Heterogeneous connexin43 expression produces electrophysiological heterogeneities across ventricular wall

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Poelzing, Steven, Fadi G. Akar, Elvera Baron, and David S. Rosenbaum. Heterogeneous connexin43 expression produces electrophysiological heterogeneities across ventricular wall. Am J Physiol Heart Circ Physiol 286: H2001–H2009, 2004.—Recently we found that electrophysiological (EP) heterogeneities between subepicardial and midmyocardial regions of the canine left ventricle (LV) are maintained as a result of regional uncoupling caused by inhomogeneous expression of the principal ventricular gap junction protein, connexin43 (Cx43). We hypothesized that transmural EP heterogeneities are in part produced by heterogeneous Cx43 expression across the ventricular wall. The left ventricles of eight dogs were sectioned to expose the transmural surface. To determine whether heterogeneous Cx43 expression influenced EP function, high-resolution transmural optical mapping of the arterially perfused canine wedge preparation was used to measure transmural conduction velocity (Vtmax), dV/dtmax, transmural space constant (λTM), and transmural gradients of action potential duration (APD). Relative Cx43 expression, quantified by confocal immunofluorescence, was significantly lower (by 24 ± 17%; P < 0.05) in subepicardial compared with deeper layers. Importantly, reduced subepicardial Cx43 was associated with transmural heterogeneities of EP function evidenced by selectively reduced subepicardial Vtmax (by 18 ± 9%; P < 0.05) compared with deeper layers. In subepicardial regions, dV/dtmax was fastest (by 19 ± 15%) and λTM was smallest (by 18.1 ± 2%), which suggests that conduction slowing was attributable to localized uncoupling rather than reduced excitability. The maximum transmural APD gradients occurred in the same regions where Cx43 expression was lowest; this suggests that Cx43 expression patterns served to maintain APD gradients across the transmural wall. These data demonstrate that heterogeneous Cx43 expression is closely associated with functionally significant EP heterogeneities across the transmural wall. Therefore, Cx43 expression patterns can potentially contribute to arrhythmic substrates that are dependent on transmural electrophysiological heterogeneities.

IT IS WELL ESTABLISHED that when studied in isolation, significant electrophysiological (EP) heterogeneities can be identified between cells that span the transmural ventricular wall (17). For example, a transient outward potassium current (Ito) is strongly expressed in epicardial and midmyocardial but not endocardial cells (25). Additionally, midmyocardial cells express a lower density of the slowly activating delayed rectifier potassium current (17) and a higher density of the late sodium current (36). Owing to their unique ionic composition, midmyocardial cells display longer action potential duration (APD) and enhanced sensitivity to interventions that prolong APD such as bradycardia and class III antiarrhythmic drugs (25). Previously, using high-resolution optical mapping, we found that steep APD gradients are indeed present in the intact ventricle, particularly between the subepicardial and midmyocardial layers (3). Under conditions that mimic long QT syndrome, these APD gradients were of sufficient magnitude to form a substrate for ventricular tachyarrhythmias (3).

The functional expression of transmural EP heterogeneities in intact heart depends on the variation of ionic characteristics intrinsic to cells across the ventricular wall and the extent of cell-to-cell coupling via gap junctions between transmural muscle layers (15, 31). Intercellular coupling is expected to attenuate transmural heterogeneities of APD between cell types that span the ventricular wall. Therefore, the mechanism responsible for maintaining EP heterogeneities under conditions of apparently normal cell-to-cell coupling remains a major unresolved question (4, 31).

Gap junctions are composed of intercellular channels that permit the transfer of electrical currents and small molecules between neighboring cells. Connexin43 (Cx43), the principal gap junction protein found in ventricular myocardium (29), plays a critical role in impulse propagation and electrical synchronization between myocytes. It is unclear to what extent APD gradients between subepicardial, midmyocardial, and endocardial cell layers should become manifest under conditions of normal intercellular coupling. Previously, Yan et al. (34) estimated that tissue resistivity may be substantially increased between subepicardial and midmyocardial regions, which suggests that APD gradients between those two layers could be in part attributed to altered cell-to-cell coupling. Therefore, we hypothesized that transmural EP heterogeneities are maintained as a result of regional uncoupling caused by inhomogeneous expression of the principal ventricular gap junction protein Cx43 across the transmural wall. To test this hypothesis, we developed a technique for comparing in detail the spatial distribution of Cx43 protein expression to membrane voltage across the transmural wall of the canine left ventricle (LV). We found that heterogeneous Cx43 expression was associated with functionally significant EP heterogeneities across the transmural wall. Therefore, Cx43 expression patterns can potentially contribute to arrhythmic substrates that are dependent on transmural inhomogeneities of repolarization.

