Architectural Correlates of Myocardial Conduction
Changes to the Topography of Cellular Coupling, Intracellular Conductance, and Action Potential Propagation with Hypertrophy in Guinea-Pig Ventricular Myocardium

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Background—We tested the hypothesis that alterations to action potential conduction velocity (CV) and conduction anisotropy in left ventricular hypertrophy are associated with topographical changes to gap-junction coupling and intracellular conductance by measuring these variables in the same preparations.

Methods and Results—Left ventricular papillary muscles were excised from aortic-banded or sham-operated guinea-pig hearts. With intracellular stimulating and recording microelectrodes, CV was measured in 3 dimensions with simultaneous conductance mapping with subthreshold stimuli and correlated with quantitative histomorphometry of myocardial architecture and connexin 43 distribution. In hypertrophied myocardium, CV in the longitudinal axis was smaller and transverse velocity was greater compared with control; associated with similar differences of intracellular conductance, consistent with more cell contacts per cell (5.7±0.2 versus 8.1±0.5; control versus hypertrophy), and more intercalated disks mediating side-to-side coupling (8.2±0.2 versus 10.2±0.4 per cell). Intercalated disk morphology and connexin 43 immunolabelling were not different in hypertrophy. Hypertrophied preparations showed local submillimeter (=250 μm) regions with slow conduction and low intracellular conductance, which, although not affecting CV on the millimeter scale, were consistent with discontinuities from increased microscopical connective tissue content.

Conclusions—With myocardial hypertrophy, altered longitudinal and transverse CV, and greater nonuniformity of CV anisotropy correspond to changes of intracellular conductance. These are associated with alteration of myocardial architecture, specifically the topography of cell–cell coupling and gap-junction connectivity. (Circ Arrhythm Electrophysiol. 2014;7:1198-1204.)

Key Words: action potentials connexin 43 gap junctions left ventricular hypertrophy slowed conduction velocity

In intact myocardium the 3-dimensional (3D) distribution of gap-junctions is a function of cellular architecture and distribution of cell–cell interactions, so that the topography of intracellular conductance is a major determinant of action potential (AP) propagation. Computational,1 cell-culture,2 and transgenic models3 support this concept. The relationship between longitudinal and transverse conduction (conduction anisotropy) and small-scale nonuniformities are critical determinants of the arrhythmogenic substrate in ventricular and atrial myocardium.4,5 Although not yet measured, the relationship between intracellular conductance, AP conduction, and myocardial architecture, and how they change in myocardial pathologies, is considered to be important to characterize the myocardial substrate for complex arrhythmias and the mechanistic understanding of fibrillation in particular. Such an integrated approach requires an appropriate model when changes to myocardial structure and AP conduction occur. Myocardial hypertrophy represents such a pathology, whereby alterations to connexin expression, cell and tissue architecture and AP propagation have been separately reported in different experimental models using various techniques to measure conduction.6–12 However, these studies are often difficult to compare because of experimental differences. One study, using a model of nonischemic heart failure, did relate conduction velocity (CV) changes to myocyte dimensions and predict electrocardiographic changes.13 We used a well-characterized model of myocardial hypertrophy to address the hypothesis that conduction anisotropy and nonuniformity is determined by changes of intracellular conductance, in turn determined by the cellular topography and distribution of gap junctions on the submillimeter and millimeter scale.

Clinical Perspective on p 1204
Methods

Methods are described in the Data Supplement.

Model of Ventricular Hypertrophy

Ventricular hypertrophy was induced in male Dunkin–Harley guinea pigs (4 to 6 months), by placing a constricting clip around the ascending aorta. Sham controls underwent identical operations, but without clip placement.14 Left ventricular papillary muscles were excised for electrophysiological recordings, and adjacent muscles placed in Zamboni fixative for morphological examination. These preparations were used because of their longitudinal fiber arrangement and less interstitial tissue. The investigation and surgical procedures conformed to the Guide for the Care and Use of Laboratory Animals (UK Animals Act, 1986, and the US National Institutes of Health; NIH publication No. 85-23, revised 1996), the study was approved by the Local Ethical Review Committee.

1D Intracellular Conductance and CV

Left ventricular preparations (0.5–to 0.9-mm diameter) were superfused (4 mL/min) with Tyrode solution at 36°C. Longitudinal CV, \( CV_L \), was measured by stimulating the preparation at one end and recording multiple intracellular conducted AP at distances, \( d \), > 1 mm from the stimulating electrode. Conduction delay was the time, \( t \), between stimulus artifact and \( d \)-V, the AP upstroke. \( CV_L \) was calculated from the slope of linear \( d \)-versus-\( t \) plots. Specific intracellular conductance was calculated from \( G = \frac{CV_L \times \tau_y}{a} \), where \( a \) = myocyte radius, \( C_m \) = specific membrane capacitance, and \( \tau_y \) = time constant of the conducting AP subthreshold phase. Transverse \( CV, CV_T \), was measured by stimulating with large Ag/AgCl electrodes on either side of the preparation; intracellular recordings were made perpendicular to the longitudinal axis15 (Figure I in the Data Supplement).

