Conduction of the cardiac impulse is dependent on both active sarcolemmal ionic currents and passive electrical properties determined by the myocardial tissue architecture and specialized junctions responsible for electrical coupling, the gap junctions (GJs). The influence of myocardial architecture on conduction is determined by the size and shape of individual cardiomyocytes and their packing geometry in the myocardium. The role of GJs in cardiac conduction is dependent on their constituent connexin isoforms, as well as on the size, number, and spatial distribution of these junctions. Connexin isoforms of GJs have been shown to vary in different cardiac tissues with the following specific conduction properties: the nodes, a specialized conduction system, and atrial and ventricular myocardium. The size, number, and spatial distribution of GJs vary also distinctly across the different types of myocytes with disparate conduction properties and therefore are considered an important structural determinant of conduction.

Examples of tissues with disparate structural patterns of GJ interconnections are the atrial and ventricular myocardium. However, virtually nothing is known about the mechanisms of establishment and regulation of specific patterns of GJs in atrial myocytes (AMs) and ventricular myocytes (VMs) in vivo. Although different loading conditions imposed on these cardiac chambers may influence the structural patterns of cellular interconnections and their GJs, we hypothesized that intrinsic factors within cardiac myocytes may also play a role in determining specific patterns of GJ organization in AMs and VMs. To verify this hypothesis, we used a model of disaggregated and externally unloaded adult rat AMs and VMs in vitro and in vivo. Using transmission electron microscopy (TEM) and confocal scanning laser microscopy (CSLM), we have quantitatively compared the structural features of GJs in AMs and VMs in adult rat hearts in vivo with those of developing GJs in cultured AMs and VMs in vitro. The size, number, and spatial distribution of GJs vary also distinctly across the different types of myocytes with disparate conduction properties and therefore are considered an important structural determinant of conduction.

Materials and Methods
Experiments were performed according to protocols approved by Regierungspärisdium, Darmstadt, Germany. Adult hearts were ob-
GJs in Adult Atrial and Ventricular Myocardium

We used TEM to study GJs in adult rat AMs and VMs in vivo. Representative micrographs of AMs in LP of section denote relatively simple intercalated disks (IDs) and GJs with a profile length <1 μm (Figure 1A). Figures 1B and 1C show representative GJs in AMs in TP of section.

VMs sectioned in LP showed highly tortuous IDs and numerous GJs (Figure 1D). Large GJs (profile length >3 μm) in LP of section were infrequent, but when observed (online Figures 1A and 1B, available in an online data supplement at http://www.circresaha.org), they were more common between VMs connected in a side-to-side orientation (following the definition of Luke and Saffitz). Large GJs were observed more frequently in VMs sectioned in TP than in LP. This type of GJ appeared in TEM as long (up to 10 μm) ribbonlike junctions (following the original description of Hoyt et al.), exhibiting highly folded and tortuous contours (Figure 1E). Further examples of VMs in TP of section displaying large GJs at the IDs are shown in online Figure 2. Large GJs were found in VMs to be mainly confined to the periphery of the IDs (Figure 1F) in close proximity to or being continuous with the external sarcolemma. Further examples of peripherally located large GJs are provided in online Figure 3. It is worthy to note that large GJs were observed even in small-sized IDs (online Figure 4) at the lateral cellular branches that are numerous in VMs.

Quantitative Analysis of GJs In Vivo

We used TEM to quantify GJ size in adult rat AMs and VMs in vivo. The advantage of TEM resides in the high-resolution identification of GJs as a structure that can be directly measured with precision. Quantitative TEM analysis revealed that AMs are interconnected by GJs having a profile length of 0.53 ± 0.03 μm in TP and 0.51 ± 0.03 μm in LP, whereas in VMs the mean GJ length was significantly greater in TP than in LP (1.04 ± 0.04 μm versus 0.65 ± 0.06 μm, respectively).

Histograms comparing the frequency distribution of different GJ size classes in AMs versus VMs in both LP and TP (online Figure 5) show no significant differences in this parameter between AMs and VMs in LP. However, VMs sectioned in TP showed significantly higher frequency values of large GJs than those in VMs in LP or in AMs in both LP and TP. Thus, from the total 1346 GJs measured in VMs in TP of section, 138 (10.2%) had a profile length >3 μm, whereas such GJs identified in VMs in LP and AMs in LP and TP comprised, respectively, only 1.5%, 0.8%, and 0.9% from the total number of GJs. This difference is reflected in the relative contribution of large GJs to the total GJ length (online Figure 6). Thus, large GJs encompassed 49.3% of the total GJ length in VMs in TP and <15% in VMs in LP. The contribution of large GJs to the total GJ length in AMs averaged 5.5% in LP and 6.2% in TP.