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METHODS

Canine Wedge Preparation

To assess the functional consequences of Cx43 expression patterns across the transmural wall, we developed a system for optically mapping action potentials from cells that span the entire transmural wall of the arterially perfused canine wedge preparation as described previously (3, 34). Briefly, hearts were excised from eight male mongrel dogs (body wt, 20–25 kg). The procedures used conform to the guidelines set forth by the Institutional Animal Care and Use Committee. Wedges of myocardium measuring ∼3 × 1.5 × 1 cm were dissected from the anterior and anterolateral or posterior walls of the LV in proximity to secondary branches of the left anterior descending and circumflex coronary arteries, respectively. Wedges were perfused with oxygenated Tyrode solution [that contained (in mmol/l) 129 NaCl, 25.0 NaHCO3, 0.5 MgSO4, 4.0 KCl, 5.5 dextrose, and 1.8 CaCl2]. Perfusion pressure was maintained at 50–60 mmHg. Wedges were discarded if collateral arteries shunted significant flow away from the preparation as evidenced by a coronary resistance <1.2 mmHg·ml−1·min−1 (3). Preparations were completely immersed in temperature-controlled (36 ± 1°C) perfusate to prevent the formation of intramyocardial temperature gradients. Wedges were stabilized against a flat imaging window by application of a gentle constant pressure via a movable piston, which obviated the need for pharmacological suppression of contraction. Preparations were determined to be stable for >4 h of perfusion as judged by the stability (±5%) of coronary resistance, APD, and QT interval.

Transmural Optical Mapping System

Previously we developed an optical action-potential mapping system (2, 14, 22) that is capable of resolving membrane potential changes as small as 0.5 mV with 1 ms of temporal resolution from 256 sites simultaneously across the entire transmural surface of the wedge preparation (3). After the preparation was stained with the voltage-sensitive dye 4-(6-[2(di-n-butylamino)-6-naphthyl]vinyl)pyridinium (di-4-ANEPPS, 15 μmol/l) by direct arterial perfusion for 10 min, dye was excited by 514 ± 5 nm light emitted by a 250-W tungsten-filament lamp. Fluoresced light was long-pass filtered at 610 nm and focused onto a 16 × 16-element photodiode array (model C4675; Hamamatsu) through high numerical aperture photographic lenses using the tandem-lens configuration (Nikon; 85 mm, F/1.4 and 105 mm, F/2.0; Ref. 14). A 768 × 493-pixel charge-couple device video camera (TMC-74; PULNIX; Sunnyvale, CA) was used to view and localize the mapping field relative to the anatomical features of the preparation. The anatomical reference points on the wedge were used to precisely align action potential maps with sections of tissue obtained for postoptical mapping measurements of Cx43 distribution.