3D Electrophysiological Measurements

Supra-threshold or subthreshold pulses were passed between 2 intracellular microelectrodes to measure CV or intracellular conductance (g), respectively. Interelectrode tip distance was measured within 20 \( \mu \)m (Figure II in the Data Supplement). The current-passing electrode remained in 1 cell and the recording electrode moved to 40 to 60 different sites to obtain 3D conduction or conductance maps. Impalement depth was measured to determine accurately interelectrode distance and incorporated into a 2D data display: the x axis corresponded to the longitudinal fiber axis and the y′ axis to the distance \( V \) (y′z′), that is, y (transverse) and z (depth) axes were assumed equivalent.

Data are displayed in \( x \)- and \( y \)-planes as conduction delay iso-chrones or lines of equal conductance. The direction with the fastest CV and largest conductance per unit distance was the \( x \) axis. Perpendicular values were measured to estimate anisotropic ratios of conduction and conductance. In regions where conductance was relatively homogeneous the time course of membrane potential responses to subthreshold stimuli were fitted to a 3D solution of the cable equation (Figure 2A, see Results section).16

Western Blotting

Proteins from whole-tissue homogenates were resolved by 12% polyacrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were incubated with connexin 43 (Cx43) primary antibody (polyclonal antimouse, Millipore; 1:1000 dilution) and subsequently with horseradish peroxidase–conjugated secondary antibody (goat antimouse, Millipore; 1:10000 dilution). Protein bands were quantified by densitometric analysis, and normalized to corresponding actin protein densities, which were unaltered between control and left ventricular hypertrophy (LVH) animals.17,18

Connexin Immunohistochemistry

Preparations in Zamboni fixative were wax embedded. Hematoxylin and eosin–stained sections identified: tissue orientation, quantified tissue morphology, and cell dimensions. Myocyte cross-sectional areas were measured in 210 cells per section, from 25 sections per specimen.19 Sections (10 \( \mu \)m) were labeled with a rabbit polyclonal E12H anti-Cx43 antibody,20 dilution (1:50), Cx40 and Cx45 immunolabelling used antibodies,21,22 diluted 1:10 for Cx40 and 1:20 for Cx45. Secondary incubation was with antirabbit Cy3 (Chemicon International Inc., 1:500). TRITC-conjugated wheat-germ agglutinin (Sigma, 1:100) labeled cell membranes. Nuclei were labeled with the fluorescent nucleic acid–binding dye TO-PRO-3 (Invitrogen, 1:250).

Myocyte and Tissue Topology

A previously published method was used.23 Preparations were fixed in Na cacodylate buffer (0.08 mmol/L), glutaraldehyde (2%), and CaCl2 (2 mmol/L); postfixed in 1% osmium tetroxide; dehydrated and embedded in araldite resin. A series of 100 1-\( \mu \)m serial sections were cut in the plane longitudinal to the fiber axis, mounted sequentially and stained with methylene blue. For each sample, 6 separate index myocyte profiles were randomly selected in the 50th section and followed through the entire section series to identify the number of cells with which each index cell was coupled. Connective tissue percentage was estimated from sections fixed with 4% paraformaldehyde, stained with picrosirus red; relative areas of connective tissue and muscle were measured.

Quantification of Cx43 Signal Area

Confocal images of 6, randomly selected high-power (\( \times 630 \)) fields of transversely sectioned tissue and an optical projection image of each en-face disk were examined to derive Cx43 signal area per disk and unit disk area. Cx43 signal density was total Cx signal area divided by total area occupied by myocytes. The Cx43 area per cell was calculated as total Cx43 signal area divided by cell dimensions.

Statistical Analyses

Values are medians (25, 75% interquartiles) without assumption about the normality of the data sets, n=number of hearts. Differences between sets were tested with Wilcoxon rank-sum tests, the null hypothesis was rejected at \( P<0.05 \). Specific \( P \) values are quoted except when \( P<0.0001 \). The association between 2 variables was assessed from the Spearman rank correlation coefficient, \( r \).

Results

Heart-to-body weight ratio was greater in the aortic constriction (LVH) compared with the sham (control) group (4.8 [4.3, 5.3] versus 3.8 [3.5, 3.9] g/kg; \( n=13, 13 ; P<0.0001 \)). There were no signs of LV failure (lung-to-body weight ratio 4.9 [4.2, 5.0] versus 4.6 [4.3, 4.8] g/kg; \( P=0.28 \)) and no liver ascites. The greater heart-to-body weight ratio was mirrored by a larger cell diameter but no change to cell length (Table 1).

Quantitative Western blotting showed that overall Cx43 expression was similar in myocardium from control and LVH hearts (Figure 1A). The topography and 3D distribution of gap junctions was measured to correlate with changes to possible electrophysiological properties in hypertrophied myocardium.

