Altered Right Atrial Excitation and Propagation in Connexin40 Knockout Mice

Suveer Bagwe, MD, MSc; Omer Berenfeld, PhD; Dhananjay Vaidya, MPH, PhD; Gregory E. Morley, PhD; José Jalife, MD

Background—Intercellular coupling via connexin40 (Cx40) gap junction channels is an important determinant of impulse propagation in the atria.

Methods and Results—We studied the role of Cx40 in intra-atrial excitation and propagation in wild-type (Cx40+/+) and knockout (Cx40−/−) mice using high-resolution, dual-wavelength optical mapping. On ECG, the P wave was significantly prolonged in Cx40−/− mice (13.4±0.5 versus 11.4±0.3 ms in Cx40+/+) in Cx40+/+ hearts, spontaneous right atrial (RA) activation showed a focal breakthrough at the junction of the right superior vena cava, sulcus terminalis, and RA free wall, corresponding to the location of the sinoatrial node. In contrast, Cx40−/− hearts displayed ectopic breakthrough sites at the base of the sulcus terminalis, RA free wall, and right superior vena cava. Progressive ablation of such sites in 4 Cx40−/− mice resulted in ectopic focus migration and cycle length prolongation. In all Cx40−/− hearts the focus ultimately shifted to the sinoatrial node at a very prolonged cycle length (initial ectopic cycle length, 182±20 ms; postablation sinus cycle length, 387±44 ms). In a second group of experiments, epicardial pacing at 10 Hz revealed slower conduction in the RA free wall of 5 Cx40−/− hearts than in 5 Cx40+/+ hearts (0.61±0.07 versus 0.94±0.07 m/s; P<0.05). Dominant frequency analysis in Cx40−/− RA demonstrated significant reduction in the area of 1:1 conduction at 16 Hz (40±10% versus 69±5% in Cx40+/+) and 25 Hz (36±11% versus 65±9% in Cx40+/+).

Conclusions—This is the first demonstration of intra-atrial block, ectopic rhythms, and altered atrial propagation in the RA of Cx40−/− mice. (Circulation. 2005;112:2245-2253.)

Key Words: atrium ■ conduction ■ connexins ■ Fourier analysis ■ sinoatrial node

Intercellular coupling via gap junction channels is an important determinant of impulse propagation in the heart.1 Alterations in expression of cardiac connexin proteins may lead to abnormal conduction and arrhythmia.2 Therefore, knowledge of the role of connexins in impulse propagation is essential in the understanding of arrhythmic mechanisms. Polymorphisms in the regulatory genes for connexin40 (Cx40) have been shown to be linked with familial atrial standstill and increased vulnerability to atrial fibrillation,3,4 both related to conduction abnormalities. Immunohistochemical studies in the mouse have indicated that of the 3 connexins (Cx40, Cx43, and Cx45) known to be expressed in myocytes, Cx40 is found mainly in the atrial myocardium and His-Purkinje system.5-7 Recently, it has been shown that strands of Cx43- and Cx40-positive atrial cells protrude into the Cx45-positive sinus nodal area in the mouse.8

Previous studies have demonstrated that P-wave duration, PQ interval, and QRS duration are significantly prolonged in Cx40 knockout (Cx40−/−) mice.9-12 It has been speculated that P-wave prolongation may result from a prolongation of intra-atrial conduction time caused by local block with consecutive prolongation of activation path length. However, in the absence of detailed activation studies, the exact role of Cx40 in intra-atrial propagation remains poorly understood. Our objective was to study the role of Cx40 in intra-atrial excitation and propagation in wild-type (Cx40+/+) and knockout (Cx40−/−) mice using high-resolution optical mapping. Our results demonstrate for the first time that null mutation of Cx40 impairs sinoatrial propagation and results in the development of atrial ectopic pacemakers, which maintain the overall cardiac rhythm in these mice.

Methods
See the online-only Data Supplement (http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.104.527325/DC1) for additional information about Methods.

Mice
The mouse colony was founded by a breeding pair (129Sv/C57BL6 strain) heterozygous for the Cx40 knockout mutation.9 Data were obtained from adult (aged 11 to 20 weeks) wild-type (Cx40+/+; n=22) and knockout (Cx40−/−; n=22) mice obtained after at least 15 generations of traditional backcrosses.9 Separate groups of mice

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were studied for the microelectrode recordings (n=8) and right atrial (RA) mapping during basal rhythm (n=12), of which 4 mice of each genotype were used for ablation experiments. Five mice from each genotype were used for pacing. Animals for different experiments were chosen randomly. All animal care protocols conformed to institutional and National Institutes of Health guidelines. All analyses were done on littermates generated by interbreeding mice heterozygous for Cx40.

Optical Mapping and Motion Correction Algorithms
Hearts were isolated and Langendorff-perfused as previously described. Optical mapping of the RA epicardial surface was performed with the use of a novel high-resolution, dual-wavelength video imaging approach to measure changes in potentiometric dye fluorescence (Di-4-ANEPPS) in the absence of motion artifacts. The approach enabled us to nearly eliminate the mechanical artifacts associated with the contraction of the tissue and to accurately quantify apparent conduction velocity at the spatiotemporal scale of the mouse heart. (The technique is explained in detail in the online-only Data Supplement.) Briefly, by using simultaneous recordings with 2 CCD cameras, our approach relies on the offline application to all movies of a translational motion tracking-and-correction algorithm based on the template-matching technique. This is followed by voltage signal amplification and local motion subtraction. No electromechanical uncouplers or physical restraint procedures were used in any of the experiments.

Other Recording and Data Acquisition Techniques
Six-lead surface ECG recordings were obtained from conscious mice as described in detail elsewhere. Optical pseudo-ECG, volume-conducted ECG, and microelectrode recordings were obtained from the Langendorff-perfused hearts. Four Cx40+/− and Cx40−/− hearts were used for Western blot and immunofluorescence analysis of Cx43 protein. (See online-only Data Supplement for details.)