Functional differences in intercellular coupling between transmural muscle layers were assessed by measuring five EP parameters within each layer of myocardium that were each dependent on cell-to-cell coupling: APD, APD gradient, conduction velocity, and rise time to precisely align action potential maps with sections of tissue obtained for postoptical mapping measurements of Cx43 distribution. Complementary immunolabeling techniques were used to quantify the magnitude of Cx43 protein expression in each muscle layer across the transmural wall and hence identify Cx43 heterogeneities between muscle layers. Paraffin-embedded blocks of canine myocardium that were optically mapped were then sectioned parallel to the epicardium from epicardial to midmyocardial to endocardial layers every 2 mm to allow for selective Cx43 analysis of each layer. Immunofluorescence (IF) analysis of Cx43 was performed as previously described (5). Briefly, paraffin-embedded tissue layers were sectioned at a thickness of 5 μm and mounted on gelatin-coated slides. Sections were deparaffinized, placed in citrate buffer, and boiled in a microwave oven for 10 min. The sections were incubated overnight with anti-Cx43 antibodies (1:400 dilution; Zymed) and then incubated with Cy3-conjugated goat anti-rabbit IgG (1:800 dilution; Zymed) before being examined by laser scanning confocal microscopy (×40 oil immersion lens, Airy 1 pinhole). The degree of confocality was kept constant (depth of focus, ∼602 nm) for each experiment to minimize overlap of the Cx43 label. Each layer was analyzed from eight fields to obtain an average Cx43 quantity within a layer. Measurements of 1.325 myocytes yielded the finding that 80% of left ventricular myocytes had length-to-width ratios >4:1. To eliminate artifactual quantification of Cx43 protein, samples were discarded when the imaging plane was not parallel to the long axis of the fibers; this was judged to occur when the myocyte

layers. The average APD within each layer was calculated to generate a transmural APD profile from epicardium to endocardium. The derivative of the APD profile was used to determine the transmural APD gradient as a function of depth from the epicardium. We used a previously described algorithm (2) to calculate conduction velocity selectively (±15°) in the transmural direction of propagation (θTM) as a function of distance from the epicardium of each of the 16 transmural muscle layers. Maximum action potential upstroke velocity (dV/dtmax) was calculated after action potential amplitudes were normalized to 100 mV.

Measurement of Space Constant

Previously we developed a method for estimating the cardiac space constant (Λ) as an empirical index of intercellular coupling in intact tissues (2). Briefly, a subthreshold unipolar stimulus (0.8-mA amplitude, 15-ms duration square wave, 0.1 Hz; model 2200; A-M Systems) was applied to the myocardial surface through a platinum electrode, and the reference electrode was placed in the bath. The extent of the membrane voltage (Vm) decay in space from the site of stimulation was measured simultaneously in the transmural direction from each optical pixel. Previously we showed that ΛTM derived from the exponential decay of Vm in space provides an index of intercellular coupling between transmural layers, because ΛTM is independent of electrical loading caused by variations of fiber angle orthogonal to the transmural surface (i.e., rotational anisotropy; Ref. 21). The ΛTM values were measured from three separate sites within each muscle layer and averaged to determine the average ΛTM value for each layer.

Histological Analysis

After each preparation was optically mapped, wedges were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Sections were cut from the most superficial regions of the transmural surface (corresponding to the optically mapped region) and stained with hematoxylin and eosin as described previously (34). Tissue sections were imaged with a Nikon Eclipse E600 microscope for offline histology assessment. Substantial variations in the extracellular matrix such as invasion of inflammatory cells and fiber separation were qualitatively assessed at positions where EP parameters were measured. Additionally, myocyte size was measured along the transmural axis from 50 myocytes within each layer of myocardium where relative Cx43 was also quantified.

Relative Transmural Cx43 Quantification by Immunofluorescence

The magnitude of Cx43 protein expression in each muscle layer across the transmural wall and hence identify Cx43 heterogeneities between muscle layers. Paraffin-embedded blocks of canine myocardium that were optically mapped were then sectioned parallel to the epicardium from epicardial to midmyocardial to endocardial layers every 2 mm to allow for selective Cx43 analysis of each layer. Immunofluorescence (IF) analysis of Cx43 was performed as previously described (5). Briefly, paraffin-embedded tissue layers were sectioned at a thickness of 5 μm and mounted on gelatin-coated slides. Sections were deparaffinized, placed in citrate buffer, and boiled in a microwave oven for 10 min. The sections were incubated overnight with anti-Cx43 antibodies (1:400 dilution; Zymed) and then incubated with Cy3-conjugated goat anti-rabbit IgG (1:800 dilution; Zymed) before being examined by laser scanning confocal microscopy (×40 oil immersion lens, Airy 1 pinhole). The degree of confocality was kept constant (depth of focus, ∼602 nm) for each experiment to minimize overlap of the Cx43 label. Each layer was analyzed from eight fields to obtain an average Cx43 quantity within a layer. Measurements of 1.325 myocytes yielded the finding that 80% of left ventricular myocytes had length-to-width ratios >4:1. To eliminate artifactual quantification of Cx43 protein, samples were discarded when the imaging plane was not parallel to the long axis of the fibers; this was judged to occur when the myocyte
length-to-width ratio was <4. For all IF images, the high-intensity immunoreactive signal that arose from Cx43 localized in gap junctions was clearly separated from the background signal for all transmural muscle layers as demonstrated in a representative example (Fig. 1A). The separation of the high-intensity signal from the background allowed for the prospective application of a constant threshold (>90% signal intensity) to distinguish between the two. The relative Cx43 quantity was defined as the proportion of total myocardial tissue area occupied by the Cx43 IF signal as described previously (8). To compare Cx43 expression across experiments, each relative measurement of Cx43 was normalized to the mean of the total Cx43 quantity measured for an entire experiment.