Taken together, quantitative data of adult rat hearts indicate that myocytes forming atrial and ventricular tissue are interconnected by GJs that differ in a tissue-specific manner in terms of their size and spatial distribution. In particular, these data show that large ribbonlike GJs are a structural peculiarity of VMs but not of AMs in vivo.

To investigate whether GJs are reestablished in tissue-specific patterns in vitro, we conducted experiments using isolated AMs and VMs from adult rat hearts and maintained in long-term culture.

Immuconfocal Characterization of Cell Cultures

Figures 2A through 2D are 3-dimensional views showing the difference in the architecture of IDs and in the cell size between AMs and VMs isolated from respective adult tissues. By immunolabeling for N-cadherin, randomly selected AMs showed 5.5 ± 1.5 IDs per cell, whereas VMs possessed 9.8 ± 2.1 IDs per cell (P<0.01, n=100 cells). These data are consistent with previously published values.

During subsequent maintenance in culture, isolated AMs and VMs disassemble ID structures, spread on the substratum, and tend to rapidly reestablish new IDs, confirmed...
by immunolabeling for N-cadherin and α-catenin (Figures 2E and 2F).

**Evaluation of GJ Formation by CSLM**

CSLM revealed that in AMs cultured for 6 days, connexin43 (Cx43) reappeared along the newly redeveloped IDs as clusters of distinct small dots (Figures 3A and 3B). From day 9 to day 15, AMs showed a gradual increase in the size and number of GJs per ID (Figures 3C through 3E). The majority of redeveloped GJs were oval or discoid in shape. Figures 3F through 3I show Cx43 GJs in VMs at different developmental stages ranging from 6 to 15 days in culture. At day 6, GJs were small and sparsely distributed along the IDs (Figure 3F), whereas at day 9, GJs appeared larger and more numerous as shown in LP at low and high magnifications (Figures 4I through 4L). Similar patterns of GJs were found in TP (Figures 4M and 4N).

AMs at 12 days exhibited myofibrils with distinct and regular Z-bands (Figure 5A). GJs connecting these cells appeared as linear or curvilinear junctions as shown in LP (Figures 5B through 5D) and TP (Figures 5E and 5F).

AMs at 15 days contained compactly arranged myofibrils and secretory granules (Figure 5G). Although the overall pattern of GJs at 15 days (Figure 5H) did not differ substantially from that at 12 days, occasional GJs exceeding 1 μm in length tended to have folded contours (Figure 5I). Typical patterns of GJs observed in TP of section in AMs at day 15 are shown in Figure 5J.

TEM data of cultured VMs at early stages of GJ formation (6 to 9 days in culture) were described previously. Here, we

**Ultrastructural Assessment of GJ Formation**

We used TEM to examine in further detail the sequences of GJ formation in relation to phenotypic changes of AMs and VMs in culture. As in tissue sections, we have analyzed GJs in cultured myocytes in LP and TP to the substratum.

TEM of AMs at 6 days in culture revealed dedifferentiating cells almost lacking myofibrillar structures while abundantly containing secretory granules and Golgi complex (Figure 4A). Reassembled IDs between AMs at 6 days showed the presence of zipperlike adhesion junctions (Figure 4B) and very close cell-to-cell appositions (Figure 4C), representing typical GJs, as observed at high magnifications in LP (Figures 4D and 4E). Similar patterns of GJs were found in TP (Figures 4F and 4G).

At day 9, as development further proceeded, AMs showed nascent myofibrils with irregular Z-densities (Figure 4H). As compared with 6 days, GJs at 9 days appeared larger and more numerous as shown in LP at low and high magnifications (Figures 4I through 4L). Similar patterns of GJs were found in TP (Figures 4M and 4N).

AMs at 12 days exhibited myofibrils with distinct and regular Z-bands (Figure 5A). GJs connecting these cells appeared as linear or curvilinear junctions as shown in LP (Figures 5B through 5D) and TP (Figures 5E and 5F).

AMs at 15 days contained compactly arranged myofibrils and secretory granules (Figure 5G). Although the overall pattern of GJs at 15 days (Figure 5H) did not differ substantially from that at 12 days, occasional GJs exceeding 1 μm in length tended to have folded contours (Figure 5I). Typical patterns of GJs observed in TP of section in AMs at day 15 are shown in Figure 5J.

TEM data of cultured VMs at early stages of GJ formation (6 to 9 days in culture) were described previously. Here, we

**Figure 2.** Three-dimensional images of freshly dissociated adult myocytes triple-immunolabeled for N-cadherin (green), TRITC-phalloidin (red), and myomesin (purple) in AMs (A and C) and VMs (B and D). Reestablished IDs in AMs (E) and VMs (F) at 12 days in culture show a colocalization of N-cadherin (blue) with α-catenin (red), rendering them purple. Myofibrils are stained green with FITC-phalloidin.