Pacing Protocol
The RA was paced at progressively higher frequencies of 10, 12, 16, and 25 Hz with the use of 5-ms stimuli at 1.5× diastolic threshold. Activity during pacing was recorded for 4 seconds. The conduction velocity in the slowest direction was quantified for the RA free wall of the 2 genotypes at 10 Hz. The time taken for conduction of the action potential to reach the junction of the RA free wall, sulcus terminalis, and right superior pulmonary vein was also calculated.

Data Analysis
Optical mapping data are presented as maps of activation time of a single experiment or as mean and SD of activation time in multiple experiments. In addition, dominant frequency maps were generated by applying for each pixel a fast Fourier transformation (resolution, 0.2 to 0.5 Hz). The frequency with the highest power was considered the dominant frequency. Conduction time and apparent conduction velocity were measured by a vectorial approach, as described in detail in the online-only Data Supplement.

Ablation Protocol
In 4 Cx40+/− and 4 Cx40−/− mice, consecutive ablations of the initial breakthrough site of activation were performed with the use of an Aaron-Ram high-temperature, fine-tip (0.75 mm) cautery (Aaron Medical Industries). The final ablation was of the sinoatrial node high-temperature, fine-tip (0.75 mm) cautery (Aaron Medical Industries). The final ablation was of the sinoatrial node (SA) node. The final ablation was of the SA node.

Statistical Analyses
Values are reported as mean±SEM. An unpaired t test was used for comparing observations obtained by ECG and microelectrodes and for comparing the conduction velocities, cycle lengths, and areas of 1:1 response to pacing at specific frequencies. A 2-way ANOVA was used to test the dependency of area with 1:1 response to pacing in the 2 types of mice. P<0.05 was considered significant.

Results
Figure 1 shows representative surface (Figure 1A and 1B) and volume-conducted (Figure 1C) ECG recordings obtained from Cx40+/− and Cx40−/− hearts. Although the RR intervals were similar (Cx40+/−, 162.6 ms; Cx40−/−, 170 ms), the ECGs clearly demonstrate that the P wave is aberrant and prolonged, together with the PR interval, in the Cx40−/− mouse. The Table summarizes the ECG data from Cx40+/− (n=8) and Cx40−/− (n=8) mice. These data extend previous data obtained in anesthetized Cx40−/− animals, suggesting a delayed or altered intra-atrial conduction during spontaneous rhythm. We also confirm the increased PR and QRS duration previously seen in these mice.

RA Activation in Wild-Type Hearts
To determine the mechanisms underlying the surface ECG alterations, RA epicardial activation patterns were recorded in...
ECG Data From Cx40+/+ and Cx40−/− Mice

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<th>Cx40+/+</th>
<th>Cx40−/−</th>
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<tr>
<td>RR</td>
<td>187.3±4.5</td>
<td>190.3±6.6</td>
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<tr>
<td>P</td>
<td>11.4±0.3</td>
<td>13.4±0.5*</td>
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<tr>
<td>PR</td>
<td>41.9±0.9</td>
<td>49.9±1.5†</td>
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<tr>
<td>QRS</td>
<td>15.2±1.1</td>
<td>19±1.1*</td>
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Data are mean±SEM, expressed in milliseconds; n=8 for each genotype. *P<0.05. †P<0.01.

Cx40+/+ and Cx40−/− mouse hearts. Figure 2A shows the RA anatomy, including the right superior vena cava and left superior vena cava as well as the opening of the inferior vena cava. The veins open into the smooth part of the RA, which is separated on the surface from the RA free wall by the sulcus terminalis.

Spontaneous electric activity was confirmed for the Cx40+/+ and Cx40−/− hearts, following which 4-second movies were obtained from the epicardial surface of the RA. The activation sequence during spontaneous electric activity was similar in 12 Cx40+/+ hearts. Therefore, superimposing the individual activation maps allowed construction of the composite color activation map. In Figure 2B, the 1-ms isochrone map shows that RA activation is characterized by a focal breakthrough at the junction of the right superior vena cava and RA free wall, which corresponds to the location of the sinoatrial node. The breakthrough leads to wave fronts that activate the entire field of view within 6 ms. Propagation was relatively slow across the sulcus terminalis, taking ~3 to 4 ms to reach the RA appendage. There was also slowing of the impulse as it traversed the intercaval region medial to the crista terminalis toward the septum and atroventricular node. The SD of ~1 ms over the vast majority of pixels on the RA (Figure 2C) emphasizes the very high reproducibility of the data. Our results demonstrate preferential propagation in the RA of Cx40+/+ hearts from a unifocal sinoatrial node toward the atroventricular node along the sulcus terminalis, consistent with previous results obtained with the use of microelectrodes and optical mapping.

Abnormal RA Activation in Knockout Hearts
In all Cx40−/− hearts (n=12), spontaneous electric activity was characterized by an ectopic focal breakthrough and an abnormal sequence of intra-atrial activation. Figure 3A shows a representative map from 1 such Cx40−/− mouse. An ectopic breakthrough (red) is seen in the RA appendage, with subsequent activation of the entire field of view in ~7 ms. The 1-ms contour lines superimposed on the activation map highlight the substantially altered local propagation velocity pattern in the RA of this knockout heart. In 3 Cx40−/− mice, areas with extremely delayed activation were observed, suggesting conduction block. One such example is shown in Figure 3B, in which an area overlying the sinoatrial node region showed delayed activation of up to 14 ms after the first focal breakthrough on the RA appendage. Similar areas of extreme delay were found at different locations on the RA free wall of 2 additional Cx40−/− hearts (not shown) but were never observed in the Cx40+/+ hearts.