Relative Transmural Cx43 Quantification by Western Immunoblotting

To confirm Cx43 quantification by confocal IF, Cx43 measured by IF was compared with Cx43 measured by Western immunoblotting in each muscle layer from seven additional wedges. Before formalin fixing of ventricular wedges for confocal IF analysis, a section of tissue was carefully separated (using a razor blade) from the transmural surface. This transmural surface was sectioned from epicardial to midmyocardial to endocardial layers every 2 mm to allow for selective Western immunoblotting analysis of each layer, which is similar to the IF sectioning technique described above. Each layer was divided into three tissue samples with approximate dimensions of 2 × 3 × 2 mm and snap-frozen in liquid nitrogen. Western immunoblotting analysis was performed on ventricular tissue samples as previously described (12) using a primary rabbit polyclonal anti-Cx43 antibody (Zymed) and anti-rabbit horseradish peroxidase-linked whole antibody (Amersham). Lanes were loaded with 7 μg of total protein. An immunoblot for actin was used as a control for equal protein loading. Actin immunoblots had similar intensities in all lanes compared with protein samples from multiple animals. Immunoreactivity was detected by chemiluminescence (Rennaissance; NEN Life Science).
A

endocardial (Fig. 3 transmural conduction patterns during planar stimulation of the region indicate faces. Relative crowding of isochrone lines in the subepicardial midmyocardial and subendocardial Cx43 signals. To confirm this result, a subset of experiments (n = 7) was performed using both confocal IF and Western immunoblotting. The density of Western immunoblot bands corresponding to Cx43 protein expression in the subepicardium was reduced compared with midmyocardial and subendocardial Cx43 signals. To confirm this result, a subset of experiments (n = 7) was performed using both confocal IF and Western immunoblotting. The density of Western immunoblot bands corresponding to Cx43 protein expression in the subepicardium was reduced compared with midmyocardial and subendocardial (Fig. 1A, right). In all experiments, relative Cx43 quantities measured by confocal IF were highly correlated with Cx43 measured by Western immunoblotting (r = 0.88) and fell along the line of identity thereby confirming the quantitative IF results (Fig. 1C).

Relative Cx43 expression was quantified by IF because IF 1 has been shown to measure Cx43 protein localized within the gap junctions (24), 2) yields important information on the spatial distribution of Cx43, and 3) allows for high spatial resolution measurements of Cx43. Figure 2 summarizes the results of the eight experiments where regional Cx43 expression was compared directly to functional EP changes. Although relative Cx43 quantity was similar in midmyocardial and endocardial layers, there was a significant reduction (by 24 ± 17%) of Cx43 expression (Fig. 2A) in subepicardial layers (0–20% of the transmural distance). Similar patterns of Cx43 expression were observed in wedges taken from the anterior and posterior walls of the LV.