Myocardial Topography and Distribution of Interolated Disks

The general topographical arrangement of cells was preserved in hypertrophy: myocytes retained approximately cylindrical shapes, with a major intercalated disk at each end, but more lateral connections in LVH tissue (Figure 1B). Overall packing geometry and connectivity between adjacent cells were estimated from the number of adjacent cells with which an individual cell made intercalated disk contact. This was
significantly greater in hypertrophied samples, compared with control. Figure 1C shows a representative photomicrograph of the middle of 3 serial sections, with cell outlines drawn in adjacent panels, to illustrate the number and pattern of cell contacts with which a particular index cell (cell A in this example) makes contact. Cell A had 2 end-to-end abutments (cells D and G in the middle section); the number of end-to-end abutments per cell was unchanged in hypertrophy. The greater number of cells to which an individual myocyte makes contact in LVH myocardium was mirrored by more intercalated disks per cell (Table 1) from more lateral abutments. The greater number of cell-to-cell contacts in LVH tissue was not because of cell elongation (Table 1). However, the number of intercalated disks coupling any 2 adjacent cells was fewer in hypertrophy (1.48 [1.37, 15.5] versus 1.26 [1.07, 1.39]; \( P < 0.0135 \)) because of the greater number of separate cell contacts. Overall, with LVH tissue, a particular cell made side-to-side contact with more cells, but via fewer intercalated disks between any 2 cells.

### Connexin Quantification and Distribution

The number of intercalated disks per cell was greater in LVH myocytes (Table 1). However, individual intercalated disks showed no significant architectural changes: disk area was similar, as was the proportion of intercalated disk labeled for Cx43. In addition, Cx43 labeling as a percentage of cell perimeter, the latter measured with wheat-germ agglutinin, was not different in LVH. However, the total area labeled for Cx43 was greater (Table 1) and reflects the larger membrane area of LVH myocytes and more intercalated disks. However, the amount of lateral Cx43 labeling was =5-fold more because of the greater number of lateral intercalated disks. Thus, myocyte hypertrophy was accompanied by more intercalated disks and Cx43 per myocyte. There was no significant difference to Cx43 density, commensurate with the above Cx43 expression data, but the greater absolute amount of Cx43 per cell was confined largely to lateral myocyte boundaries.

Cx40 immunolabelling was not detected in control or LVH hearts, but as a positive control was demonstrated in atrial tissue. Cx45 immunolabelling was indistinguishably sparse in control and hypertrophy myocardium.

### Table 1. Cell Dimensions, Distribution, and Morphology of Intercalated Disks and Cx43 Distribution in Control (n=12) and LVH (n=8) Myocardium: n=11 for Control Myocyte Diameter and Length

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LVH</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter, ( \mu m )</td>
<td>17.5 (15.7, 18.1)</td>
<td>21.1 (19.4, 21.8)</td>
<td>0.00019*</td>
</tr>
<tr>
<td>Cell length, ( \mu m )</td>
<td>154 (116, 178)</td>
<td>147 (134, 166)</td>
<td>0.971</td>
</tr>
<tr>
<td>Cell contacts per cell</td>
<td>5.5 (5.4, 6.1)</td>
<td>8.6 (7.0, 9.1)</td>
<td>0.0039*</td>
</tr>
<tr>
<td>Intercalated disks per cell</td>
<td>8.3 (8.1, 8.6)</td>
<td>10.1 (9.1, 11.4)</td>
<td>0.00028*</td>
</tr>
<tr>
<td>Intercalated disk area, ( \mu m^2 )</td>
<td>30.9 (21.8, 38.5)</td>
<td>32.1 (21.6, 38.3)</td>
<td>1.000</td>
</tr>
<tr>
<td>Cx43 area per unit area of disk</td>
<td>0.30 (0.25, 0.32)</td>
<td>0.30 (0.22, 0.41)</td>
<td>0.972</td>
</tr>
<tr>
<td>Cx43 labeling, % WGA perimeter</td>
<td>2.6 (2.2, 3.3)</td>
<td>2.8 (2.7, 3.1)</td>
<td>0.851</td>
</tr>
<tr>
<td>Cx43 labeled area cell, ( \mu m^2 )</td>
<td>199 (189, 212)</td>
<td>252 (236, 335)</td>
<td>0.00031*</td>
</tr>
<tr>
<td>Lateral Cx43 labeling, % total</td>
<td>5.0 (4.2, 5.6)</td>
<td>24.0 (18.5, 26.2)</td>
<td>0.00025*</td>
</tr>
</tbody>
</table>

Median values (25, 75% interquartiles). Cx43 indicates connexin 43; and LVH, left ventricular hypertrophy.

*\( P < 0.05 \) (LVH vs control).