After the initial ectopic breakthrough, a variable number of secondary breakthrough sites were also seen in the Cx40−/− hearts. This phenomenon was not observed in the Cx40+/+ hearts. In Figure 3C, a schematic shows the sites of the ectopic breakthrough in all 12 Cx40−/− mice. Importantly, the sinoatrial node region was never the site of initial breakthrough in any of the knockout hearts. In 7 cases the initial site of activation was located at the base of the sulcus terminalis and around the opening of the inferior vena cava.

Figure 4 illustrates the distribution of the apparent conduction velocities over the entire mapped area measured during basal rhythm in 8 Cx40+/+ and 8 Cx40−/− mice. Figure 4A shows that the percentage of the optically mapped areas at certain velocities are different for the 2 genotypes. In Figure 4B, the same data have been plotted as a percentage of the optically mapped area for a given range of apparent conduction velocity. During basal rhythm there is a significant increase in the very low velocities (<0.3 m/s) in the Cx40−/− mice compared with the Cx40+/+ mice, with concomitant reduction of velocities between 0.3 and 1 m/s.

Ablation of Breakthrough Sites
The differences in the activation patterns of Cx40+/+ and Cx40−/− hearts were further studied in focal ablation experiments. In all Cx40−/− mice, after the ablation of the first site of activation, the sinoatrial activation pattern was immediately replaced by a much slower rhythm with a broad wave front traveling from the left superior vena cava/inferior vena cava region to the rest of the RA in a caudocranial direction (not shown). Completely different patterns were observed in all Cx40−/− hearts. In Figure 5A, the top portion shows 3 activation maps obtained during consecutive focal ablations...
in a knockout heart; the corresponding pseudo-ECGs are shown below. In basal rhythm (left), the initial breakthrough activation was located in the lower portion of the RA appendage (asterisk); impulses generated at a constant cycle length of 227 ms rapidly propagated through the rest of the atrium. On ablation of this site, the leading pacemaker focus shifted (middle) to the base of the sulcus terminalis and activated the atrium at a slower rate (cycle length, 285 ms). On ablation of the second site, the leading pacemaker shifted again, but this time to the sinoatrial node region (right). Under these conditions, the sinoatrial activation sequence appeared relatively normal. However, as shown by the pseudo-ECG, the cycle length was markedly prolonged. Similar to the Cx40+/− hearts, ablation of the sinoatrial node resulted in an additional pacemaker shift (not shown), wherein a broad wave front from the region of the left superior vena cava and inferior vena cava activated the RA.

Figure 5B shows composite ablation data from 4 Cx40+/− and 4 Cx40+/+ hearts. The basal cycle lengths of the Cx40+/− (182±20 ms) and Cx40+/+ hearts (188±12 ms) were not significantly different. In both cases, ablation resulted in cycle length slowing, regardless of leading pacemaker location. In the Cx40+/− hearts, 2 to 3 ablations were required before the leading pacemaker was observed at or near the

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**Figure 3.** RA activation in Cx40−/− hearts. A, One-millisecond isochronal activation map in representative RA. An ectopic discharge in the RA free wall results in an altered pattern with prolonged atrial activation time. B, In another RA, the pattern of activation is characterized by extremely delayed activation of an area corresponding to the sinoatrial node. C, Locations of ectopic sites in the RA of 12 Cx40−/− mice. Abbreviations are defined in Figure 2 legend.

**Figure 4.** A, Spatial distribution of apparent conduction velocities (CV) over the entire optically mapped area during basal rhythm in both Cx40+/− (n=8) and Cx40−/− mice (n=8). B, Percentage of the area with a given range of apparent conduction velocity. Note larger percentages of slower velocities in the Cx40−/− mice.

**Figure 5.** A, RA activation maps and pseudo-ECGs of knockout mouse before (left) and after first (middle) and second (right) ablations. In control, an ectopic discharge (white star) resulted in altered activation. On first ablation (ABL), the ectopic focus migrated, and cycle length (CL) was prolonged. The second ablation shifted the focus to the sinoatrial node (right). B, Cycle length changes induced by consecutive ablations in 4 Cx40+/+ (left) and 4 Cx40−/− (right) hearts. Other abbreviations are defined in Figure 2 legend.
sinoatrial node. In these hearts, consecutive ablation of ectopic sites resulted in a shift of the leading pacemaker to the sinoatrial node, with significant prolongation of the cycle length (321 ± 26 ms; P < 0.05). Cycle length prolongation occurred in both genotypes after ablation of the sinoatrial node (Cx40+/+, 387 ± 44 ms; Cx40−/−, 432 ± 56 ms). Taken together, the aforementioned data clearly demonstrate that the abnormal P wave in the ECG of the Cx40−/− mice is associated with altered atrial conduction patterns resulting partially from sinoatrial node impairment leading to slowing of sinoatrial node cycle length, with consequent development of ectopic pacemaker activity.

**Atrial Pacing and Intra-Atrial Propagation**

Figure 6A presents 1-ms activation maps obtained while pacing the RA appendage at a basic cycle length of 100 ms (red arrows). The anisotropic conduction pattern in the Cx40−/− heart (right) was similar to that of the Cx40+/+ heart (left). However, in the former conduction was uniformly slow over the entire RA. In contrast to the spontaneously active hearts (Figure 3), we did not find any patchy areas of delayed atrial activation during pacing. In Figure 6B, the local epicardial velocities in the direction of slowest propagation during pacing of the RA at a basic cycle length of 100 ms (10 Hz) are shown. Mean apparent conduction velocity in the RA was reduced in the Cx40−/− mice (0.61 ± 0.07 m/s; n=5; P < 0.05) compared with the Cx40+/+ mice (0.94 ± 0.05 m/s; n=5). Overall, the conduction time from the pacing site to the junction of the right superior vena cava and sulcus terminalis was 2.4 ± 0.2 ms in the Cx40+/+ and 3.6 ± 0.2 ms in the Cx40−/− mice (P < 0.05). These data indicate that Cx40 contributes significantly to impulse propagation within the atrial myocardium. This is in agreement with previous multielectrode and immunolocalization studies.5,8,11

