Reduced Content of Connexin43 Gap Junctions in Ventricular Myocardium From Hypertrophied and Ischemic Human Hearts

N.S. Peters, MRCP; C.R. Green, PhD; P.A. Poole-Wilson, MD, FRCP; N.J. Severs, PhD

Background. Gap junctions are a determinant of myocardial conduction. Disturbances of gap-junctional content may account for abnormalities of impulse propagation, contributing to the arrhythmic tendency and mechanical inefficiency of ischemic and hypertrophied myocardium. The aim of this study was to characterize gap junction organization in normal human ventricular myocardium and to establish whether abnormalities exist in myocardium of chronically ischemic and hypertrophied hearts.

Methods and Results. Cardiac gap-junctional connexin43 antibodies and confocal microscopy were used in a quantitative immunohistochemical study of surgical myocardial samples to explore the structural basis of electromechanical ventricular dystunction in chronic ischemic and hypertrophic heart diseases. Normal adult human left ventricular myocardium had a gap-junctional surface area of 0.0051 μm²/μm² myocyte volume; gap junctions were confined to intercalated disks, of which there was a mean of 11.6 per cell. The right ventricle showed similar gap junction surface area. Left ventricular myocardium from ischemic hearts (distant from any fibrotic scarring), despite normal numbers of intercalated disks per cell, had a reduced gap junction surface area (0.0027 μm²/μm²; P=.02), as did hypertrophied myocardium (0.0031 μm²/μm²; P=.05). The cardiac myocytes in the pathological tissues were larger than normal, and estimated gap-junctional content per cell was reduced in ischemic ventricle (P=.02) compared with normal.

Conclusions. Gap junctions in normal adult human working ventricular myocardium occupy an area of 0.0051 μm²/μm² myocyte volume. This surface area is reduced in ventricular myocardium from hearts subject to chronic hypertrophy and ischemia, despite a normal number of intercellular abutments, and this alteration may contribute to abnormal impulse propagation in these hearts. (Circulation. 1993;88:864-875.)

Key Words • gap junctions • structure • hypertrophy • ischemia • microscopy

Gap-junctional organization is an important determinant of intercellular conductance and the conduction properties of myocardium. The normal pattern of anisotropic conduction in ventricular myocardium, by which conduction parallel to the myocyte long axis is up to four times more rapid than that transverse to it, is dependent in part on the low resistivity of the gap-junctional membranes, their distribution, and their abundance.

The underlying mechanism of many clinical cardiac arrhythmias is a myocardial reentrant electrical circuit, perpetuation of which requires areas of slowed conduction within the circuit, so that the advancing wave front encounters excitable tissue and initiation of which is enhanced by a local dispersion of recovery of excitability of the constituent myocytes. These factors are thought to provide the substrate for clinical reentrant arrhythmias. Efforts in basic cardiac electrophysiology have focused on how alterations of active membrane ionic properties can lead to slowing of electrical propagation and affect recovery of excitability, but this approach has not provided a full explanation of arrhythmogenesis. Indeed, there are reports of normal membrane potential characteristics in myocytes from myocardium with markedly abnormal electrophysiological properties and manifest arrhythmias. There has been increasing recognition that changes in the passive resistivity encountered by a propagating impulse can reduce conduction velocity and increase heterogeneity of conduction, irrespective of changes in active membrane properties. Despite the recognition of this key influence on the behavior of the propagating impulse, little information is available on the quantity and distribution or the function of gap junctions in human myocardium in health or disease.

In this study, immunohistochemical detection of the principal cardiac gap-junctional protein, connexin43, by confocal laser scanning microscopy and electron microscopic techniques were used to examine the structural organization of gap junctions in normal human ventricular myocardium and to establish whether changes in gap junction content and organization occur in diseased ventricular tissue in which there are recognized alterations of myocyte form and electromechanical interaction. The results provide new insights into a possible
anatomic factor in the pathogenesis of electromechanical dysfunction and arrhythmias.

Methods

Myocardial Samples

Ventricular specimens were obtained from patients fulfilling the study criteria (defined below), selected at random from those undergoing cardiac surgery having given prior written consent, or from transplant donor hearts. The biopsies were taken from either the left ventricular apex or the mid right ventricular free wall as appropriate in each case, either by excision of a 3-mm ellipse of epimyocardium or by use of a transmural needle biop tome (diameter, 1.5 mm) from which the epimyocardial end was used for study. All biopsies obtained at routine surgery were taken immediately after the heart was arrested on cardiopulmonary bypass.