Figure 1. Connexin 43 (Cx43) and myocardial architecture. A, Cx43 protein expression, normalized to actin, for control and left ventricular hypertrophy (LVH) myocardium (\( n=8, 5; P=0.378 \)). B, Confocal sections (left, control; right, LVH) immunolabelled for Cx43 (red), mainly confined to intercalated disks at ends of cells (arrows), or at lateral sites (arrowheads) especially in LVH. Cell membrane, green; nuclei, blue; cell membrane/Cx43 overlaps, yellow. C, Photomicrograph (\( \times 400 \)) of an LVH myocardium section; shown is 1 from the middle of a consecutive series of one hundred 1-\( \mu m \) thick sections. Cell-to-cell interactions between an index cell (A) and its neighbors (B–G) traced through the whole cell thickness. Arrowheads mark intercalated disks and ends of cell A. Cartoons of this section and those immediately above and below are shown.
evident when both CVL and transverse CV, CVT, were measured together; CVL was less (61.1 [57.9, 65.2] versus 76.6 [75.7, 79.3] cm/s; n=7, 8; P=0.00062) and CVT greater (24.5 [21.7, 27.9] versus 18.7 [17.5, 19.8] cm/s; P=0.0114) in LVH. This resulted in a smaller conduction anisotropy ratio in LVH (CVL/CVT; 2.36 [2.22, 2.91] versus 4.20 [3.71, 4.61]; P=0.00075). τap was significantly smaller in transverse compared with longitudinal conducted APs, but in either axis were similar in LVH and control preparations (longitudinal, 0.27 [0.25, 0.28] versus 0.26 [0.26, 0.28] ms; P=0.820; transverse, 0.23 [0.22, 0.25] versus 0.24 [0.22, 0.26] ms; P=1.00). This excludes changes of ionic currents flowing during the AP upstroke as a cause of altered CV in LVH. Total intracellular specific conductivity, Gi, was estimated from longitudinally conducted APs and the 1D solution of the cable equation (see Methods section). Gi was significantly smaller in LVH (1.04 [0.64, 1.47] versus 2.17 [2.02, 2.47] mS/cm; P=0.00029) using values of ai, τap, and CVL.

3D CV, CV_dip, and Intracellular Conductance, g_3D

Figure 2 shows intracellular recordings from a preparation with uniform conduction and conductance characteristics, to test if data could be analyzed assuming a homogeneous spatial structure. A subthreshold stimulus response conformed to the transient solution of the 3D-cable equation (part A). The relationship between steady-state response, v_0, and stimulating current magnitude (i) was linear for subthreshold responses (part B). Values of v_0 as a function of interelectrode distance, were fitted to a 3D solution of the cable equation (part C) and in 4 experiments yielded values for the 3D space constant of 0.31, 0.21, 0.19, and 0.16 mm. Part D shows the upstroke phase of APs from 2 intracellular recording sites a known distance (d) apart, and their differentials (dV/dt). CV was calculated from the ratio of d and difference in delays (t2-t1).

Figure 3 shows anisotropy of conduction (part A) and intracellular conductance (part B) in control myocardium, with a primary (horizontal) axis of higher CV and conductance, and a perpendicular axis of minima. Table 2 shows CVs with an anisotropy ratio in control tissue (3.48 [3.11, 5.23]) similar to that measured with 1D conduction (3.84 [3.60, 4.07]), where the latter values were recorded on a supramillimetre scale. The 3D conductance ratio from subthreshold pulses was (3.95 [3.03, 5.29]). In LVH, CV in the primary axis was significantly smaller, but greater in the transverse axis (Table 2), corresponding with findings with 1D propagation. Intracellular conductance anisotropy was similarly smaller in LVH tissue.

Figure 4 shows the relationship between paired CV and conductance anisotropy ratios for control (open squares) and LVH (closed circles) data. For the control and LVH data combined, there was a significant association between these anisotropy ratios (r=0.95; n=15; P<0.0001). These data are therefore consistent with the hypothesis that variation in CV anisotropy depends on changes to anisotropy of intracellular conductance, with LVH values at the smaller region of the spectrum of values.

Figure 5 shows a phenomenon in several LVH preparations, namely small-scale conduction (part A) and conductance (part B) discontinuities—where isochronal and isoconductance lines are bunched, indicating local regions of low intracellular conductance. This was not observed in sham-operated preparations, but was in

Figure 2. Intracellular stimulation and recording. A, subthreshold response to a 0.1 nA current; v_0, steady-state response: line is a fit to the 3D cable equation:

\[ V(r, t) = \frac{k}{r} \left( e^{-\frac{r}{2\lambda}} \text{erfc} \left( \frac{r}{2\lambda \sqrt{t}} \right) + e^{\frac{k}{r}} \text{erfc} \left( \frac{r}{2\lambda \sqrt{t}} + \sqrt{t} \right) \right); \]

r, interelectrode distance; k, constant; erfc, complementary error function. B, current–voltage relationship of v_0 as a function of subthreshold current. A and B, r=200 μm. C, plot of v_0 (0.1 nA stimulation) as a function of interelectrode distance, r. Line is a plot of v_0 = \frac{k}{r} \exp(-\frac{r}{2\lambda}), \lambda=3D space constant, K=constant. D, upstroke phases of 2 action potentials and their first derivative with suprathreshold stimulation: t1 and t2 are delays for conduction to the 2 recording sites from the stimulus site, S. Distance between 2 recording electrodes 480 μm.