It has been suggested that the atria of Cx40−/− mice have an increased susceptibility to tachyarrhythmias because of reduced intercellular coupling.18 Although we did not find any tachyarrhythmias in the atria of knockout mice during basal conditions, we hypothesized that the absence of Cx40 would increase the degree of inhomogeneous propagation at high excitation frequencies. Thus, we paced the RA at various frequencies and used dominant frequency analysis to determine the spatial changes in intra-atrial conduction at rates of 10 to 25 Hz. Representative examples for each genotype are shown in Figure 7A. At 10 Hz, almost the entire RA was...
activated in a 1:1 manner in both Cx40\(^{+/+}\) and Cx40\(^{-/-}\) hearts. However, at 25 Hz, although the majority of sites in the Cx40\(^{+/+}\) responded 1:1, in the Cx40\(^{-/-}\) heart the area of 1:1 response was greatly reduced. In this case, areas (domains) of 2:1, 3:1, and other complex subharmonic frequency combinations, including an alternans pattern, became apparent. Composite data from all the experiments in the Cx40\(^{+/+}\) (n=6) and Cx40\(^{-/-}\) (n=6) hearts are presented in Figure 7B. The percentage of the mapped RA that responded in a 1:1 manner is plotted as a function of the pacing frequency. Clearly, at >12 Hz there was a reduction in the 1:1 domain in the Cx40\(^{-/-}\) hearts. Concomitantly, in all experiments the number of domains in which individual frequencies were lower than the pacing frequency increased (not shown). The apparent small reduction in the RA area after 1:1 in the Cx40\(^{-/-}\) was an artifact due to a reduction in signal-to-noise ratio at the periphery of the preparation. Overall, statistical comparison of the percent space occupied by the 1:1 domain indicated significant effects of genotype (Cx40\(^{+/+}\) versus Cx40\(^{-/-}\); P<0.01) and pacing frequency (P<0.01). Significant genotype-dependent differences were noted for the 1:1 domain at 16 Hz (Cx40\(^{+/+}\), 40±10%; Cx40\(^{-/-}\), 69±5%; P<0.05) and 25 Hz (Cx40\(^{-/-}\), 36±11%; Cx40\(^{+/+}\), 65±9%; P<0.05). Thus, the absence of Cx40 reduces RA conduction velocity and decreases the safety factor for maintaining 1:1 conduction at faster pacing frequencies.

Substrate Considerations

Figure 8A shows data obtained from 4 mice in which Cx43 protein was measured in the whole heart and both atria of Cx40\(^{+/+}\) and Cx40\(^{-/-}\) mice. We observed no differences in the amount of protein when wild-type mice were compared with knockout mice. Similarly, Figure 8B demonstrates no differences in immunostaining of Cx43 antibody in the RA of wild-type and knockout mice. We also excluded genotype-specific reduction in the excitatory currents by recording transmembrane potentials in the RA free wall using standard microelectrode techniques. Figure 8C shows representative action potentials, and Figure 8D summarizes the electrophysiological properties of cells from 8 Cx40\(^{+/+}\) and 8 Cx40\(^{-/-}\) hearts. There were no significant differences in resting membrane potential, dV/dT\(_{max}\), action potential amplitude, or action potential duration between genotypes.

Discussion

The most important results of this study are as follows: (1) In Cx40\(^{+/+}\) mice, leading pacemaker activity is manifested as an epicardial focal breakthrough at the sinoatrial node. In contrast, Cx40\(^{-/-}\) hearts display ectopic breakthrough sites at the base of the sulcus terminalis, RA free wall, and right superior vena cava. (2) Consecutive ablation of such sites resulted in ectopic focus migration and cycle length prolongation. In all Cx40\(^{-/-}\) hearts, the focus ultimately shifted to the sinoatrial node at a prolonged cycle length. (3) Epicardial pacing revealed conduction slowing in the RA free wall of Cx40\(^{-/-}\) hearts. (4) Dominant frequency analysis demonstrated a significant reduction in the spatial distribution of 1:1 conduction at 16 and 25 Hz. These data provide a detailed mechanism for the alterations in the P wave recorded on surface ECGs from Cx40\(^{-/-}\) mice\(^{9–11,18}\) and provide important insight into basic mechanisms underlying impaired conduction and tachyarrhythmias associated with altered intercellular coupling.

ECG Intervals

Cx40\(^{-/-}\) mouse hearts present right bundle-branch block,\(^{13,21}\) delayed AV nodal, atrionodal, and proximal His bundle conduction,\(^{22}\) and impaired left bundle conduction.\(^{21}\) Long sinus node recovery time, sinus entry block, slow atrial conduction, and atrial tachyarrhythmias have also been reported.\(^{10,11,18}\) Together, these studies provided supporting evidence that Cx40 is an important determinant of impulse propagation in the atria as well as the specialized conduction system. Our experiments extend those observations by demonstrating for the first time that the abnormal P wave in the Cx40\(^{-/-}\) mice is the result of an altered sinoatrial node function, which ultimately results in the development of ectopic pacemaker activity at varying sites within the RA.
Atrial Pacemaking in the Absence of Cx40

The present study is the first to use the highly sensitive technique of dual-wavelength optical mapping to investigate the origin and propagation of pacemaker activity in mice. The null mutation of Cx40 resulted in obvious differences in RA activation during basal rhythm with respect to the focus and pattern of activation. Our results suggest that the absence of Cx40 impairs sinoatrial node automaticity in such a way as to slow the pacemaker discharge. Concomitantly, ectopic foci are unmasked at various locations of the RA, and 1 of these foci becomes the dominant pacemaker that drives the entire heart.