**Figure 3.** Development of Cx43 GJs in AMs (A through E) and VMs (F through I) cultured for 6 to 15 days. A, Double labeling for N-cadherin (green) and Cx43 (red). For clarity of GJ plaques, N-cadherin signal has been omitted from panels B through I.
report more detailed data of GJs in VMs at 12 to 15 days. Redifferentiating VMs at these intervals showed mature myofibrils (Figure 6A) and reorganized T-tubules (Figure 6B), comparable with those found in vivo or in freshly dissociated adult VMs. VMs at day 12 developed large GJs with a ribbonlike appearance observable in LP (Figures 6C and 6D) and TP (data not shown). VMs at day 15 showed long ribbonlike GJ profiles discernible in LP at low (Figures 6E and 6F) and high (Figures 6G through 6I) magnifications. Similar configurations of GJs were found in TP (Figures 6J and 6K). Some ribbonlike GJs had exceptionally long profiles (>10 \( \mu \text{m} \)) as shown in LP (Figures 6F and 6H) and TP (Figure 6J).

Figure 4. Phenotype of AMs at days 6 (A) and 9 (H) in culture. B, Zipper-like structures within the reassembled IDs are denoted by arrowheads. C through G, GJs (arrows) at day 6. I through N, GJs (arrows) at day 9. Panels A through E and H through L are LP of section; panels F, G, M, and N are TP. NUC indicates nucleus; Mf, myofibrils. Panels A through C, F, H through J, and M, Bars=1 \( \mu \text{m} \); panels D, E, G, K, L, and N, Bars=0.5 \( \mu \text{m} \).

Figure 5. Phenotype of AMs at days 12 (A) and 15 (G) in culture. B through F, GJs (arrows) at day 12. H through J, GJs (arrows) at day 15. Panels A through D and H through I are LP of section; panels E, F, G, and J are TP of section. A, B, G, H, and J, Bars=1 \( \mu \text{m} \). C through F, I, Bars=0.5 \( \mu \text{m} \). Abbreviations as in Figure 4.

Figure 6A to 6K. Regardless of the time in culture in both AMs or VMs, no significant changes were observed in TEM parameters of GJs measured in LP versus TP of section. For example, in VMs at day 15, the average size of the total 81 GJs selected at random from sections cut in LP was 1.15 \( \mu \text{m} \) and did not differ from that of the total 75 GJs observed in TP (1.05 \( \mu \text{m} \)). In the corresponding AM cultures, the size of an average GJ profile was 0.57 \( \mu \text{m} \) in LP and 0.52 \( \mu \text{m} \) in TP.

Similar to TEM data, GJ morphometric parameters obtained by CSLM in 12- to 15-day cultures and expressed as GJ profile length and area were respectively 2- and 4.5-fold greater in VMs than those in AMs (Table).

Figures 7A and 7B compare the frequency distribution of GJ size classes as determined by CSLM and TEM in AMs versus VMs in 12- to 15-day cultures. Although the histograms of GJ size distribution showed a skew toward the smaller junctions in both types of myocytes, statistical analysis revealed significant differences between these cultures due mainly to the higher proportion of larger GJs in VMs than in AMs. Thus, in VMs, from the total 2238 GJs measured by CSLM, 332 GJs (14.8%) had a profile length >3 \( \mu \text{m} \). By contrast, AMs showed a marked paucity of larger GJs, such that only 20 GJs of 2076 (0.96%) exceeded 3 \( \mu \text{m} \) (Figure 7A). The results obtained using CSLM showed a close resemblance with those obtained by TEM in that 14.1% of GJs in VMs (42 of 298) had a profile length >3 \( \mu \text{m} \) (Figure 7B). In AMs, such GJs comprised only 1% (3 of 286).

Quantitative Analysis of GJ Formation

We determined the size of individual GJs by TEM and by CSLM using immunolabeling for Cx43. The results given in the Table show that a progressive increase in the number and size of GJs occurred in both AMs and VMs with increasing time in culture. However, at 12 and 15 days, when comparable total lengths of IDs were examined by TEM, the percentage of the total GJ length per ID was \( \approx 2 \)-fold greater in VMs than in AMs. This difference is mainly attributable to the 2-fold higher GJ profile length in VMs than in AMs, whereas the total number of GJs per unit ID length was comparable between these groups.
The difference between AMs and VMs in the frequency distribution of GJ sizes is reflected in the contribution of different size classes to the total GJ length (Figures 7C and 7D) and area (Figure 7E). Thus, in marked contrast with the symmetrical configuration of the histograms of the relative contribution of different GJ sizes to total GJ length and area as observed in AMs, the configuration of the corresponding histograms in VMs was markedly skewed toward the higher values of the contribution of larger GJs to the total GJ length and area. Compared with VM cultures, in which the contribution of GJs with a length >3 μm to the total GJ length averaged 64.7% and 60.4% (as determined respectively by CSLM and TEM), the contribution of such GJs to the total GJ length in AMs was respectively 9.5- and 7.2-fold lower. Furthermore, when the contribution of the different GJ size classes was related to the total GJ area (Figure 7E), GJs with a length >3 μm encompassed 87.4% and 25% of total GJ area in VMs and AMs, respectively. Finally, when the contribution of the different GJ size classes was added to plot the cumulative contribution of different size classes to the total GJ length (Figure 7F), GJs with a profile length >3 μm constituted 93.8% of the cumulative GJ length, whereas in the corresponding VM cultures, these GJs constituted only 40.6%.