Relationship Between Cx43 and EP Function

Transmural conduction velocity. Wedges were stimulated with a plate placed against the endocardium or epicardium to simplify the geometry of propagation and facilitate interpretation of \( \theta_{TM} \) differences. A representative contour map shows transmural conduction patterns during planar stimulation of the endocardial (Fig. 3A, left) and epicardial (Fig. 3B, left) surfaces. Relative crowding of isochrone lines in the subepicardial region indicate \( \theta_{TM} \) slowing compared with deeper myocardial layers. Slowed subepicardial conduction was also evidenced by the discrete separation of action potential upstroke traces (Fig. 3A, right) between intraepicardial sites A and B. To rule out the possibility that the selective subepicardial \( \theta_{TM} \) slowing during endocardial stimulation was due to breakthrough conduction, \( \theta_{TM} \) was also measured during epicardial plane wave stimulation. Importantly, as shown in Fig. 3B, subepicardial \( \theta_{TM} \) remained slower relative to deeper transmural layers during epicardial stimulation as evidenced by persistent crowding of isochrones and separation of action potential upstrokes between sites A and B. Importantly, slowing of subepicardial \( \theta_{TM} \) was independent of the type of stimulation (planar or point) as well as the location of the stimulus.

The rate of rise of action potential upstrokes recorded from evenly spaced cells across the transmural wall was fastest in subepicardial regions (Fig. 3, right). Specifically, the \( \frac{dV_m}{dt_{max}} \) (horizontal bars) measured from subepicardial cells were
clearly larger than midmyocardial and subendocardial values. For all experiments, \( \frac{dV}{dt}_{max} \) of subepicardial cells (35 ± 11 V/s) was significantly larger compared with deeper muscle layers (28 ± 6 V/s). This finding suggests that \( \lambda_{TM} \) slowing in the subepicardium was attributable to a localized reduction of intercellular coupling in epicardial layers and not depressed excitability. Moreover, \( \lambda_{TM} \) was significantly smaller in subepicardium compared with midmyocardial layers (\( \lambda_{TM} \) 0.69 ± 0.07 and 0.82 ± 0.06 mm; \( P < 0.05 \)), which further suggests that heterogeneous Cx43 expression produced relative intercellular uncoupling between these muscle layers.

It is also evident from transmural contour maps (Fig. 3, left) that there is some variation in conduction patterns and velocity within each transmural muscle layer. However, when \( \theta_{TM} \) was averaged across each transmural muscle layer, a clear and highly reproducible \( \theta_{TM} \) profile was evident in all experiments. The representative example shown in Fig. 4A reveals a significant and selective \( \theta_{TM} \) slowing within the epicardial zone. Moreover, relative Cx43 expression in each layer follows the \( \theta_{TM} \) profile measured across the transmural wall. The variability of relative Cx43 expression and \( \theta_{TM} \) within each layer (standard deviation bars), when \( \theta_{TM} \) and corresponding relative Cx43 expression data were classified by cell type (Fig. 4B), subepicardial cells exhibited a distinct Cx43 protein expression and \( \theta_{TM} \) that was significantly lower than midmyocardial and endocardial cell types. The heterogeneities in relative Cx43 expression and the resulting \( \theta_{TM} \) profiles between transmural muscle layers were significantly greater than the variations of these parameters within each muscle layer as is evidenced by the significant differences in mean values relative to their standard deviations. The relationship between the relative Cx43 distribution and \( \theta_{TM} \) across the transmural wall for all experiments is summarized in Fig. 2, A and C. Importantly, relative Cx43 expression was significantly lower in subepicardial regions where \( \theta_{TM} \) was also reduced compared with deeper layers (by 18 ± 9%). These data demonstrate a close relationship between relative transmural Cx43 expression patterns and EP function across the transmural wall.

Transmural APD gradients. In contrast with isolated myocytes, intercellular coupling in intact heart might be expected to minimize repolarization gradients between epicardial, midmyocardial, and endocardial cell types. Figure 5, which shows the average transmural APD profile from eight experiments, reveals that the sharpest transition in transmural APD occurs

**Fig. 3.** Transmural conduction velocity is reduced in subepicardial muscle layers. Left: isochrone maps (3 ms) of transmural activation during planar stimulation. A: stimulation of endocardium. B: stimulation of epicardium. Crowding of isochrones in the subepicardium indicates slowed conduction. Right: action potential upstrokes from equally spaced sites. Separation of upstrokes between points A and B also indicates slowed subepicardial conduction. Values of \( \frac{dV}{dt}_{max} \) for each upstroke are plotted below \( V_m \) traces, which indicate that sites with the slowest conduction exhibit the most rapid rate of rise. Rapid subepicardial rate of rise was consistent regardless of the direction of pacing; this indicates a mechanism of conduction slowing related to intercellular uncoupling rather than altered excitability.
between the subepicardium and deeper layers of tissue, which is consistent with previous reports (34). As shown in Fig. 5B, the APD gradient is largest between 13 and 20% of the transmural distance; this corresponds exactly with regions of reduced Cx43 protein expression and altered cell-to-cell coupling.