Role of Cx40 in Normal and Ectopic Pacemaker Activity

Ectopic discharges have been shown previously in Cx40−/− mice but not as the dominant pacemaker. Here stable pacemaker activity was demonstrated in 11 of 12 Cx40−/− hearts at ectopic locations far removed from the sinoatrial node.

In the embryonic mouse atrium, initial expression of Cx40 begins at about the same time as the maturation of the sinoatrial node (9.5 days post coitum).7,23 On the other hand, Cx43 is expressed in the atria only from 12.5 days post coitum onward.24 It has been shown that the epicardial area surrounding the primary pacemaker zone in the mouse is composed of Cx40 and Cx43,19 with Cx45 also being present in the central sinoatrial node. Thus, it is possible that heterotypic gap junction channels in the epicardial transition zone between the sinoatrial node and the RA act as current rectifiers, as has been previously suggested.25 Although Cx43+/− mice have not been shown to have any altered atrial parameter,26 we can only speculate on the role of Cx43 and Cx45 in this transition zone. It thus seems plausible that Cx43, along with Cx45, is unable to maintain the necessary coupling in the transition zone and that the high conductance of Cx40 is probably required for successful sinoatrial coupling.

Molecular Basis for Ectopic Activity

Areas of automaticity have been previously observed in the sulcus terminalis and coronary sinus27,28 Nodal and extranodal pacemakers have been described in rabbits,29 dogs,30 and humans27 both in sinus rhythm and under autonomic or pharmacological modulation. Clearly, cells capable of developing automatic pacemaker activity may be widely distributed throughout the atria, outside the sinoatrial node.23,28,31,32

However, an important question that remains unanswered is whether the shift of the dominant pacemaker from the sinoatrial node region to an ectopic location in the Cx40−/− animals (Figure 3) is the result of slowing of the sinoatrial node or acceleration of the ectopic sites or both. Mechanistically, a more specific question would be whether the appearance of ectopic activity in the Cx40−/− mice is a consequence of alterations in genes encoding for or regulating membrane ion channels involved in pacemaker activity in the sinoatrial node and/or atria. Indeed, recently it has become clear that connexins are in many ways similar to scaffolding proteins that can bind cytoskeletal proteins, protein kinases, and cellular scaffolds.32,33 Recent evidence has even suggested that some of the biological properties of connexin are channel independent.34 Recent evidence has even suggested that some of the biological properties of connexin are channel independent.34,35 On the basis of the complex regulation and interactions of connexins, it would be expected that manipulations of connexins may result in alterations in other genes. A case in point is the recent demonstration by Gu et al13 that Cx40−/− mice have a high incidence of conotruncal abnormalities, which raised the possibility of interference of neural crest interaction with the developing heart. Currently, the genetic modifiers needed for the expression of ectopic pacemaker sites remain undetermined. Our experiments suggest that reduced coupling enables the expression of these ectopic foci, which would otherwise have been prevented from becoming spontaneously active by the hyperpolarizing influence of surrounding nonpacemaking atrial tissue. It follows that the absence of Cx40 reduces the sinoatrial coupling sufficiently to prevent the sinoatrial node from being the dominant pacemaker. However, the molecular mechanisms responsible for these changes are well beyond the scope of the present study and will require further investigation.

Conduction Slowing in the RA of Cx40−/− Mice

Qualitatively, the activation pattern of the Cx40−/− RA paced at 10 Hz was similar to that in the wild-type mouse. However, RA apparent conduction velocity was reduced by ≈36% in the Cx40−/− mouse. Although spatial heterogeneity in the remaining coupling may possibly explain the appearance of local areas of conduction block and/or heterogeneities in propagation (Figures 3 and 4), in general, the lack of Cx40 in the RA results in intra-atrial conduction slowing, which, together with the ectopic nature of the activity, explains in part the P-wave prolongation observed in the ECG. Additional slowing in the left atrium would be expected to play a role as well. Conduction velocity is dependent on the interaction of several parameters, including cell excitability,34 intercellular coupling,35 and tissue geometry.36 To our knowledge, no structural malformations have been reported for the RA of the Cx40−/− mouse heart,33 and therefore the latter parameter would not be expected to be responsible for the observations. In addition, our microelectrode recordings, together with previous work showing no changes in the excitatory current of the Cx43−/− mouse heart,37 indicate that a reduction in excitability is unlikely. Thus, the reduced conduction is mainly due to loss of coupling. Nevertheless, a possible role of differences in tissue structure, sodium channels in the intercalated discs,38 and fibroblasts in conduction38 cannot be ignored when one attempts to fully understand our results.

Frequency Dependence in the Absence of Cx40

The atria in Cx40−/− mice seem more susceptible to arrhythmias.18 This, along with our finding of slow conduction, prompted the need to understand the frequency dependence of impulse propagation in these mice. Pacing the Cx40−/− RA at frequencies >12 Hz resulted in areas of intermittent block (eg, 2:1; 3:2), demonstrating that the degree of electric coupling is an important parameter determining frequency dependence of propagation. In Cx40−/− mice, the other
candidate connexins maintaining coupling in the atria are Cx43 and Cx45. Data suggest that Cx45 is expressed in very small amounts in the canine atrium but not at all in the adult mouse. Our Western blot and immunolocalization results revealed no changes Cx43 protein in the atria of Cx40−/− mice compared with Cx40+/+ mice (Figure 8). Furthermore, no upregulation of the transcript or protein amounts was observed for Cx43, Cx37, or Cx45 in Cx40−/− mice. Cx43 thus seems likely to be maintaining the conduction during basal rhythm in the absence of Cx40. However, the presence of Cx43 seems inadequate at high pacing rates, possibly because of the heterogeneous distribution and/or the lower unitary conductance of Cx43 compared with Cx40. On the other hand, it appears that Cx40 provides the atrial myocardium with a high safety factor in maintaining 1:1 conduction even at fast rates.