In summary, quantitative TEM and CSLM data substantiate and extend the conclusion that AMs and VMs in vitro differ markedly in the size of their GJs and in the proportion of large ribbonlike GJs. These data are comparable with the results obtained in vivo. Another obvious difference was a marked contribution of large GJs (>3 μm) to the total GJ length in VMs both in vitro and in vivo as compared with a very modest contribution of large GJs to the total GJ length in AMs both in vitro and in vivo.

Discussion

Previous quantitative data of adult canine hearts have clearly demonstrated marked structural differences between atrial and ventricular tissues in the patterns of intercellular connections and in the size, number, and spatial distribution of their GJs.2,20 The present quantitative study performed in adult rat hearts confirms these findings and demonstrates that myocytes forming atrial and ventricular myocardium are interconnected by GJs that differ in a tissue-specific manner in terms of their size and 3-dimensional distribution. In particular, we

<table>
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<tr>
<th>Quantitative TEM and CSLM Analysis of GJ Formation in Vitro</th>
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<td>Mean GJ profile length, μm</td>
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*P<0.01, †P<0.05, VM vs AM.
Figure 7. Histograms showing frequency distributions of different GJ size classes (A and B), their relative contribution to total GJ length (C and D) and area (E), or their cumulative contribution to total GJ length (F) in AM vs VM cultures as determined by CSLM (n=5 cultures) and TEM (n=5 cultures). *P<0.05, **P<0.01, VM vs AM.

show that a specific subclass of extremely large ribbonlike GJs represents a structural peculiarity of adult rat ventricular tissue, previously characterized in detail in adult canine ventricle.28,31

The specific aim of the present study was to verify the hypothesis that atrial and ventricular tissue-specific patterns of GJs in vivo are determined and regulated by intrinsic factors within the myocytes forming these cardiac tissues, rather than extrinsic factors, such as load or wall tension. The following findings support our hypothesis: (1) the observation that isolated and externally unloaded AMs and VMs in long-term culture in vitro reestablish patterns of GJ interconnections that are similar with disparate atrial and ventricular tissue-specific patterns of GJ interconnections as we documented quantitatively in adult hearts in vivo and (2) the demonstration that VMs in vitro consistently redevelop a specific subpopulation of large ribbonlike GJs, closely resembling those observed in adult VMs in vivo. These conclusions are based on direct measurements of GJs by TEM in adult AMs and VMs in vivo and in AMs and VMs in long-term culture in vitro. In addition, quantitative TEM data of GJ formation in vitro were confirmed by CSLM measurements of Cx43 GJs.

It should be emphasized that the data on the spatial organization of GJs in vitro cannot be entirely extrapolated to the 3-dimensional situation in vivo, which is undoubtedly much more complex. Such examples include (1) the presence in adult VMs in vivo of an anisotropic pattern of GJ distribution at the IDs with higher GJ length in TP than in LP of section, as reported in canine hearts28,31 or rat hearts (present study) and the lack of this pattern, with almost no differences in GJ size in LP and TP, as documented in our in vitro experiments, and (2) higher frequencies of large GJs in VMs in vitro than in vivo. A plausible explanation for the observed differences is a continuous process of remodeling of the intercellular connections and GJs that occurs in the course of postnatal heart development. This process is not species-specific but has conclusively been described in a number of mammalian species,37,38 including rats,1,39,40 Moreover, this process continues over a relatively extended time after birth41; in the human heart, the adult pattern of GJ distribution is achieved at ~6 years of age.42 Certainly, these distal steps in the establishment of adult patterns of GJ distribution cannot be achieved in vitro. Nonetheless, our data showing that the major structural differences in GJs between AMs and VMs in vivo are regained in vitro strongly indicate intrinsic myocyte-specific mechanisms in control of GJ size.