Histological Analysis

In addition to gap junctions, the extracellular matrix and myocyte dimensions can also influence intercellular coupling. Representative histological sections of transmural tissue from the subepicardium and midmyocardium are shown in Fig. 6A. The dimensions of myocytes in the transmural direction are similar between different layers of tissue as illustrated by the white bars in the figure. Across all experiments (Fig. 6B), the average transmural cell dimensions for epicardial, midmyocardial, and endocardial myocytes did not differ (12.82 ± 3.31, 12.83 ± 2.39, and 12.67 ± 2.16 μm, respectively). In addition, no substantial variations in the extracellular space or invasions of inflammatory cells were observed. Taken together, these data strongly suggest that regional uncoupling between the epicardial and deeper transmural layers could not be explained by the regional variations of cell dimension or the extracellular matrix.

DISCUSSION

Cardiac gap junctions play an important role in impulse propagation and arrhythmias. In this report, we demonstrate that relative Cx43 expression is selectively reduced in the subepicardium of the left ventricular wall, which is closely associated with correspondingly selective uncoupling between subepicardial and midmyocardial cells as evidenced by localized \( \theta_{TM} \) reductions despite faster \( dV/dt_{max} \) and smaller \( \lambda_{TM} \) values. Importantly, the largest transmural APD gradients occurred in areas that exhibited reduced Cx43 expression and intercellular coupling, which suggests that Cx43 expression patterns can potentially contribute to arrhythmic substrates that are dependent on transmural dispersions of repolarization.

To our knowledge, this is the first report to describe both the transmural heterogeneities and functional consequences of Cx43 protein expression across the ventricular wall. Uniform analysis of Cx43 expression across all transmural layers was accomplished by only including imaged fields where the majority of fibers had a length-to-width ratio >4. The selective sectioning of tissue parallel to the epicardium combined with a novel method for determining whether myocytes were parallel to the imaging plane allowed for precise correlation of regional Cx43 expression with function. Importantly, the relative subepicardial Cx43 quantity was reduced by 24% compared with...
all other myocardial layers in adult canine LV. There were no significant differences in relative Cx43 expression between midmyocardial and endocardial layers. Moreover, the reduction of relative subepicardial Cx43 was observed regardless of the sectioning or immunochemistry techniques used. Cx43 primarily localized to the ends of myocytes in all transmural muscle layers, from epicardium to endocardium in the typical pattern previously reported (20). Localization of Cx43 around myocytes was similar for samples obtained from posterior, anterior, basal, and apical portions of the LV across the transmural wall, which indicates that the regional differences in cell-to-cell coupling between transmural muscle layers that we observed were associated with reduced Cx43 expression rather than variable localization of Cx43 around myocytes. Although it seems unlikely, one cannot rule out from our data that subepicardial gap junctions may have reduced conductances compared with gap junctions within deeper layers of myocardium either due to the state of Cx43 phosphorylation (6) or the heteromeric composition of Cx43 and Cx45 hemichannels (18).

To date there are little data available that describe detailed patterns of Cx43 expression in the LV. Yamada et al. (33) demonstrated a 50–60% reduction in subepicardial Cx43 expression in adult mouse LVs, which corresponds nicely with our results. They also reported that the selective reduction of subepicardial Cx43 was consistent between basal and apical portions of the LV. The magnitude of subepicardial Cx43 reduction reported by these investigators was somewhat larger than that observed in the present study, which is likely due to species differences. In contrast, Patel et al. (19) found no significant Cx43 expression differences between epicardial, midmyocardial, and endocardial layers of the canine LV. Importantly, only one measurement from each layer was obtained from each experiment; this possibly accounts for the lack of correspondence with our study. The variations in Cx43 expression seen within each transmural layer (Fig. 4B, standard deviation bars) could potentially mask the systematic and larger variations in Cx43 expression between transmural layers that were clearly evident with our method for sampling tissue with high spatial resolution.

