrical propagation, to identify other connexins in impulse propagation, and to evaluate the possibility of field effect in cell-to-cell transmission of electrical impulses. Because germline Cx43−/− mice die soon after birth, ventricular strands were synthesized from hearts excised 1 day before birth. Immunohistochemical analysis showed complete absence of Cx43 signal in Cx43−/− cells and confirmed ∼50% reduction of Cx43 signal in gap junctions in Cx43−/− cells. This is different than Cx43 conditional knockouts in which deletion of Cx43 by the Cre/lox system occurs in ∼90% of myocytes. As observed previously in neonatal and adult mouse hearts, the area occupied by Cx45 immunofluorescent signal varied in cells with different Cx43 expression levels. Cx45 signal was reduced by 49% in Cx43+/− cells and apparent only as small fluorescent spots in Cx43−/− strands. These changes probably reflect a decrease in the size of gap junctional plaques caused by deficiency of Cx43 (which is, by far, the predominant connexin expressed in both adult and neonatal ventricular myocytes), rather than a real decrease in Cx45 content in gap junctions.

Dual-voltage clamp experiments were performed to quantify the decrease of $g_j$ in Cx43+/− cells and characterize properties of remaining connexins. We found that $g_j$ in Cx43−/− cell pairs was reduced by 96% compared with Cx43+/− cells. This is similar to results in pairs of adult myocytes from conditional Cx43 knockouts, but larger than the decrease reported for Cx43−/− perinatal myocytes. In our study, the absolute value of $g_j$ was significantly smaller than in myocytes of adult conditional knockout animals. A likely explanation is that neonatal cells form cell–cell con-
tacts via small cytoplasmic processes (see Figure 2), whereas adult myocytes are coupled via larger junctions.

Both the dependence of $g_j$ on transjunctional voltage $V_j$ and recordings of single-channel events strongly suggested that Cx45 was the sole connexin responsible for cell-to-cell communication in Cx43/−/− strands. Boltzmann parameters $V_{j0}$ and $g_{j,min}$ in Cx43/−/− cell pairs were comparable to those of perinatal Cx43/−/− myocytes and closely resembled those of mouse Cx45 channels expressed in HeLa cells. However, they were somewhat larger than those of murine Cx45 expressed in N2A cells. The larger values of $V_{j0}$ and $g_{j,min}$ could be caused by background currents in cardiac cells that are absent in transfected cells. Single-channel recordings in Cx43/−/− cell pairs showed a consistent, small main state, conductance of 33 pS, which is highly characteristic of Cx45. In contrast, single-channel recordings in Cx43+/− preparations showed a wide range of conductances that were dependent on the $V_j$ polarity. This suggests the presence of different conductance states (main state, residual state, sub-state) and channel types (homotypic Cx43, heteromeric/heterotypic Cx43/Cx45) and confirms that gap junction channels in Cx43+/− ventricular myocytes can be heterotypic and heteromeric.

We measured propagation in strands of Cx43/−/− ventricular myocytes for the first time. Propagation in Cx43/−/− ventricular strands differed significantly from propagation in Cx43/−/+ and Cx43+/−/+ strands in 2 respects. First, conduction velocity was reduced by 25-fold to $\approx$2 cm/s, a value described previously as “very slow conduction.” In the heart in vivo, such slow velocity is observed only in the AV node. Second, propagation was remarkably discontinuous, with virtually simultaneous excitation within individual cells and a mean conduction delay of $\approx$2 ms between cells. In synthetic strands of neonatal rat ventricular myocytes with normal Cx43 expression, delays across gap junctions during continuous steady-state propagation are typically $\approx$80 $\mu$s. Thus, a $\approx$20-fold increase in conduction time across gap junctions occurs in the Cx43/−/− phenotype. This discontinuous type of propagation occurs at a higher margin of safety than normal propagation, caused by recurrent alternations of low and high electrical resistances formed by the low cytoplasmic resistance in series with the high resistance of Cx45 gap junctions. The same type of conduction, leading to a comparable degree of conduction slowing, has been produced experimentally in neonatal rat myocyte strands by the presence of structural discontinuities and/or by partial uncoupling with drugs. However, one unavoidable limitation of chemical uncoupling agents is that development of uncoupling leading to very slow conduction is transient and unstable. In the present studies, a steady-state level of uncoupling, sufficient to cause very slow but stable conduction, was produced experimentally for the first time, allowing comparison with $g_j$ measurements in corresponding cell pairs and with theoretical simulations of low coupling states. Our results agree with theoretical computations and prove that stable impulse propagation can be maintained, albeit slowly, despite a $>95\%$ decrease in $g_j$.

Although our measurements of $g_j$ and propagation velocity agree closely, propagation velocity measured in intact ventricles in Cx43/−/− conditional knockouts was decreased to only $\approx50\%$ of normal. The reason for the marked discrepancy in conduction velocity in CKO hearts versus germline Cx43-null neonatal myocyte strands is not clear, and any explanations remain speculative. One important reason may be that gene deletion is incomplete in conditional knockout Cx43-null neonatal myocyte strands is not clear, and any explanations remain speculative. One important reason may be that gene deletion is incomplete in conditional knockout hearts. The consequences of this unavoidable heterogeneity in Cx43 expression in conditional Cx43 knockout hearts must be considered in terms of normal cellular connections in ventricular myocardium. For example, an individual left ventricular myocyte in adult canine myocardium is connected to an average of 11.3 neighboring myocytes. A comparable degree of intercellular connectivity has been found in murine myocardium. In synthetic strands identical to those used in the present study, an individual neonatal mouse myocyte is connected to 6.5 neighbors, which extrapolates to 16.6 neighbors in 3-dimensional tissue. Thus, an average myocyte in a conditional Cx43 knockout heart may be connected to at least 1 neighbor that expresses wild-type levels of Cx43. This extent of low resistance connections within a poorly coupled syncytium should be considered as a potential explanation of the relatively high velocity values observed in Cx43 conditional knockout hearts.

As recently shown in computer simulations, field effect transmission can theoretically occur in the absence of cell-
to-cell coupling by gap junction channels. However, successful field effect transmission required localization of >95% of Na\(^+\) channels at cell poles and the presence of very high resistance intercellular clefts. It is difficult to test this mechanism in an experimental setting that faithfully reproduces these conditions. Moreover, the presence of Na\(^+\) channels associated with T-tubules speaks against localization of the totality of Na\(^+\) channels at intercalated disks. Results of the present experiments clearly ruled out field-effect transmission as a mechanism contributing to slow propagation in strands of neonatal murine myocytes.

The specific biological roles of each of the cardiac connexins are not completely understood. In the normal heart, Cx45 may contribute to slow propagation in the AV node and protect the sinus node from the large impedance load exerted by atrial tissue.\(^{44}\) Although Cx45 is the dominant connexin in early heart development,\(^{49}\) its role in the neonatal and adult myocardium.

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