Currently, the precise function of the large ventricular GJs, both in vivo and in vitro, is poorly understood. However, as established in the present study, as well as in others,7,15,19,35,43–47 the position of large GJs at the periphery of the IDs, ie, directly in the path of the action potential, would predict an important role of these junctions in intercellular current transfer between VMs, probably contributing to the efficient anisotropic pattern of impulse conduction. New insights into the function of large GJs have recently emerged from studies carried out in Cx43-deficient mice. These showed that when Cx43 is diminished, it is functionally more advantageous for VMs to maintain GJ size rather than GJ number per ID.30 These data, together with the results of computer simulation studies of conduction under conditions in which GJ plaque size is varied,28 suggest an important role of large GJs in supporting safe conduction that is critical for normal ventricular conduction.

Apart from the function of large GJs, many experimental31 and clinical34,46,47 studies documented a striking and selective disruption of large GJs in diseased ventricular tissue. The recognition that these junctions are extremely vulnerable structures under different pathophysiological settings that are prone to arrhythmias emphasizes the clinical relevance of understanding the mechanisms of formation and stabilization of large ventricular GJs.30 We propose that isolated adult rat AMs and VMs in long-term culture differing markedly in the size of their GJs can serve as reliable models for studying the role of GJ size in myocardial tissue-specific intercellular communication and conduction, and merit further investigations.

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References


Tissue-Specific Patterns of Gap Junctions in Adult Rat Atrial and Ventricular Cardiomyocytes In Vivo and In Vitro
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Sawa Kostin and Jutta Schaper

**Structural determinants of myocyte communication**

**Online Supplement Data** (available at http://www.circresaha.org)

**Materials and Methods**

**Tissue Preparation**

Adult hearts were obtained from nine Wistar rats (90 days old). The animals were deeply anesthetized with ether. The abdominal aorta was cannulated and the right atrium was opened. After 2 min of retrograde myocardial perfusion through the abdominal catheter (80 mm Hg perfusion pressure) with oxygen-saturated Tyrode’s solution, hearts were perfused for 15 min with 2% glutaraldehyde in 0.1 mmol/L natrium cacodylate (6 hearts) for transmission electron microscopy (TEM) as described,\(^1,^2\) or with 1% paraformaldehyde in 0.1 mmol/L phosphate-buffered saline (PBS) for immunohistochemistry (3 hearts) and confocal scanning laser microscopy (CSLM) as described.\(^3\) Perfusion-fixed hearts for TEM were additionally immersed in 2% glutaraldehyde for 2 more hours. Perfusion-fixed hearts for immunohistochemistry were additionally immersion-fixed in 4% paraformaldehyde for 1 h, then sequentially cryoprotected overnight with 20% sucrose, embedded in Tissue-Tek O.C.T Compound (Sakura Finetek) and quick-frozen in methylbutane at −130°C and stored at −80°C till further use.

**Isolation and Culture of Cells**

Wistar rats (90 days old) were deeply anesthetized with ether. After injection of heparin (300 IU/kg) into the inferior vena cava, the heart was excised and the
aorta was cannulated. The heart was perfused retrogradely for 5 min with a Ca\textsuperscript{2+}-free perfusion buffer (PB) containing (mmol/L) NaCl 110, KCl 2.6, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 11 and HEPES 10 (at 37°C, pH 7.4, gassed with 95% O\textsubscript{2}/5% CO\textsubscript{2}). Then perfusion was switched for 25 min to 0.03% collagenase solution (CLS 2, Worthington Biochemical Corp.) containing 0.004% pronase (Boehringer, Mannheim), 0.005% trypsin (Sigma) and 0.04 mmol/L CaCl\textsubscript{2} in PB. The atria were separated from the ventricles above the level of the atrioventricular groove using a dissecting microscope. Atrial (AM) and ventricular myocytes (VM) were separately minced in the collagenase solution containing 1.2% bovine serum albumin (BSA) at 37°C for 10 min, filtered through a nylon mesh and centrifuged at 8g for 3 min. The pellets of AM and VM were washed in PB containing 0.1 mmol/L CaCl\textsubscript{2} followed by separation in 33% Percoll (Pharmacia). Calcium was then added stepwise to a concentration of 1.0 mmol/L. The cells were resuspended in medium 199 (Sigma) containing 5 mmol/L creatine, 2 mmol/L L-carnitine, 5 mmol/L taurine, 0.1 mmol/L insulin, 10 mmol/L cytosine arabinoside, 100 IU/mL penicillin-streptomycin and 10% fetal calf serum, plated on culture chamber slides (Nunc, Illinois) coated with 5 μg/mL laminin (Sigma) at approximativaly 2.5x10\textsuperscript{4} cells/well and incubated in a 95% O\textsubscript{2}/5% CO\textsubscript{2}-incubator at 37°C. After 3 hours, the medium was replaced with the same fresh medium. AM and VM were maintained under identical conditions in long-term culture according to methods extensively described\textsuperscript{4-7}. The cells were investigated immediately after myocyte dissociation and at day 6, 9, 12 and 15 in culture. The results reported are based on 14 long-term cultures of AM in parallel with 14 cultures of VM: 6 cultures of each type of myocytes were used for CSLM, 5 cultures for TEM, and 3 cultures for quantitative immunofluorescence.
Immunofluorescent Labeling