Normal ventricular myocardium. Completely normal human myocardium for immediate fixation is not available for experimentation. For the purposes of this study, "normal" myocardium was obtained from the following sources:

1. Cardiac transplant donor hearts from which a single right ventricular biopsy was obtained on arrival at the implanting hospital after the heart was transported in cardioplegic solution on ice (for up to 4 hours). Light and electron microscopy have established that no detectable disruption of the intercalated disk structure occurs under these conditions of cardioplegia, a finding consistent with separate studies on myocardial preservation and cardioplegia. Samples of both ventricles of normal donor hearts that for technical reasons were not used for transplantation as intended were also studied.

2. The left ventricular apical region from patients with the Wolff-Parkinson-White syndrome (WPW). This group of patients experienced occasional but symptomatic tachyarrhythmic episodes but had no other detected cardiac dysfunction on routine preoperative assessment and were undergoing surgical division of the accessory pathway. Ventricular myocardium from hearts such as these, with infrequent arrhythmic episodes, has not been shown to have any structural abnormality and is generally considered normal.

By these criteria, five "normal" left ventricular samples (two transplant donor hearts and three WPW hearts) and five right ventricular samples (transplant donors) were studied.

Myocardium from ischemic ventricle. Left ventricular myocardium was obtained from five patients with three-vessel coronary artery disease undergoing coronary artery bypass graft surgery for symptomatic relief from angina. All patients had had documented anterior myocardial infarction (with or without Q waves on ECG) at least 3 months previously and were normotensive. Preoperative 201TI perfusion scintigraphy demonstrated symptomatic exercise-induced reversible ischemia in the remainder of the anteropapical territory, from which the biopsies of viable epimyocardium were obtained in each case. Myocardium immediately abutting scar from past infarction, shown in an earlier study to have a highly disrupted gap junction distribution (to a mean distance of 123 μm from the scar tissue border), was not included for analysis in the present study. For the same reason, subendocardial myocardium, which may contain wide-spread small scars in patients with chronic coronary disease, both disrupting gap-junctional distribution and distorting tissue architecture, was also avoided. This strategy allowed sampling of viable myocardium associated with a region of recurrent reversible ischemia but distant from detectable histological abnormalities to establish whether alterations of gap junction organization, in the absence of more gross histological disruption, exist in these regions. A detectable reversible thallium perfusion defect with associated angina pectoris is suggestive of ischemia affecting the full myocardial thickness, but direct demonstration that the epimyocardial samples taken had themselves been subjected to ischemia was not feasible.

Hypertrophied left ventricle. Left ventricular apical biopsies were taken during replacement of stenosed aortic valves in five patients with ECG and echocardiographic evidence of left ventricular hypertrophy.

Processing of Specimens

All biopsies required immediate fixation on removal, and the specimen was therefore divided as required in the operating theater, keeping note of tissue orientation to allow matching of areas analyzed by the different microscopic techniques described below.

Immunohistochemistry. Tissue was put in Zamboni's fixative (2% paraformaldehyde, 0.2% picric acid, 0.1 mol/L phosphate-buffered saline [PBS], pH 7.4) for 2 to 6 hours. After fixation, all samples were washed in tap water, dehydrated in alcohol, placed in chloroform, and embedded in wax according to standard histological procedures.

The primary antiserum used to localize cardiac gap-junctional protein was raised in rabbits against a synthetic peptide matching residues 131 through 142 of the cytoplasmically exposed segment of the cardiac gap-junctional protein connexin. The peptide, supplied by Dr N.B. Gilula (Research Institute of the Scripps Clinic, La Jolla, Calif) was prepared by the simultaneous multiple peptide synthesis procedure of Houghten and was coupled by use of glutaraldehyde to the carrier protein, keyhole limpet hemocyanin, for subcutaneous injection into Sandy half-lop rabbits. Full details of the production and characterization of the polyclonal antiserum are published elsewhere.

Sections (10 μm) of wax-embedded tissue were de-waxed, rehydrated, and incubated in a trypsin solution (containing 0.1% trypsin [Sigma T-8128], 0.1% CaCl₂, 20 mmol/L Triza base, pH 7.4) for 10 minutes at room temperature to reexpose antigenic sites masked by fixation. The sections were washed and treated with 0.1 mol/L L-lysine (as blocking agent) in PBS containing 0.1% Triton X-100. Incubation with the primary antiserum (dilution, 1:10 in PBS) was carried out for 1 hour at 37°C. After washing, secondary antibody treatment with swine anti-rabbit fluorescein isothiocyanate (Dako; 1:20 dilution) was given for 1 hour in the dark at room temperature. After final washing in PBS, the slides were mounted with Citifluor mounting medium (Agar Scientific Ltd, Stansted, England). Under the conditions described, the immunolabeling procedure produces even and consistent staining through the depth of the sections.