Figure 3. Conduction and conductance maps, normal myocardium (A) isochrones of conduction delays (ms). The black circle marks the stimulation point. B, lines of equal conductance (mS) for the same preparation.
5 of 12 LVH preparations. The submillimetre scale of the discontinuity would not affect macroscopic CV. Data from these high resistance regions were not included in Table 2, but they would accentuate conduction slowing and nonuniformity, and contribute further to a smaller intracellular conductance in the primary axis of LVH preparations. There was also more connective tissue in LVH preparations (11.7 [9.1, 16.0] versus 3.5 [3.2, 3.7]%; \(P=0.00025\); n=8, 12), appearing sometimes as longitudinally orientated septae between myocyte bundles (arrow, Figure 5C).

**Discussion**

The principal finding with this well-characterized model of myocardial hypertrophy was that both CV and intracellular conductance anisotropy were reduced and associated with changes to myocyte architecture, that is, more side-to-side Cx43 intercalated disks between larger myocytes, but each cell making contact with more neighboring cells.

Previous studies quantifying Cx43 in hypertrophic myocardium are conflicting, with increased, unchanged or reduced immunolabelled Cx43 per cell in different models, including human myocardium secondary to aortic stenosis,10,17,24–26 These disparities probably result from differences in the duration and stimulus of hypertrophy, as well as species variability, and emphasize the need to study the relationship between AP condu-
duction, intracellular conductance, and myocardial architecture in a single model and at greater resolution, as here.

There was a close correspondence between AP CV and intracellular conductance in both normal and hypertrophied myocardium. Previous studies using separate preparations to measure these variables only inferred this relationship,8,9 but experiments directly demonstrated this and provides an electric basis for myocardial conduction anisotropy.

An additional notable finding was the change to the topography of cell contacts and gap junctions in hypertrophied myocardium. Although the number of intercalated disks per cell increased, the proportionately greater number of cell-to-cell contacts resulted in fewer intercalated disks between any 2 myocytes. The larger number of disks per cell has also been measured in human myocardium with concentric hypertrophy.27 Ventricular myocardial cells are approximately cylindrical, with large intercalated disks at each end and smaller lateral disks. The basic cardiomyocyte shape, a cylinder with intercalated disks at both ends, is preserved in hypertrophy: thus, more intercalated disks per cell represents a greater number along the sides of the cell. The greater side-to-side connectivity and cell diameter are both consistent with the reduction in anisotropy of both conduction and intracellular conductance, as hypothesized by others.28 This structural remodelling has also been observed in human hypertrophied myocardium,17,21 and suggests that the observed electrophysiological changes may also occur in human tissue. Increased myocyte diameter in LVH (≈20%, Table 1) per se would enhance transverse CV at most by ≈9%. The actual increase of transverse CV by 48% (Table 2, CV\text{max}) must therefore result mainly from gap-junction remodeling.

Although enhanced transverse coupling reduces charge available for longitudinal propagation, the architectural findings will not entirely explain the reduced longitudinal CV and associated reduction of intracellular conductance. With LVH intracellular longitudinal conductance, \(g_{\text{long}}\), was reduced to ≈55%. Assuming cytoplasm and gap-junction conductance are in the ratio 3:1, and cytoplasm conductance is unaltered,4,29 this represents more than a halving of unit gap junction conductance. There was no evidence of altered gap-junction structure, with respect to Cx43 immunolabelling, and so a reduced unit gap-junction conductance is proposed, including contributions from lowered intracellular pH30,31 and dephosphorylation of gap-junction proteins.32

Reduced gap-junction conductance, and hence longitudinal current flow and AP propagation, could also contribute to enhanced transverse CV, by raising local intracellular current density. With a nonischemic model of heart failure longitudinal and transverse conduction velocities, as well as myocyte diameter, were increased,13 suggesting that morphological changes alone could support these conduction changes, in contrast to data from this study that also requires changes to the electrophysiological properties of gap junctions.