**Antibodies:** Monoclonal (clone GC-4) and polyclonal anti-N-cadherin, polyclonal anti-α-catenin, TRITC- or FITC-phalloidin for F-actin staining (Sigma), monoclonal anti-Cx43 (clone 1E9, Biotrend), polyclonal anti-Cx40 (Chemicon), monoclonal anti-myomesin (clone B-4, a generous gift from Dr Eppenberger, Institute of Cell Biology, Zürich) were used. Anti-mouse IgG-conjugated with Cy2, Cy3 or Cy5 and anti-rabbit IgG-conjugated with Cy2 or Cy3 (Dianova) were used as detection systems. Dilutions of primary and secondary antibodies were used as described. A concentration of 5 μg/mL of anti-Cx40 antibody produced the highest signal-to-noise ratio. Intense labeling of Cx40 in this dilution in rat endothelial cells of intramural arteries and Purkinje fibers is shown in Supplementary Figure 7.

**Immunolabeling:** AM and VM cultures were fixed for 10 min with 4% paraformaldehyde and permeabilized for 15 min with PBS containing 0.05% Triton X-100. For immunolabeling of the tissue samples, the hearts were oriented and serially sectioned in order to visualize four cardiac chambers and Purkinje fibers as detailed previously by Gourdie and coworkers. Frozen heart tissue sections 10 μm thick were placed on gelatine-coated slides, permeabilized with 0.05% Triton X-100 in PBS for 10 min. Cells and tissue sections were washed and blocked in 0.5% carboxylated BSA and 100 mmol/L glycine in PBS for 10 min, followed by sequential incubation with the primary antibodies and corresponding detection system in a moist chamber at 4°C for 12 h each step.

The following controls in the double-labeling procedure were used: 1) omission of both primary antibodies, 2) comparison with single labeling patterns obtained with each antibody, 3) alternating of the detection system in
single-labeling experiments (eg, using mouse monoclonal followed by anti-rabbit secondary antibodies) and 4) reversing the order of primary antibodies.

**Confocal Scanning Laser Microscopy**

The cells and tissue sections were examined by CSLM using Leica TCS-NT, equipped with argon/krypton and helium/neon lasers. Collected series of confocal images were transferred to the Silicon Graphics workstation for three-dimensional (3-D) image reconstructions and quantitation of the fluorescence intensity (FI) using Imaris® processing software (Bitplane, Zürich) as previously described.7,13,14

**Transmission Electron Microscopy**

Cells and tissue samples were fixed and processed for TEM as widely described.1,15-17 Tissue sections and cell cultures were analyzed qualitatively and quantitatively by TEM both in a longitudinal plane (LP) parallel to the long cell axis and in a transverse plane (TP) as previously described.15,18,19 The ultrastructural components of the intercalated disc (ID) were described following the terminology of various authors.18-23

**Quantitation of GJ Size by TEM**

TEM morphometry of gap junctions (GJ) in AM and VM in vivo and in vitro was performed as previously described.7,18,19,24,25 For the purpose of comparison and discussion of our data with those published, we define a large GJ as one whose greatest extent is over 3 μm as described by Luke and Saffitz.15

For quantitative TEM, five to ten tissue blocks of either subepicardium of the left ventricular free wall or left atrial free wall were selected per each rat. Fiber orientation was delineated using semi-thin sections (0.5 μm) stained with
toluidine blue. The sections were viewed in a Leica DM microscope to verify that myocytes had been cut in LP and if so, ultrathin sections from technically satisfactory tissue blocks were cut first in LP and then, after reorienting the block by 90° in the microtome, additional sections were prepared in a TP as described. Randomly selected ID, containing at least two plicate segments in LP and at least one plicate segment in TP, were inspected at low magnifications (below the resolution of GJ) and their X-Y spatial position was stored in the TEM computer. Then ID and GJ were rephotographed at higher magnifications. Solitary GJ not associated with clearly discernible plicate segments were excluded from the measurements. High power TEM micrographs were transferred to a Macintosh computer using a ScanJet 4C scanner (Hewlett-Packard) and Adobe® Photoshop® 5.5 software. GJ were inspected and measured using NIH image software using a high zoom to clearly delineate individual GJ. From the total 2498 GJ identified in AM in six rats in vivo, 1263 (210±12 per rat) were measured in LP and 1235 (205±13 per rat) in TP. Accordingly, from the total 2559 GJ identified in VM in six rats in vivo, 1213 (202±11 per rat) were measured in LP and 1346 (224±18 per rat) in TP of section.