Immunostained sections were examined by conventional epifluorescence and confocal laser scanning mi-
croscopy. Phase contrast microscopy was used to obtain details of tissue structure, and adjacent sections stained with hematoxylin and eosin were routinely examined by standard light microscopy. Control specimens in which the antiserum was substituted by preimmune rabbit serum or buffered saline or the second antibody by buffered saline were routinely run in parallel. The specificity of the antiserum for the peptide, for isolated cardiac gap-junctional protein, and for ultrastructurally defined gap junctions was demonstrated as part of the characterization of the antiserum.31,33

For confocal microscopy, immunolabeled sections were examined with a Bio-Rad Lasersharp MRC-500 running on standard Bio-Rad software.

**Quantitative Analysis**

For quantitative assessment of myocardial gap junctions in the normal and diseased groups, all aspects of tissue processing, labeling, and image acquisition and analysis were strictly standardized as described below.

**Connexin43 gap junction sizes.** Measurements of the longest dimension of the fluorescent spots representing gap junctions were made by use of the "length" composite command from the digital images of en face intercalated disks, which are viewed as characteristic clusters of gap junctions in transversely sectioned tissue. To confirm that the measurement of the fluorescent spots by this technique provided a reliable guide to the true size of gap junctions, despite undulating topology and different planes of orientation at the ultrastructural level,36,37 we have compared fluorescent spot sizes with gap junctions measured on freeze-fracture electron micrographs of the same tissue and demonstrated a close correspondence.33,34

**Myocardial gap junction surface area.** To determine the total quantity of connexin43 gap junction present in a volume of myocardium, the tissue section was cut transverse to the long axis of the constituent myocytes. The confocal microscope was configured with a small aperture (aperture control withdrawn 1.2 mm) so that the confocality of 10 optical slices of data taken at 1-μm intervals through the depth of the tissue permitted minimal overlap between consecutive images, ensuring acquisition of all fluorescence present (Fig 1). These series of images were collected using the ×60 objective lens and the zoom 1 computer setting (field, 180×120 μm²) with a neutral density filter to eliminate significant fading of fluorescence. The "black level" was constant (at 4.0) such that the outlines of the individual cross-sectioned cells were visible, and the "gain" control was adjusted so that the spectrum of label intensities spanned the full 255-level gray scale.

The image data from the volume of tissue of 180×120×10 μm³ were then analyzed with the standard Bio-Rad software and the more versatile PC IMAGE (Foster-Findlay Assoc, Newcastle-upon-Tyne, England) image analysis software. First, the number of cross-sectioned myocytes in the fifth optical slice was counted, and the total area of the field occupied by myocytes was determined by subtracting from the total sample field all regions that were either completely devoid of signal (interstitial fibrosis) or recognizable as blood vessels. A composite image (or projection) was constructed for each 10-image series by superimposition (Fig 1), and a binary overlay was created automatically, in which each pixel was either on or off, as determined by a set threshold of 60 on the 255-point gray scale, to eliminate the background cell outlines. The binary image was then edited by hand to reject all extraneous lipofuscin and blood vessel autofluorescence, which were easily identifiable, and an automatic count was made of the total number of pixels in the on state. This area represented the total area of labeled gap junction in the sampled volume of myocytes. Five randomly selected fields of each tissue sample were analyzed.

To minimize errors introduced by variations between immunohistochemical labeling runs, samples of each of the disease groups and a control were included in every run. The resultant mean binary image area of three sampling sites from this positive control tissue was used to ensure consistency of the technique between labeling runs.

The analysis of the optical section series by this technique allowed determination of the amount of gap junction in a defined volume of myocardium expressed in the standard manner for stereological assessment of this sort, square micrometers per cubic micrometer.38

In an effort to relate this conventional measure of surface area of gap junctions per unit volume to the size of the myocytes composing the tissue from which they were obtained, a relative index of mean cell volume of
each of the sampled fields was derived. The volume index was obtained from the ratio of the number of cross-sectioned cells to the number of complete disks present (as an index of cell length) multiplied by the average cross-sectional area of the myocytes in the sample volume. If myocytes were cylindrical in shape with an intercalated disk at each end, the product of cell length derived by this method and the cross-sectional area would provide an absolute measure of cell volume. In reality, myocytes are not simple cylinders; they possess multiple intercalated disks, all of which lie in the plane approximately transverse to the long axis of the cell. Provided that this basic structural arrangement is present in all groups, however, with equivalent numbers of intercalated disks per cell, this index of cell volume is suitable for comparative purposes. The number and mean cross-sectional area of transected myocytes and the number of complete or nearly complete (>75% circumference) collections of gap junctions representing intercalated disks were determined from the projection image. The validity of this derivation of an index of cell volume has been confirmed in an experimental animal system by comparison with precise values for cell volume determined in isolated myocytes obtained from the same myocardium (unpublished observations).