There was also close concordance between directly measured intracellular conductance changes in hypertrophy and calculations using cable theory. Furthermore, cable theory accurately described

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**Table 2. Maximum and Minimum 3D CV or Conductance, \(g\), and Anisotropy Values in Control (n=8) and LVH Myocardium (n=7)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LVH</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV\text{max}, cm/s</td>
<td>54.5 (48.4, 64.7)</td>
<td>30.5 (27.6, 37.0)</td>
<td>0.00062*</td>
</tr>
<tr>
<td>CV\text{min}, cm/s</td>
<td>11.2 (10.4, 11.9)</td>
<td>16.6 (14.7, 19.6)</td>
<td>0.0208*</td>
</tr>
<tr>
<td>CV anisotropy ratio</td>
<td>4.93 (4.37, 5.57)</td>
<td>1.81 (1.58, 2.52)</td>
<td>0.00015*</td>
</tr>
<tr>
<td>(g_{\text{max}}), mS/cm</td>
<td>6.27 (4.80, 6.62)</td>
<td>3.50 (3.41, 4.76)</td>
<td>0.00257*</td>
</tr>
<tr>
<td>(g_{\text{min}}), mS/cm</td>
<td>1.20 (0.99, 1.58)</td>
<td>1.70 (1.33, 1.96)</td>
<td>0.0247*</td>
</tr>
<tr>
<td>(g) anisotropy ratio</td>
<td>3.95 (3.03, 5.29)</td>
<td>1.87 (1.55, 2.42)</td>
<td>0.00062*</td>
</tr>
</tbody>
</table>

Median values (25, 75% interquartiles). CV indicates conduction velocity; and LVH, left ventricular hypertrophy.

*\(P<0.05\) (LVH vs control).

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**Figure 4.** The relationship between conduction velocity (CV) and intracellular conductance anisotropy ratios. Data from control (open squares) and left ventricular hypertrophy (LVH; closed circles) myocardium, each point is a separate preparation.
the time- and distance-dependent spread of subthreshold currents in a 3D continuum. However, in several hypertrophied preparations small-scale conduction and conductance discontinuities (<1 mm) were measured (Figure 6), which would not alter net CV, or be observable using extracellular recording techniques. However, the magnitude and abruptness of these changes will create nonuniformities of conduction that are recognized as important components of the arrhythmogenic myocardial substrate.33

Limitations
Papillary muscles were used because their arrangement of parallel myocardial fibres facilitated comparative electrophysiological and morphological measurements. However, extrapolation to other regions of the ventricle should be done. Moreover, the presence of Cx43 immunolabel at the cell membrane does not confirm localization at intercalated discs or functional gap junctions.

Conclusions
AP CV in normal and hypertrophied myocardium is determined primarily by the conductance of the intracellular pathway. Changes to CV and intracellular conductance anisotropy in hypertrophy may be explained by altered myocyte and intercalated disk topography.

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Disclosures
None.

References

CLINICAL PERSPECTIVE
Left ventricular hypertrophy (LVH) is associated with an increased risk of arrhythmias but the electrophysiological substrate in myocardium that underlies this significant clinical condition remains unclear. We have addressed the question using a well-validated guinea-pig model of LVH, after partial occlusion of the thoracic aorta. Action potential conduction anisotropy is a phenomenon whereby conduction is more rapid in a primary axis compared with perpendicular axes and in normal myocardium is a significant factor that minimizes arrhythmic re-entrant circuits. The data with LVH myocardium show that conduction anisotropy—measured with intracellular stimulating and recording electrodes—is reduced, slowed in the primary axis and unchanged or even increased in orthogonal axes. This electric remodeling occurs on a submillimeter scale and so would be less evident using extracellular recording methods. The loss of conduction anisotropy was mirrored by similar changes to the electric conductance of the intracellular space, the latter determined mainly by gap-junction electric properties. We attributed these electric changes in LVH myocardium to structural remodeling of the myocardium, with more transverse intercalated disks, along with reduced electric conductance of individual gap junctions. These data provide evidence that changes to the distribution and electric properties of gap junctions in LVH are a major contributor to changes of the electrophysiological substrate of myocardium that makes it prone to the development of arrhythmias.
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SUPPLEMENTAL MATERIAL

Methods

Model of ventricular hypertrophy: Ventricular hypertrophy was induced in male Dunkin-Hartley male guinea pigs, (n=18; age 4-6 months), by constriction of the thoracic aorta with a clip, internal diameter 2mm. The control group (n=23) underwent identical operations, but without clip placement: full details have been published previously.1 Hypertrophy was induced by leaving the animals for 52±5 days and produced a spectrum of increased heart-to-body weight ratios. Hearts were immediately dissected and left ventricular papillary muscles or trabeculae were excised for electrophysiological recordings, and an adjacent muscle placed in Zamboni’s fixative for morphological examination. These preparations were chosen because of their longitudinal fibre arrangement and smaller amount of fibrous, interstitial tissue. The investigation and surgical procedures conformed to the Guide for the Care and Use of Laboratory Animals (UK Animals Act, 1986 and the US National Institutes of Health; NIH publication No. 85-23, revised 1996). This study was initially approved by the Local Ethical Review Committee (University College London) and then submitted to the UK Home Office who granted a license to carry out the above procedures.