Quantitation of GJ Size by CSLM

Quantitation of GJ size by CSLM was performed according with protocols of the technical foundation of this method described by Gourdie and coworkers. In brief, confocal sections were acquired using a x63/1.32 objective lens. The confocal settings had been standardized and were kept constant in all measurements. A zoom factor of 3.92 with a 1024x1024 pixel image yielded a pixel size of 40x40 nm. The smallest GJ was defined by a size of ≥2 pixels (≥80 nm). After 3-D reconstruction, the images were transferred to a Macintosh computer and GJ were inspected and measured in NIH image software using high zoom to clearly
delineate individual GJ. To eliminate background fluorescence, a pixel intensity threshold was adjusted as described.8,16,26,27 For each time point, ten to fifteen randomly selected ID, containing the entire population of GJ were investigated.

**Quantitative analysis of fluorescence intensity**

Three consecutive AM and VM cultures at day 6, 9, 12, 15 *in vitro* and three adult rat hearts were used for quantification of the FI of Cx40 in individual GJ. After fixation and permeabilization (see: Immunolabeling) the cells and tissue samples were exposed to 0.5% carboxylated BSA and 100 mmol/L glycine in PBS for 15 min at room temperature to suppress non-specific fluorescence and then incubated with anti-Cx40 antibody in different concentrations ranging from 0.1 μg/mL to 10 μg/mL, followed by anti-rabbit IgG-conjugated with Cy3. Repeated washes with PBS were done after each step of the immunolabeling procedure. The use of anti-rabbit IgG-conjugated with Cy2 had no effect on the result (see: Supplementary Figure 7). Cell cultures and tissue sections exposed to PBS instead of primary antibodies, but incubated sequentially with the detection system served as negative controls and were run in parallel during each quantitative experiment. All processing and immunolabeling procedures were done under identical conditions for all investigated groups. The confocal settings had been standardized for all experimental groups to ensure that the image collected demonstrates a full range of FI from 0 to 255 intensity levels and were kept constant for recording of data in all measurements. The value of FI was expressed as mean FI (in arbitrary units) per voxel. The average FI of Cx40 per GJ was calculated from measurements of the total 103±4 individual GJ per each type of cardiac cells per rat *in vivo* or per each type of cells per selected intervals in culture.
Statistical Analysis

Results are reported as means ± SD. Paired t test was used to estimate the significance of differences between groups. The minimum level of statistical significance was p<0.05.

Legends for Supplementary Figures Online

Supplementary Figure 1 Online. ID in adult VM in vivo. (A) TEM micrograph that shows a portion of the ID connecting two VM at the level of the nucleus (NUC), indicating a side-to-side connection (following the terminology of Luke and Saffitz)\(^{15}\). A long GJ profile (up to 20 μm in length) is marked with arrows. The arrowhead shows an interplicate GJ, whereas open arrowheads denote annular GJ profiles. Panel B is an enlargement of Panel A with identical markers. (C) A slightly oblique section showing large GJ indicated with arrows. Small arrows denote numerous small GJ at the plicate segments (PL) of the ID. Scale bars represent 2 μm in all micrographs.

Supplementary Figure 2 Online. Micrographs made at progressive magnification of TEM showing typical ID in TP of section through left VM from a 90 days old Wistar rat (body weight 305 grams). Large GJ are indicated with arrows. (A) Low magnification survey view of an ID showing multiple plicate segments (PL) and large GJ localized at the periphery of ID or being contiguous with the peripheral non-junctional sarcolemma (sm). Panel B is a higher magnification of two of the large GJ shown at the right bottom in Panel A. (C) A portion of the ID at higher magnification shows numerous plicate segments and large GJ. (D) A fragment of the ID showing two large GJ: the left one encircles the plicate segment, the second GJ originates from a plicate segment and continues to the peripheral sarcolemma. (E) Multiple large GJ at the periphery of an ID. (F) Micrograph representing an enlargement of the GJ shown at the right side in Panel D. Scale bars represent 2 μm in all micrographs.
**Supplementary Figure 3 Online.** Examples of large GJ (arrows) localized at the periphery of the ID in adult rat VM seen in TP of section at different magnifications of TEM. The points of transition of GJ membranes into the peripheral sarcolemma (sm) are indicated with small arrows. A and B are survey images of the edges of ID showing multiple large GJ. Panels C through F are examples of large peripheral GJ seen at higher magnifications of TEM. In Panel G, the arrowhead denotes a desmosome, PL indicates a plicate segment. Panels I and J show subsarcolemmal large GJ in close contact with desmosomes (arrowheads). K – A chain of large GJ isolated from each other by desmosomal plaques (arrowheads) and circumscribing the periphery of an ID in close spatial proximity to the peripheral sarcolemma. Scale bars: 1 μm in Panels A, B, D, E, and G through K. In Panels C and F, bars represent 0.5 μm.