**Intercalated disks per myocyte: myocardial cell arrangement.** To determine the number of intercellular abutments in normal ventricular myocardial architecture and to detect alterations in disease, counts were made of the number of cell-surface clusters of gap junction label, each representing an intercalated disk, in 100 randomly selected isolated left ventricular myocytes from the normal and ischemic hearts. Myocytes were isolated by the modified enzymatic tissue dissociation method,\(^9\) as described by Harding et al.\(^40\) The cells produced were immediately fixed and processed for connexin43 labeling by use of a procedure similar to that for whole tissue, and confocal microscopy was used to make disk counts. All focal clusters of label were considered to represent intercalated disks.

**Electron microscopy: gap junction ultrastructure and connexon packing density.** Glutaraldehyde-fixed specimens from the normal and the ischemic hearts were prepared for thin-section electron microscopy and freeze-fracture electron microscopy according to standard procedures.\(^41\) Glycerinated samples for freeze-fracture were frozen in Freon, and replicas were prepared in a Balzers BAF400T apparatus. Replicas and sections were examined in a Philips EM 301 microscope.

An electron micrograph with a final magnification of \(\times70000\) of each gap junction plaque was used to determine the junctional area, using ViDS in software (Analytical Measuring Systems, Cambridge, England). The connexon packing density was determined by counting the number of connexons within a hole in a mask placed randomly on the micrograph.

**Statistical analysis.** All results are expressed as mean±SD. Group data are compared by two-sample \(t\) tests of mean values for each heart. Statistical significance is defined as a value of \(P<.05\).

**Results**

Details of the 18 patients studied are summarized in Tables 1 through 3. The ages of the patients from whom normal left ventricular tissue was obtained were 43.4±12.6 years and those from whom right ventricular tissue was obtained, 35.4±10.1 years. The five patients with ischemic heart disease were 49.6±3.7 years old. The patients with aortic stenosis were 59.6±4.6 years old.

**General Ultrastructure**

The ventricular myocardium from the transplant donor and WPW subjects had normal ultrastructural ap-

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**TABLE 1. Details of Normal Patients**

<table>
<thead>
<tr>
<th>Heart</th>
<th>Type</th>
<th>Age (years)</th>
<th>Ventricle sampled</th>
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<tr>
<td>1</td>
<td>WPW</td>
<td>37</td>
<td>L</td>
</tr>
<tr>
<td>2</td>
<td>Donor</td>
<td>46</td>
<td>L+R</td>
</tr>
<tr>
<td>3</td>
<td>WPW</td>
<td>28</td>
<td>L</td>
</tr>
<tr>
<td>4</td>
<td>Donor</td>
<td>44</td>
<td>L+R</td>
</tr>
<tr>
<td>5</td>
<td>WPW</td>
<td>62</td>
<td>L</td>
</tr>
<tr>
<td>6</td>
<td>Donor</td>
<td>26</td>
<td>R</td>
</tr>
<tr>
<td>7</td>
<td>Donor</td>
<td>37</td>
<td>R</td>
</tr>
<tr>
<td>8</td>
<td>Donor</td>
<td>24</td>
<td>R</td>
</tr>
</tbody>
</table>

WPW, patient with the Wolff-Parkinson-White syndrome; Donor, transplant donor; L, left ventricular sample; R, right ventricular sample.

**TABLE 2. Details of Patients With Ischemic Heart Disease**

<table>
<thead>
<tr>
<th>Heart</th>
<th>Age (years)</th>
<th>ECG</th>
</tr>
</thead>
<tbody>
<tr>
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<td>No Q</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>Q</td>
</tr>
<tr>
<td>11</td>
<td>46</td>
<td>Q</td>
</tr>
<tr>
<td>12</td>
<td>48</td>
<td>Q</td>
</tr>
<tr>
<td>13</td>
<td>53</td>
<td>No Q</td>
</tr>
</tbody>
</table>

Q and No Q, Q waves present/absent in anterior leads on preoperative ECG. All patients had documented anterior myocardial infarction at least 3 months previously and exercise-induced ischemia in the anteroposterior territory on thallium perfusion scan.