Limitations
From gross anatomic inspection, it is difficult to rule out any structural alterations in the sinoatrial transition zone in the Cx40−/− hearts. In addition, detailed histochemical studies using antigens like HNK will be needed to ascertain the origin of the ectopic sites in Cx40−/− mice and to determine whether they are present in Cx40+/+ mice. The distribution pattern of gap junctions in the sinoatrial node and the transition zone as well as the RA free wall in the absence of Cx40 remains to be determined. The role played by heteromic/heterotypic gap junctions in sinoatrial and intra-atrial propagation in Cx40−/− mice atria is uncertain. The precise mechanism by which Cx40 deletion results in slowing of the sinoatrial node pacemaker function and generation of ectopic pacemaking sites remains to be elucidated. Finally, we do not at this point know the effect of absence of Cx40 on interatrial and left atrial propagation.

Conclusion
We have characterized the role of Cx40 in intra-atrial propagation during both sinus rhythm and pacing. We show for the first time that, because of sinoatrial dysfunction, ectopic foci become the leading pacemakers in the heart of Cx40−/− mice. We also demonstrate that the location of such foci along with slowing of apparent conduction velocity in the RA is responsible in part for the prolonged and deformed P waves observed in these mice. Our results also attribute a protective role to Cx40 in that it helps the RA to sustain faster cycle lengths and prevents breakdown of 1:1 conduction.

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References


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EXPANDED MATERIALS AND METHODS

Mouse Langendorff-Perfused Heart Preparations

Mice were heparinized (heparin sodium [0.5U/g]), anesthetized by brief CO$_2$ inhalation and sacrificed by cervical dislocation. Hearts were surgically removed via a thoracotomy and placed in a custom-built perfusion / superfusion apparatus. While the heart was fully immersed in Tyrode solution (containing in mmol/L: NaCl, 130; NaHCO$_3$, 24; NaH$_2$PO$_4$, 1.2; MgCl$_2$, 1; glucose, 5.6; KCl, 4; CaCl$_2$, 1.8 saturated with 95% O$_2$: 5% CO$_2$ gas mixture), the aorta was cannulated using a 22g stainless steel cannula and perfused with warm (37-38 ºC) Tyrode solution at a constant pressure of 68-74 mm Hg (~1-2 ml/min). The perfusion/superfusion apparatus was composed of an outer water-jacketed dish (Radnoti Inc.) and inner custom-built perfusion chamber (Figure 1). The custom-built perfusion chamber allowed for horizontal positioning of the heart. All hearts were allowed to equilibrate for 15 minutes following perfusion. No pharmacological or mechanical motion reduction technique was used during any of the experimental protocols.

Electrocardiographic Recordings

Volume-conducted ECG’s were recorded during Langendorff perfusion from Ag-AgCl electrodes (0.5mm × 1mm). Signals were amplified and low-pass filtered with a differential amplifier (CyberAmp 380, Axon Instruments), digitized (Digidata 1200) at 5 kHz and stored for offline analysis. Interval measurements were performed on signal averaged ECG recordings. Signal averaging (10-15 beats) provided average interval
duration measurements and improved the signal-to-noise for each mouse ECG trace. ECG intervals were defined as described previously.²

Optical Mapping of Voltage-Sensitive Dye Fluorescence

High-resolution optical mapping studies were performed on an upright custom-made macroscope equipped with two CCD cameras (Dalsa, Inc) as illustrated in Figure 1. Excitation light from a 100-W mercury arc lamp (Olympus, Inc) was passed through an inline IR filter and a 420-490 nm bandpass filter (Oriel, Inc). A 500 nm longpass dichroic mirror reflected the blue (420-490 nm) excitation light onto the heart. Emitted fluorescent light (>520 nm) from the heart was transmitted towards the CCD cameras. Another dichroic mirror (short reflecting, 600 nm long pass) splits the emitted wavelengths into two components. Wavelengths longer than 600 nm were further filtered by a long pass (600 nm) filter and captured by one of the CCD cameras (CCD1). The other CCD camera (CCD2) captured wavelengths between 520-590 nm. An electronic shutter (Uniblitz, Inc.) placed in the excitation light path was used to limit exposure time during mapping studies. This technique allowed for multiple recordings with minimal photobleaching and phototoxicity. Images were acquired at 912 frames/s with 12-bit resolution from a 64×64 pixels array, which provided a final spatial resolution of 67 µm and a temporal resolution of about 1 ms.

Motion Tracking and Correction Algorithm

A retrospective motion-correction algorithm based on the template matching technique³⁻⁵ was applied to all the movies. In short, template-matching techniques are
based on the assumption that a local displacement, \( d \), of a structure in one image \( I_0 \) can be estimated by defining a certain window, \( W \), containing this structure, and by finding the corresponding position of that window in a second image \( I \) containing also that structure, by means of correlation. We applied the technique as follows: First, bilinear interpolation was used to achieve subpixel resolution in space.\(^6\) Next, control grid points were chosen on the surface of image \( I_0 \); i.e., frame 0 in the movie. Each grid point was surrounded by a constant control area, \( W \), of 5×5 pixels. Thereafter, the displacement vector, \( d \), for each of the grid points in time was determined by searching for a new position of \( W \) at frame \( I \), which gave the maximum cross correlation between the template \( W \) at frame \( I_0 \) and the \( W \) at frame \( I \). In our algorithm the search distance for the template matching was not constant and could be changed interactively to find the best estimate of the displacements of the corresponding control points.

To obtain a complete vectorial field of displacement for all pixels, spatially weighted interpolation was applied to the movement of the control grid points. Using the complete displacement field so obtained for each pixel in each frame, a complete track of the motion from the reference frame \( I_0 \) onwards was thus obtained and corrected for each pixel.