**Supplementary Figure 4 Online.** Large GJ (arrows) at the small-sized ID in adult VM in vivo (Panel A through E). Panel B is an enlargement of the upper GJ shown in Panel A. Panel D is an enlargement of the GJ from the central part of Panel C. PL indicates plicate segments. Scale bars: 2 μm in Panels A, C and E. In Panels B and D, bars represent 1 μm.

**Supplementary Figure 5 Online.** Frequency distribution of different GJ size classes in AM versus VM in LP and TP in vivo. The number from the upper part of each bar represents total number of GJ obtained from six rats.

* p<0.05, ** p<0.01 (VM versus AM).

**Supplementary Figure 6 Online.** The relative contribution of different GJ size classes to the total GJ length in AM versus VM in LP and TP in vivo. * p<0.05, ** p<0.01 (VM versus AM).
**Supplementary Figure 7 Online.** Cx43 and Cx40 in adult rat hearts *in vivo* and in AM and VM *in vitro*. (A) Adult rat left AM double labeled for Cx43 (Cy2, green) and Cx40 (Cy3, red). Note that Cx43 is confined to AM, whereas Cx40 conspicuously labels GJ in endothelial cells of a intramural artery. L - indicates the lumen of an artery. (B) Cx43 and Cx40 in an adult rat left ventricle. Strong Cx43-immunofluorescence is detectable in both „working“ VM and in a subendocardial strand of Purkinje cells (P). (C) Identical section as in Panel B showing intense positive Cx40 signals in Purkinje cells, while a Cx40-signal is totally absent in subjacent VM. These data of Cx40 in adult rat hearts are in close agreement with other studies of Cx40 protein\textsuperscript{12,28,29} and mRNA\textsuperscript{30,31} for Cx40 in adult rat AM, VM,\textsuperscript{10,28,32-34} and Purkinje cells,\textsuperscript{10-12,28,32,34-36} (D and E) Sequential immunolabeling for Cx43 detected with anti-mouse IgG-coupled with Cy3 (red) followed by immunolabeling for Cx40 (with 5 μg/mL of the primary anti-Cx40 antibody) detected with anti-rabbit IgG-conjugated with Cy2 (green) in the re-developed GJ in AM cultured for 15 days. (F) Representative recordings of the FI from the boxed region of an individual GJ showing the distribution of the FI of Cx43 and Cx40 per voxel. Notice that all values of the FI per each voxel are in the range of 0-255 level scale units. (G and H) Similar GJ in AM cultured for 15 days and double labeled for Cx43 and Cx40 in an alternative experiment in which the order of primary antibodies and the fluorochromes of the secondary antibodies had been changed in that anti-Cx40 and the detection system were applied first, followed by anti-Cx43 and respective detection system, while keeping constant confocal settings to the values of the previous experiment shown in Panels D through F. (I) Representative recordings of the FI of Cx43 and Cx40 from the boxed regions in Panels G and H showing no major difference in the distributions of individual values of FI for Cx43 and Cx40 with those shown in Panels F. (J) Results of the quantitation of FI of Cx40 in individual GJ *in vivo* and *in vitro*. For the measurements, Purkinje fibers served as internal positive control, and the omission of anti-Cx40 antibody in all types of myocytes
in vivo and in vitro, served as negative controls. Each value represents mean±SD of the FI obtained from 3 rats in vivo and 3 cultures of AM and VM at day 9 and 15 in vitro. The difference in the mean values of FI of Cx40 per individual GJ between Purkinje cells and those in AM and VM in vivo or in AM and VM at day 9 and 15 in culture was highly significant (*** - p<0.001). There was no statistical difference between the mean values of FI in negative controls with those using anti-Cx40 antibody in AM and VM both in vivo and in vitro.

**Supplementary Figure 8 Online.** Representative patterns of immunolabeled GJ for Cx43 in adult rat AM and VM in vivo. Confocal images represent projections from 10 optical sections taken at 0.5 μm interval through transversely sectioned ID of atrial (A) and ventricular (B) myocardium. Note that the majority of GJ in AM are oval, discoid or slightly elongated in shape. A prominent chain of large and elongated immunostained GJ (arrows) cirumscribing the periphery of the ID is obvious in VM.
References for Online Supplement


Frequency distribution of GJ size classes

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- Atrial myocytes (n=6)
- Ventricular myocytes (n=6)
Relative contribution to the total GJ length

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- Atrial myocytes (n=6)
- Ventricular myocytes (n=6)
Supplementary Figure 7