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**TABLE 3. Details of Patients With Aortic Valve Stenosis**

<table>
<thead>
<tr>
<th>Heart</th>
<th>Age (years)</th>
<th>Valve gradient (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>60</td>
<td>77</td>
</tr>
<tr>
<td>15</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>16</td>
<td>54</td>
<td>110</td>
</tr>
<tr>
<td>17</td>
<td>63</td>
<td>80</td>
</tr>
<tr>
<td>18</td>
<td>65</td>
<td>60</td>
</tr>
</tbody>
</table>

All patients had ECG and echocardiographic evidence of left ventricular hypertrophy.
appearances, illustrated in Fig 2. The myocardium sampled from the ischemic hearts had an orderly arrangement of fibers, with no tissue distortion and no apparent fibrosis. Various degrees of hypertrophy were present, but thin-section electron microscopic appearances were otherwise normal. The myocardium from patients with aortic stenosis had the classic features of hypertrophy, with myocyte enlargement and hyperchromatic rectangular nuclei, the extent of which varied from cell to cell.

**Immunolabeled Connexin43 Gap Junction Distribution at Confocal Microscopy**

Immunolocalization of connexin43 gave consistent patterns of gap junction staining, common to left and right ventricle of normal hearts and the left ventricular myocardium from hearts with aortic stenosis and ischemia. In all these groups, gap junctions were visualized with normal adult human patterns previously described; bright fluorescent domains marking the positions of intercalated disks between myocytes, appearing as transverse lines of individually resolved spots in longitudinally sectioned myofibers (Fig 3, a) and discoid arrays delineated peripherally by large gap junctions in transversely sectioned myocardium (Figs 3, c and 2, b).

Controls in which antiserum treatment was omitted or substituted with preimmune serum showed no specific fluorescent labeling (Fig 4, g). Grapelike collections of lipofuscin (Fig 4, g) with characteristic pink autofluorescence were present in normal and to a greater extent in diseased myocardium.

**Quantitative Characterization of Gap Junctions**

**Analysis of connexon density in freeze-fractured gap junctions.** Analysis of data from freeze-fractured gap junctions (Fig 5) shows that there is no correlation between gap junction size and the density of its component connexons ($r=-.22, P=.13$). Fig 6 shows the plot of these variables for gap junction plaques of <1 $\mu m^2$ surface area from the ischemic hearts. There was no difference in density distribution between myocardium from the normal and ischemic hearts. Measurement of total label present in the immunohistochemical images will therefore be proportional to the number of connexons and the quantity of connexin regardless of any variation in spot size distribution between different samples.

**Size of immunolabeled gap junctions.** Measurement of the longest axis of the labeled spots of 25 intercalated disks selected at random from the five normal left ventricular specimens revealed a mean length of 0.52±0.30 $\mu m$, with a distribution as shown in Fig 7, A. The larger junctions at the periphery of the disk had a mean length of 0.67±0.32 $\mu m$ (maximum, 2.8 $\mu m$); the junctions in the central area of the disk, 0.36±0.17 $\mu m$ (minimum, 0.1 $\mu m$). The distribution of the labeled

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**FIG 2. Thin-section electron micrographs of left ventricular myocardium from a patient with Wolff-Parkinson-White syndrome, showing normal features. a, Low-power electron micrograph showing an electron-dense intercalated disk (large arrow) traversing between abutting myocytes in the characteristic step-like manner. The striations of the sarcomeres are aligned in this healthy tissue (small arrow). Scale bar=5 $\mu m$. b, Higher-power electron micrograph showing gap junctions of normal appearance (arrows).** Note the characteristic long stretch of gap-junctional membrane at the ends of the disk, consistent with the peripheral ring of large junctions on immunolabeling (see Fig 3, c). Scale bar=1 $\mu m$. 

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This page contains a detailed analysis of myocardial tissue, focusing on the distribution and characteristics of gap junctions, as well as the immunolocalization of connexin43. The text discusses the appearance of myocardium from normal and ischemic hearts, detailing the orderly arrangement of fibers and the characteristic features of hypertrophy. Immunolabeling studies are described, including the consistent patterns of gap junction staining and the presence of typical pink autofluorescence in diseased myocardium. Quantitative analysis of connexon density and gap junction size are presented, revealing no correlation between gap junction size and connexon density. The size distribution of immunolabeled gap junctions is measured, with a mean length of 0.52±0.30 $\mu m$ across normal left ventricular specimens.
FIG 3. Confocal immunolocalization of connexin43 gap junctions in left ventricular myocardium from a patient with Wolff-Parkinson-White syndrome, showing normal features. a, A single optical section of longitudinally sectioned myocardium revealing each individual gap junction domain as a separate spot (arrow) grouped within intercalated disks. Scale bar=50 μm. b, A projected optical series from transversely sectioned myocardium showing the characteristic features of a disk viewed en face (arrow), with a peripheral ring of large junctions and smaller central domains. Scale bar=50 μm. c, High-power view of en face disks, showing the entire gap junction population of each disk. Scale bar=10 μm.
FIG 4. A confocal optical section series of 1-μm intervals through the depth of transversely sectioned normal human myocardium labeled for connexin43 (compare with Fig 1). Scale bars=50 μm. a through e, The first, third, fifth, seventh, and ninth optical sections in an optical series acquired for the quantification procedure. There are progressive changes in the image content throughout the series. f, The projection of the 10 images of the optical section series (a through e), showing some completely defined intercalated disks with their entire gap junction population displayed in focus. g, A wax section adjacent to that in a through f, in which preimmune serum was substituted for the HJ antiserum in the processing procedure. This control section contains no fluorescent label, demonstrating the specificity of the procedure, and shows the lipofuscin autofluorescence, which can readily be distinguished from labeling (when present) for the purposes of editing the image.
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FIG 5. Electron micrograph of freeze-fracture replica showing the densely packed connexon particles (arrow) of an entire junctional plaque (gj) from a patient with ischemic heart disease. Scale bar=0.5 μm.