Intracellular conductance and conduction velocity measurements: Left ventricular preparations up to 5 mm long and 500-900 µm diameter were used. In a subset of experiments transverse conduction was also measured with wider preparations (up to 1.0 mm) but were carefully chosen not to be thicker than 300 µm. Preparations were mounted horizontally and superfused with Tyrode’s solution (mM): NaCl, 118, NaHCO₃, 24; NaH₂PO₄, 0.4; MgCl₂, 1.0; CaCl₂, 1.8; glucose, 6.1; Na pyruvate, 5.0; gassed with 5% CO₂-95% O₂ at
37°C. Conduction velocity in the longitudinal axis, $CV_L$, was measured by stimulating the preparation (1 Hz, 1.5-times threshold) at one end with Ag/AgCl-coated extracellular electrodes (125 µm diameter) and recording conducted action potentials (AP) with intracellular microelectrodes at distances, $d$, more than 1 mm from the stimulating electrode; impalments were <100 µm from the preparation surface. Maximum AP upstroke rate, $dV/dt_{max}$ was obtained by analogue differentiation of the AP signal and conduction delay, $t$, was the time between the stimulus artifact and $dV/dt_{max}$: $CV_L$ was calculated from the slope of a linear $d$ vs $t$ plot. The time constant of the AP sub-threshold phase, $\tau_{ap}$, was measured from the slope of a semi-logarithmic plot of membrane potential as a function of time, $t$, before threshold was attained. The specific intracellular conductance, $G_i=(2CV_L^2.C_m.\tau_{ap})/a$ calculated; $a$=myocyte radius (below); $C_m$=specific membrane capacitance (1µF.cm$^{-2}$). Transverse conduction velocity, $CV_T$, was measured by stimulating the muscle with large Ag/AgCl electrodes on either side of the preparation and restricting the extracellular space. Intracellular recordings were made perpendicular to the longitudinal fibre axis, as described previously. Care was taken to ensure that conduction was limited to the longitudinal or transverse planes of interest. Supplemental Figure 1 shows data from a preparations designed to measure CV in the longitudinal (part A) or transverse (part B) planes. In part A four separate longitudinal tracks were used, three at a depth of 50-100 µm from the surface and one at 100-150 µm depth. The values of $CV$ were similar in all tracks indicating that a uniform conduction plane was achieved. Furthermore, the deeper track yielded a similar value to the superficial tracks, indicating that tissue was viable in the surface and deeper tracks. Part B shows that in three separate transverse tracks $CV$ values were also similar.
**Three-dimensional (3-D) electrophysiological measurements:** Intracellular pathway conductance ($g$) was measured directly to quantify the relationship between AP conduction velocity and $g$. This was done with two intracellular microelectrodes, one to pass current and the other to record membrane potential ($E_m$) changes. Suprathreshold current pulses (10-20 µs) elicited conducting APs and subthreshold pulses (14 ms) generated electrotonic $E_m$ responses. Microelectrodes were mounted on micromanipulators with Vernier scales to allow calculation of interelectrode tip distance with and accuracy of 20 µm (Supplemental Figure 2). A current-passing electrode remained in one cell and a recording electrode was moved to different sites (40-60) in a series of linear tracks at various angles to the longitudinal axis of the preparation. At each recording site, suprathreshold and subthreshold responses were recorded to generate superimposable conduction and conductance maps. Although all impalements were limited to a depth of 50 µm, depth was taken into account to determine accurately interelectrode distance and incorporated into a two dimensional display of data, such that one axis corresponded to the longitudinal fibre axis ($x$-axis, Supplemental Figure 2) and the other to the distance $\sqrt{y^2+z^2}$ ($y'$-axis), i.e. $y$ (transverse) and $z$ (depth) axes were assumed equivalent.

Data are displayed in the $x$- and $y'$-plane as isochrones of conduction delay between stimulating and recording points, or as lines of equal conductance. The direction showing the fastest $CV$ and largest conductance per unit distance was taken as the $x$-axis. Values perpendicular to this axis were also measured to estimate the anisotropic ratio of conduction and conductance. In regions of the preparation where variation of conductance was relatively homogeneous, the time course of membrane potential responses to subthreshold stimuli was fitted to a three-dimensional solution of the cable equation.\textsuperscript{2}
Conductance values were normalised (mS.cm\(^{-1}\)) to unit interelectrode distance for comparison between preparations.