Figure 2 shows an example of motion detection in one movie. Panel A shows the grid of control points on the reference image (frame 0). In panel B, during frame 50, the same grid points moved to new coordinates. In panel C, the motion-tracking algorithm has detected the translational displacement of each of the grid points to a new position. Superimposition of the control points from frames 0 and 50 illustrates the motion of each of the points, which can be seen in more detail in the inset. As explained above, the
displacement vector for each pixel is then calculated from the grid point shifts using spatial linear weighted interpolation. Then, all pixels in frame 50 are repositioned backwards to their location in the reference frame 0, thus maintaining the structure image free of translational motion. Thus, the motion correction algorithm is designed to rectify translational motion in both the X and Y axes.

**Voltage Signal Amplification and Local Motion Subtraction**

During transmembrane voltage change there is a shift in the emission spectrum of the voltage sensitive dye Di-4-ANEPPS. As a consequence, there is an increase in the signal obtained by CCD2 (green band, see Figures 1) and a decrease in signal in CCD1 (red band). During motion however, there is also similar change in signal obtained by both CCD cameras resulting from changes in the voltage sensitive dye density. In other words, the voltage change signal is discordant and the signal corresponding to local motion is concordant in the 2 cameras. Thus, after appropriate offset and gain correction a subtraction of the signals from the 2 cameras results in amplification of the voltage change signal and a subtraction of the local motion artifact caused by contraction and expansion.

Panel A of Figure 3 shows the optical action potentials post application of the motion correction algorithm for individual pixels for CCD1 and CCD2 on the left and right, respectively. Local motion artifact is still a part of the optical action potential. Panel B shows on the left the superimposition of the signals obtained by the 2 cameras after appropriate offset and gain correction. Note the discordance in the initial phases of the action potentials due to transmembrane voltage change and the concordance of the late phases caused by local motion. Since motion at each pixel is unique, only perfect
translational alignment will result in cancellation of the local motion artifact. Our motion correction algorithm results in perfect translational motion correction. This is observed by the similarity of the motion artifact of individual optical action potentials in CCD1 and CCD2 and the resultant optical action potentials after subtraction on the right side of panel B. Thus sequential combination of translational and local motion correction using both the motion correction algorithm and dual wavelength optical mapping system eliminates the motion artifacts.

**Protocol for Motion Correction and Voltage Signal Amplification**

In all our experiments the movies obtained by the 2 CCD cameras were first corrected for translational motion using the motion correction algorithm. This was followed by offset and gain correction of the movie obtained by CCD2. The 2 movies were then subtracted to yield the final movie in which we had eliminated motion and amplified the signal corresponding to a transmembrane change in voltage. All activation maps, conduction velocity measurements and frequency analysis were carried out on the motion corrected movie.

**Validation of Motion Correction Algorithm using Microelectrode Recordings:**

Recordings were obtained using microelectrodes (15-30 MΩ) filled with 3M KCl solution. The signals were appropriately conditioned and sampled at 15-20 kHz. The resting membrane potential, action potential amplitude and maximum time derivative of transmembrane potential (dV/dt\text{max}) were analyzed during sinus rhythm. APD measures at 30, 50 and 90% were also calculated.
In 2 atria from WT mice we obtained microelectrode recordings from the site of the initial epicardial activation breakthrough and acquired recordings typical of SAN cells, which confirmed that the location of the breakthrough corresponded to the site of the SAN. Since the site was always the same we were able to acquire microelectrode recordings form the site before the optical experiment and then map the area. We also obtained the action potentials from the transitional zone and from the right atrial free wall (RAFW). In figure 4 we present a plot of the correlation between the optical and intracellular action potential durations (APDs). Note that the APDs shown in the graph were obtained from SAN, transitional zone and RAFW. A strong correlation is demonstrated between the data obtained with the two approaches, demonstrating the validity of the motion correction procedure.

Obviously, we could not do the same for the knockout mice, as we never knew in advance where the initial activation breakthrough would appear, and it was nearly impossible to map point by point during optical mapping; i.e., after the dye had been introduced. The red light needed for optical mapping experiments was too dim to allow manipulation and correct positioning of the microelectrode.

**Atrial Activation During Basal Rhythm**

After equilibration, the heart was rotated such that the dorsal aspect of the right atrium (RA) along with a part of the left atrium (LA) and interatrial space occupied by pulmonary veins faced the microscope objective. Hearts were stained with voltage sensitive dye (Di-4-ANEPPS, Molecular Probes) by injecting a 0.2 ml bolus containing 125 nmol/L into a 10 ml compliance chamber within the perfusion line. Imaging was started after 8-10
minutes when fluorescent intensity reached a steady state. Volume conducted ECG was recorded before the imaging. Normal basal rhythm was confirmed for the Cx40\textsuperscript{+/+} and Cx40\textsuperscript{/-} mice\textsuperscript{1,8} following which optical mapping of the right atrium was carried out. Optical movies during basal rhythm were recorded for 4 seconds.

**Ablation protocol**

In 4 Cx40\textsuperscript{+/+} and Cx40\textsuperscript{/-} mice used for mapping sino-atrial propagation during sinus rhythm ablation of the breakthrough site was carried out. The aim of the protocol was to locate the site of the dominant pacemaker site and subsidiary pacemakers thereafter.

Initially the site of the first breakthrough on the right atrium was mapped. This site was ablated using a Aaron-Ram high temperature fine tip (0.75 mm) 2200°F cautery (Aaron Medical industries, Fl) under visual and CCD guidance keeping the position of the preparation constant under the cameras. Figure 5 shows the epicardial surface of the right atrial free wall (RAFW), with the sinoatrial node (SAN) region, the sulcus terminalis (ST) and the inferior vena cava (IVC). Inside the small square is the area of ablation. The inset on the lower right shows a picture of the ablation probe whose tip measured 0.75mm linearly. Clearly the damaged area was very small (~0.5-0.6 mm\textsuperscript{2}; mean, 0.48 mm\textsuperscript{2}, SD, 0.14 mm\textsuperscript{2}; n = 15 burns in 5 hearts).