junction long-axis measurements pooled from the ischemic left ventricles (mean, 0.54±0.42 μm) showed no difference from normal (Fig 7, B).

**Gap-junctional surface area per unit volume of tissue.** An example of images from an optical section series of connexin43-labeled transversely sectioned ventricular myocardium prepared for quantitative analysis is shown in Fig 4. The results of the morphometric analysis of such images to determine myocardial gap junction content in the healthy and diseased hearts are shown in Table 4. Normal adult left ventricular myocardium had a gap junction surface area of 0.0051±0.0015 μm²/μm³ myocyte volume, and normal right ventricle showed no significant difference (0.0044±0.0006 μm²/μm³). Left ventricular myocardium from recurrently ischemic hearts had a significantly reduced gap junction surface area of 0.0027±0.0009 μm²/μm³ (P=.024). Hypertrophied left ventricle from patients with aortic stenosis also had a reduced gap junction surface area of 0.0031±0.0005 μm²/μm³ (P=.05) and was not significantly different from myocardium from the ischemic hearts.

FIG 6. Plot of connexon surface density vs gap junction plaque area in plaques up to 1 μm² pooled from ventricular myocardium from the ischemic hearts. There is no correlation (P=.18), and the slope is not significant.

FIG 7. Frequency distributions of long-axis measurements of labeled spots in confocal images (A) from the normal left ventricles (mean, 0.52 μm) and (B) from the ischemic left ventricles (mean, 0.54 μm). There is no difference between these distributions.
### Table 4. Results of Quantitative Morphometric Analysis

<table>
<thead>
<tr>
<th>Heart</th>
<th>Tissue type</th>
<th>Gap junction surface area/volume myocardium (µm²/µm³)</th>
<th>Cell volume index</th>
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<tbody>
<tr>
<td>1</td>
<td>LV</td>
<td>0.00476±0.00076</td>
<td>29 436±5377</td>
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<tr>
<td>2</td>
<td>LV</td>
<td>0.00751±0.00158</td>
<td>15 347±859</td>
</tr>
<tr>
<td>3</td>
<td>LV</td>
<td>0.00419±0.00072</td>
<td>23 516±5534</td>
</tr>
<tr>
<td>4</td>
<td>LV</td>
<td>0.00360±0.00117</td>
<td>24 668±6998</td>
</tr>
<tr>
<td>5</td>
<td>LV</td>
<td>0.00540±0.00113</td>
<td>20 925±1170</td>
</tr>
<tr>
<td>Mean LV</td>
<td></td>
<td>0.00508±0.00151</td>
<td>22 778±5174</td>
</tr>
<tr>
<td>2</td>
<td>RV</td>
<td>0.00501±0.00106</td>
<td>22 799±3512</td>
</tr>
<tr>
<td>4</td>
<td>RV</td>
<td>0.00349±0.00034</td>
<td>22 582±3136</td>
</tr>
<tr>
<td>6</td>
<td>RV</td>
<td>0.00464±0.00123</td>
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</tr>
<tr>
<td>7</td>
<td>RV</td>
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<td>Mean ILV</td>
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<tr>
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<td>Mean HLV</td>
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<td>31 447±3794*</td>
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</tbody>
</table>

Mean, mean±SD of means of five sample fields for each heart. Values are mean±SD. LV, normal left ventricle; RV, normal right ventricle; ILV, ischemic left ventricle; HLV, hypertrophied left ventricle.

*Significantly different from value for normal left ventricle (P<.05).