Heterogeneous expression of Cx43 may be a cardioprotective mechanism by the production of resistive barriers during ischemia between the endocardium and the epicardium, which is highly vascularized with primary cardiac arteries and veins. Additionally, the rather selective reductions in subepicardial Cx43 could be a result of the distinct developmental origin of the epicardial organ (30). Another possible explanation may arise from the finding that Cx43 expression is dependent on pulsatile stretch (35). Midmyocardial and endocardial layers undergo greater segment shortening (11) and strain (7) than epicardial fibers, which may explain the relative increase of Cx43 expression in midmyocardial and endocardial layers. An altered activation sequence has also been associated with Cx43 remodeling, which suggests that Cx43 expression is dependent on the pattern of conduction (19). However, remodeling of Cx43 proximal to the site of pacing results in a reduction of Cx43 protein. Therefore, the activation sequence cannot explain the finding that reduced Cx43 in this study was found in the epicardium, which activates last during normal sinus rhythm.

Because protein expression does not necessarily correlate with protein function, it was important to compare the distribution of Cx43 expression to functional indices of cell-to-cell coupling across the transmural wall. Discrete conduction slowing was consistently found in subepicardial regions (33 ± 5 vs. 41 ± 6 cm/s in deeper layers) exactly where Cx43 expression...
was also reduced. The magnitude of epicardial conduction slowing (by 18%) that we observed is consistent with theoretical predictions of the extent of $\theta_{TM}$ slowing expected from the 24% reduction in subepicardial Cx43 expression measured in our experiments (23). Subepicardial $\theta_{TM}$ was consistent with previous reports of transverse conduction velocity on the epicardial surface of canine LV (9, 27), which suggests that coupling transverse and transmural fibers are similar in the subepicardium. In addition, $\theta_{TM}$ in deeper muscle layers was similar to previously reported transmural conduction velocities of 44 cm/s (34).

Importantly, we found that conduction slowing, which was localized exclusively to epicardial and subepicardial layers (see Fig. 2), was associated with significant increases in $dV/d_{t_{\text{max}}}$ (by 18%). If decreased epicardial conduction velocity (in the range we observed) were caused by depressed excitability, theory would predict a substantial reduction and not the increase of $dV/d_{t_{\text{max}}}$ that we observed (23, 28). Although the extent of $dV/d_{t_{\text{max}}}$ enhancement that we observed within epicardial layers was higher than what would be predicted theoretically from the extent of $\theta_{TM}$ slowing (23, 28), the direction of change (increase rather than decrease) is completely consistent with an effect of localized uncoupling within epicardial layers of muscle. To further validate the interpretation of changes in $dV/d_{t_{\text{max}}}$ as an index of intercellular coupling, we conducted three additional experiments in the canine wedge preparation using carbamolone and flecainide (a class IC antiarrhythmic drug) to directly compare the effects of uncoupling vs. depressed excitability, respectively, on $dV/d_{t_{\text{max}}}$ values. These drugs were administered at sufficient concentrations to slow conduction equally and to the same extent (by 20%) that conduction was slowed in subepicardium relative to deeper myocardial layers. Although intercellular uncoupling by carbamolone did not change $dV/d_{t_{\text{max}}}$ values, reducing $\theta_{TM}$ to the same extent by flecainide significantly decreased $dV/d_{t_{\text{max}}}$ (by 25.9 $\pm$ 5.6%; $P < 0.05$) as predicted by computer-simulation studies (23). Hence $dV/d_{t_{\text{max}}}$ is more sensitive to changes in excitability than intercellular coupling. The fact that we found significantly faster $dV/d_{t_{\text{max}}}$ values despite slower $\theta_{TM}$ in subepicardium indicates that slowing of conduction in the subepicardium cannot be explained by depressed excitability and was likely attributable to localized intercellular uncoupling.