**Western blotting:** Left ventricular papillary muscles were snap-frozen in liquid N\(_2\). A whole-tissue homogenate was prepared with RIPA lysis buffer (with protease inhibitors, 1:100) and protein concentration determined using bovine serum albumin as a standard in a Bio-Rad (UK) protein assay. Protein (50 µg) from each sample was prepared in NuPAGE® LDS sample buffer 4X (all reagents Invitrogen Life, UK) with NuPAGE® sample-reducing agent 10X. Proteins were resolved by 12% polyacrylamide SDS-PAGE and transferred to PVDF membranes for two hours at room temperature (RT). Membranes were blocked for two hours at RT then incubated overnight with primary antibody for Cx43 (polyclonal anti-mouse, Millipore; 1:1000 dilution) in 1% BSA; Tris-buffered saline; Tween (TBS-T). Membranes were washed with TBS-T and incubated for one hour at RT with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse, Millipore; 1:10000 dilution). After a final TBS-T wash, protein bands were detected by an ECL-plus system (GE Healthcare, UK) and quantified by densitometric analysis using Image-J software (NIH, version 1.4K). All quantified Cx43 band densities were normalised to their corresponding actin protein density (polyclonal anti-mouse, Millipore; 1:1000 dilution).

**Connexin immunohistochemistry:** Preparations were placed for four hours in Zamboni’s fixative (2% paraformaldehyde, 0.2% picric acid, 0.1 M phosphate-buffered saline, pH 7.4) and wax-embedded. Haematoxylin and eosin-stained sections were examined under light microscopy to identify tissue orientation; quantify tissue morphology and measure cell dimensions. The muscle/interstitial cross-sectional area ratio was measured in ≥10 cells per section, from at least five sections per specimen.³ Sections (10 µm) were dewaxed,
rehydrated and trypsinised, and for connexin43 (Cx43) labelling blocked in 0.1 M lysine for one hour, followed by 90-minute incubation at 37°C with a rabbit polyclonal E12H anti-Cx43 antibody,4,5 (dilution 1:50). Cx40 and Cx45 immuno-labelling was carried out by overnight incubation with antibodies (generously provided by Dr DB Gros and Professor NS Severs5,6) diluted 1:10 at 4°C for Cx40 and 1:20 at room temperature for Cx45. Secondary incubation in all cases was with anti-rabbit Cy3 (Chemicon International Inc, Temacula, USA), diluted 1:500 for 45 minutes at room temperature. TRITC conjugated-wheat germ agglutinin (Sigma, 1:100 dilution) labelled cell membranes. Nuclei were labelled with the fluorescent nucleic acid binding dye TO-PRO-3 (1:250 dilution, Invitrogen, UK).

Myocyte topology: To examine the inter-relationships of individual cells a previously-published method was used.7 Small sections of preparations were fixed for high-resolution light microscopy in 0.08 mM Na cacodylate buffer (pH 7.4), containing 2% glutaraldehyde and 2 mM CaCl₂. Samples were post-fixed for two hours in 1% osmium tetroxide, dehydrated in an alcohol series and embedded in araldite resin. A series of 100 serial sections, 1 µm thick, were cut in a plane longitudinal to the long fibre axis, mounted sequentially, and stained with methylene blue. For each sample six individual index cardiac myocyte profiles were randomly selected in the 50th section and followed through the entire section series to identify the precise number of cells with which each index cell was coupled. Fibre diameter in the nuclear plane was recorded from at least ten cells in similar fields and used for electrophysiological calculations. Connective tissue content was estimated from sections fixed with 4% paraformaldehyde and with picrosirius red and the relative areas of red (connective tissue) and muscle (yellow/orange) calculated.
Quantification of Cx43 signal area: Immunolabelled sections were examined with a Leica TCS 4D confocal microscope and PC Image Analysis software (Foster-Findlay Associates, UK). Confocal images of six, randomly-selected high power (x630) fields of transversely-sectioned tissue and an optical projection image of each en-face disk were examined to derive Cx43 signal area per disk, and Cx43 signal area per unit disk area. Cx43 signal density was the total Cx signal area divided by the total area of the field occupied by cardiac myocytes. The Cx43 signal area per cell was calculated as the total Cx43 signal area divided by cell number in this field.

References


Supplemental figure legends

Supplemental Figure 1. Measurement of action potential (AP) conduction velocity. The relationship between distance, $d$, between stimulating and recording electrodes and AP conduction delay, $t$, between stimulus artifact and AP upstroke. A: control preparation; four measurement tracks in the longitudinal axis, plot colors correspond to those in the inset; S, stimulating electrode. Solid lines impalements 50-100 µm from the preparation surface; the dotted line (green) impalements 100-150 µm from the surface. CV values: blue 73.0; black 75.0; red 70.1 cm.s$^{-1}$; green 72.8. B: LVH preparation - three tracks in the transverse plane with impalements 50-100 µm deep: CV values: black 28.5; red 25.1; blue 28.2 cm.s$^{-1}$.
Supplemental Figure 2. **Estimation of interelectrode tip distance for two intracellular microelectrodes.** Micrometer positions in $x$ and $y$ planes were noted when the electrodes touched the superfusate surface, positions 1 and 2. Subsequent recording of the depth of electrode advancement to achieve cell impalement and the angles $\phi$ and $\psi$ permitted estimation of the $z$-separation and the interelectrode distance, $r^2 = \sqrt{(x^2 + y^2 + z^2)}$. 