Comparative index of myocyte volume. The cell volume index derived from the hearts with aortic stenosis demonstrated the increased ventricular myocyte volume that would be expected in hypertrophied myocardium (31 477±3794 units) compared with normal (22 778±5174 units; P=.019). The mean value for myocardium from the ischemic ventricles (31 067±6553 units), although increased, did not differ significantly from normal (P=.062), owing to the presence of a wider range of cell sizes than the aortic stenosis group.

Since the cell volume indexes were derived from the same tissue fields as the gap-junctional surface density values, these data allow approximation of an index of gap junction area per myocyte for each myocardial specimen, derived from the mean of the gap junction areas and the cell volume indexes of each of the five sample volumes. This value is reduced in myocardium from the ischemic hearts (10 758±2133 units) compared with normal (14 996±2268 units; P=.019). Hypertrophied myocardium, by comparison, has a value of 12 848±1533 units (P=.11). These results and their interpretation must be treated with caution because the ratio used in their derivation may artificially exaggerate differences.

**Intercalated disk counts.** A confocal image of a labeled isolated ventricular myocyte is shown in Fig 8. When such images were used to count the number of surface clusters of connexin43 label (each representing an intercalated disk) per myocyte, there was no difference between cells from myocardium of normal (11.55±2.20) and ischemic hearts (11.87±2.49; P=.33).

**Discussion**

Quantitative studies of gap junctions in mammalian myocardium, most of which used thin-section electron microscopy, have been reviewed by Page. These studies are confined to species other than humans, in whom characterization of myocardial gap junctions and alterations that may accompany human myocardial disease have therefore not been determined. We have published findings at the borders of healed human infarcts.
but have not previously attempted quantification of gap junction content.

The group of ischemic patients tended to be older than the group with normal left ventricles (49.6±3.7 vs 43.4±12.6 years), a difference that is difficult to overcome given the reasons for which these groups undergo surgery. However, this difference was not statistically significant (P<0.35), and there were no trends to suggest age-related changes in any parameters within any of the study groups.

The gap-junctional surface area in normal human left ventricular myocardium (0.0051 μm²/μm² myocyte volume), as determined in the present study, falls within the range of values reported from electron microscopic ultrastructural analyses of myocardium of other mammalian species. These include rabbit ventricle (0.017 μm²/μm²), rat ventricle (0.0047 and 0.0022 to 0.0154 μm²/μm²), and canine ventricle (0.0085 μm²/μm²), from which Luke et al have also estimated a value of 0.0052 μm²/μm³ by an immunohistochemical technique using light microscopy. In contrast with all these reports, the technique used in the present study enables the quantity of gap junction to be related directly to entire and completely visualized intercalated disks sampled through relatively large volumes of tissue. The quantitative spatial distribution data obtained facilitate meaningful comparisons between disease groups and could, in principle, allow direct correlation with myocardial function and electrophysiological data acquired before myocardial fixation.

The presence of fluorescent labeling must be interpreted with some caution, since the antibody is directed to a small segment of the connexin43 molecule and labeling may not necessarily represent intact or functional gap junctions. However, the agreement of our data with the values obtained by standard electron microscopic morphometry, the correlation of the staining patterns with gap junction distribution determined by ultrastructural techniques, and the confirmation by immunogold labeling at the electron microscopic level of specific binding of the antibodies to gap junctions all serve to support the validity of the approach.

Our finding of a mean of 11.55±2.2 intercalated disks per myocyte in normal human epimyocardium is consistent with data reported by Luke and Saffitz, who used a quantitative electron microscopic technique to determine the mean of 11.2 intercalated disk contacts per myocyte in normal canine ventricular myocardium. These authors showed a reduced number (6.5) of such intercellular contacts in canine myocardium associated with infarction but had specifically selected regions associated with fibrotic scarring. In the present study, however, we obtained normal intercalated disk counts in ischemic hearts, despite changes in cell size distribution, suggesting that the basic architecture of intercellular abutments was not significantly altered in regions free of scar.

Substrate for Abnormal Impulse Propagation

It has long been suggested that fibrosis plays a part in the anatomy of arrhythmogenesis, but the results of the present study suggest a mechanism by which intercellular coupling may be impaired in the ischemic heart without invoking fibrosis as the explanation.