To further establish the functional significance of heterogeneous Cx43 expression across the transmural wall and, specifically, reduced Cx43 expression in subepicardial layers, we used $\lambda_{TM}$ as an empirical index of transmural coupling between muscle layers (2). Subepicardial $\lambda_{TM}$ was significantly reduced compared with deeper layers, which nicely corresponded with our findings on subepicardial $dV/d_{t_{\text{max}}}$ and $\theta_{TM}$ and reaffirms the presence of regional intercellular uncoupling within subepicardial compared with deeper transmural layers. The biophysical principles underlying the estimate of $\lambda$ are based on one-dimensional tissue, which predicts that $\lambda = [R_{\text{m}}/(R_{\text{m}} + R_{J})]^{1/2}$, where $R_{\text{m}}$, $R_{J}$, and $R_{I}$ are the membrane, extracellular, and intracellular resistances, respectively (32). Although it is assumed that $R_{\text{m}}$ is constant during delivery of the subthreshold stimulus during diastole, the lack of observable macroscopic changes in the extracellular matrix does not preclude microscopic changes that may also substantially affect the EP measurement in this study. Our estimate of $\lambda_{TM}$ served as a useful index of intercellular uncoupling, but its absolute magnitude cannot necessarily be compared with that obtained from the classic derivation of $\lambda$ (32). However, the finding that $\lambda_{TM}$ is reduced in the subepicardium is consistent with heretofore-unexplained findings of increased tissue resistivity in the subepicardium (34).

It remains difficult to fully explain the discrepancies that frequently arise between theoretical predictions and experimental measurements that attempt to correlate Cx43 expression to function. There are several potential explanations why Cx43 protein quantity does not necessarily lead to predictable changes in intercellular coupling, including the following: 1) not all Cx43 protein is localized to the cell membrane to form gap junctions; 2) the phosphorylation state of Cx43 may influence gap junctional conductance independent of Cx43 quantity (6); 3) variations in cell size and capacitance can influence the relative contribution of gap junctions to propagation (26); 4) although the dynamic stoichiometry of connexins is poorly understood, there is evidence that colocalization of other connexins to gap junctions can be influenced by expression of Cx43 (13), and the formation of hybrid channels composed of various connexin proteins can influence unitary conductance of each gap junction; and 5) $\lambda$ is also dependent on both intra- and extracellular resistivities, and our data do not distinguish between the two (32). However, the absence of transmural differences in the extracellular matrix or cell size further suggests that transmural EP heterogeneities are dependent on reduced intercellular conductance (i.e., Cx43 expression) and not increased intracellular or extracellular resistances. It is imperative to continue to reexamine theoretical models in light of new experimental results to further improve our understanding of the underlying relationship between gap junction expression and function.

We found that APD gradients are most pronounced between the subepicardial and midmyocardial layers, which is precisely where relative Cx43 expression is most reduced. Yan et al. (34) found a similar sharp transition in APD between subepicardial and deep subepicardial layers in intact heart preparations. M cells in LV have been noted to occur at depths of 1.5–5.2 mm from the epicardium (25), thereby providing the driving force for transmural APD gradients. One would expect that transmural APD gradients arise, on the one hand, from transmural heterogeneities in ion channel expression and function, and on the other hand, are opposed by the extent of intercellular coupling at the interface between these transmural layers. Previously, we showed that large APD gradients between the subepicardial and midmyocardial layers underlie arrhythmias in a long QT syndrome variant-2 model under conditions of apparently normal coupling (3). The findings in the present study can potentially explain a mechanism responsible for maintaining transmural dispersion of repolarization because we (3) and others (25) have previously demonstrated that the epicardial-M cell interface can reside within 2 mm of the epicardial surface, which is precisely where relative Cx43 expression and intercellular coupling was found to be the lowest in the present study. It is also possible that Cx43 remodeling by diseases such as heart failure can be responsible for transmural inhomogeneities of repolarization that give rise to life-threatening cardiac arrhythmias (1). However, the functional consequences of reduced subepicardial Cx43 values are not necessarily straightforward, because there is a complex
interplay between spatial gradients of ion channels across the transmural wall and cell-to-cell coupling. Nonetheless, these data suggest that patterns of expression of Cx43 across the transmural ventricular wall can be a mechanism for maintaining physiological and pathophysiological inhomogeneities of repolarization in syncytial preparations.

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