Both ischemic heart disease and left ventricular hypertrophy are associated with ventricular arrhythmias and sudden cardiac death. The development of treatment strategies for cardiac arrhythmias that occur in these and other clinical settings requires a detailed and complete understanding of the mechanisms by which these arrhythmias are generated. One of the many adverse effects of cardiac ischemia is a decrease in conduction velocity, a factor increasing the likelihood of arrhythmias. Although gap junctions are established as the organelle determining intercellular and whole-tissue conductance and conduction velocity, and an absence of gap junctions results in electrical isolation, the relation between the relative abundance of gap junctions, intercellular conductance, and conduction velocity is poorly understood. Evidence suggests that regions of the heart with slow conduction and relatively low intercellular conductance (such as the atrioventricular node) contain fewer gap junctions than regions of more rapid conduction. The possibility exists, therefore, that a reduction in gap junction surface area per unit volume of about 40% in ventricular myocytes from ischemic and hypertrophied ventricles, as demonstrated in the present study, may significantly alter patterns and rates of impulse propagation. The distribution of labeled gap junction long-axis lengths and the number of intercalated disks per cell remained unchanged in the myocardium from the ischemic hearts compared with normal, suggesting that the reduction in gap-junctional content occurs as a result of reduction in the numbers of all sizes of junction. This is of interest because electron microscopic examination of the canine infarct border region has suggested a preferential reduction of larger junctions, but fibrotic scarring of the canine myocardium may be relevant to this difference.

The greater myocyte volume that characterizes hypertrophied myocardium associated with chronic aortic stenosis was evident in the present study. Compensatory...
hypertrophy that might be expected in the myocytes of the myocardium around a healed infarct\textsuperscript{23,53} did not occur to a statistically significant degree, despite a greater mean cell volume, owing to the large standard deviation.

**Functional Significance: Correlating Morphology With Electrophysiology**

Although electrophysiological studies of arrhythmogenic cardiac disease have documented significant abnormalities of active membrane properties,\textsuperscript{8} many have failed to do so.\textsuperscript{13,14} Boyden et al\textsuperscript{14} concluded that alteration of unspecified morphologic features of the myocardium was responsible for the electrophysiological disturbances in chronically hypertrophied canine atria. Kieval et al\textsuperscript{15} demonstrated normal action potential characteristics but abnormal gap-junctional intercellular conductance in postischemic myocyte pairs, and reduced junctional conductance in cultured myocytes infected with *Trypanosoma cruzi* is associated with a qualitative reduction in connexin\textsuperscript{43} expression.\textsuperscript{54} A reduction in gap-junctional coupling between myocytes may, therefore, be an important morphological feature that could interact with altered active membrane properties in diseased myocardium of a variety of pathogens. It is likely that arrhythmogenic states result from a combination of changes of both membrane properties and coupling characteristics of the myocardium, and it has been suggested that abnormal cellular coupling may be the predominant electrophysiological derangement at the border of a healed myocardial infarct.\textsuperscript{55}

It has not been possible to study the interaction between the action potential and myocardial resistivity during programmed stimulation,\textsuperscript{55-58} but a number of computer models simulating this interaction have been developed. Joyners\textsuperscript{59} showed that as coupling resistance increases, the “intrinsic” cellular differences of action potential duration become increasingly manifest, and Lesh et al\textsuperscript{60} showed a coincident increase in axial resistivity, leading to slowed conduction. The combination of dispersion of action potential duration and slow conduction promotes reentrant tachycardia initiation and perpetuation,\textsuperscript{12} and Quan and Rudy\textsuperscript{61} model suggests that cellular uncoupling is arrhythmogenic by just these mechanisms.

**Implications of Results**

A reduction in gap junction coupling may, therefore, change conduction velocity and unmask intrinsic differences in action potential characteristics, manifest at the clinical level by nonspecific changes in the QRS complex on the ECG, changes in the coordination of contraction and the mechanical efficiency of the heart, and a possible lowering of arrhythmogenic threshold. These phenomena are well recognized in ischemic and hypertrophied hearts. The process of uncoupling may, itself, induce changes in action potential characteristics\textsuperscript{55,61} and recovery of excitability.\textsuperscript{55} All these alterations increase the potential for spontaneous arrhythmias,\textsuperscript{62} for which there is growing evidence of the role of abnormal intercellular coupling.\textsuperscript{1,12,55,61} The results of the present study suggest that changes in resistivity, as evidenced by altered gap junction quantity in the diseased human ventricle, may indeed occur.

The resistivity of myocardium, a potentially important factor in clinical arrhythmias, has been ignored in many studies confined to relating active membrane properties to the behavior of the tissue and the heart as a whole. In the present study, normal human ventricular myocardium has been characterized with respect to gap junctions. Abnormalities of gap junction content, as demonstrated in ischemic and hypertrophied hearts, would be consistent with the clinically apparent disturbances of ventricular electromechanical function in these conditions, the explanation and treatment of which remain poorly understood. Little is known of the action of conventional antiarrhythmic agents on cellular coupling and anisotropy,\textsuperscript{63} and with a number of clinical trials questioning the conventional wisdom of pharmacological arrhythmia treatment,\textsuperscript{64} a broader approach to understanding electromechanical dysfunction may require greater attention to gap junction intercellular coupling.

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