Post ablation, movies were acquired to find out the change in location and frequency of the breakthrough. The final ablation was of the area corresponding to the SA node, namely the junction of the RSVC, the right atrial appendage and the sulcus terminalis.
Pacing Protocol

A micropipette pulled to an outer diameter of 25-100 μm with a fire-polished tip was used as a suction electrode to deliver unipolar pacing stimuli to the right atrial appendage at all time points studied, such that impulses propagated through the right atrial appendage and free wall towards the AV node and then into the ventricles. The right atrium was paced at progressively faster frequencies of 10, 12, 16 and 25 Hz using 5 ms stimuli at 1.5 × diastolic threshold. Activity during pacing was recorded for 4 seconds. An attempt was made to maintain a constant position of the pacing electrode with respect to the right atrial appendage in all the preparations.

Data Analysis

Pseudo-ECG: The fluorescent signals from all the pixels in a frame of the movie were summed and a time series of the aggregate signal from each frame in the movie was used to summarize the whole optical recording. This is a modification of the procedure described previously in detail.9

Dominant Frequency Analysis: Spectral analysis was carried out for each color-coded pixel by applying a fast Fourier transformation (Resolution: 0.2-0.5 Hz).10 The frequency with the highest power was considered the dominant frequency (DF).

Analysis of Conduction Velocity and Conduction Time: Optical movies of paced activity were signal averaged as described previously.2 In the absence of pharmacological motion reduction, the time of the optical action potential upstroke was marked at the
maximum time derivative of the fluorescence change and a diastolic baseline was established immediately prior to the upstroke. Activation times (defined as the time at which each signal reached 50% of its upstroke amplitude) were determined for each pixel. Local conduction vectors were calculated for each pixel on the basis of the activation times of surrounding pixels. Only those vectors that proceeded in an orderly centrifugal fashion from the pacing electrode were analyzed. Similarly, simultaneously activating pixels close to the stimulating electrodes were ignored. Vectors were averaged in 30-degree bins of orientation and the maximum and minimum conduction velocity was recorded from each activation sequence\[11\]. The maximum conduction velocity was termed the apparent conduction velocity.

Figure 4 of the article illustrates how the apparent conduction velocities over the entire mapped area are measured during basal rhythm in both the WT and KO. Panel A shows data from a total of 16 mice. Please note the difference in the velocity plots. In panel B, we have plotted from the same data the percentage of the optically mapped area for a given range of apparent conduction velocity. The emphasis here is on the fact that the Cx40\textsuperscript{-/-} mice have a higher component of slower velocities as compared to the WT.

**Western Blot Analysis:** Panel A of Figure 8 of the article shows data obtained from four mice in which Cx43 protein was measured in whole heart (left) and both atria (right) of Cx40\textsuperscript{+/+} and Cx40\textsuperscript{-/-} mice. We observed no differences in the amount of protein when comparing wildtype with knockout mice. Similarly, panel B demonstrates no differences in immunostaining of Cx43 antibody in right atrium of wildtype and knockout mice.

**Western Immunoblot:** Whole hearts or isolated atria were homogenized in PBS with
Complete® protease inhibitor (Roche) and 1mM PMSF. The sample was centrifuged at 3,000 G for 5 minutes to remove debris and the supernatant was collected. To this we added 6 X SDS sample buff (SSB, Laemmeli, 1970) to bring it to a final concentration of 1 X SSB, then it was incubated at 37°C for 15 minutes prior to loading 100 µg each lane of a SDS-PAGE. Zymed monoclonal anti-Cx43 (1:1000) was used to detect connexin. HRP-labeled goat anti-mouse serum (Sigma) and ECL-reagent (Amersham) was used to visualize the bands. Blots were stripped and reprobed with monoclonal anti-beta actin (Sigma) serving as a loading control. Immunofluorescence: Sections were prepared from the right atrial free wall. Tissue was imbedded in OCT and quickly frozen. The specimens were serial-sectioned along the coronal plane (ventral to dorsal) in 5-µm slices, mounted on a poly L-lysine coated slide and fixed with 20% methanol. After blocking in 10% BSA with 0.2 % Triton X-100 in phosphate-buffered saline (PBS), the sections were stained with anti-Cx43 (Chemicon) followed by FITC-labeled goat anti-rabbit antisera. All washes were with 0.2% Triton X-100 in PBS. Samples were photographed with an exposure of 1.5 ms using an Olympus IX 70 epifluorescent microscope and Q imaging, Retiga 1300 camera.
References


Figure 1. Experimental Setup. A schematic of the optical components of the upright macroscope mounted with two high-speed CCD (CCD1 and CCD2) cameras. The macroscope was mounted on a x-y stage allowing for micrometer alignment of the optical mapping system without movement of the isolated perfused heart. Volume conducted ECGs were recorded simultaneously with optical mapping from the Ag-AgCl posts in the inner dish. LS, light source (Hg Arc Lamp); C, collimator; IF, interference filter; F1, bandpass filter (420-490 nm); F2, longpass filter (600 nm); F3, bandpass filter (520-590 nm); DM1, dichroic mirror (500 nm); DM2, dichroic mirror (600 nm).
Figure 2. Motion correction algorithm. A and B, snapshots of grid points superimposed on fluorescence distribution in frames 0 and 50, respectively. C, grid points from frame 0 and 50 are superimposed. Please note the translational displacement of individual grid points as seen in Panel B.
Figure 3. Local motion correction and transmembrane potential signal amplification. A, the signals corresponding to a single action potential in a grid of nine pixels as seen by the two cameras (shown in green and red for CCD1 and CCD2, respectively) following the application of the motion correction algorithm. In B, left, superposition of the individual signals seen by CCD1 and CCD2 (left) shows that the motion component of the signals is concordant but the transmembrane potential change in voltage is discordant. In B, right, after offset and gain correction, the subtracted optical action potentials are completely devoid of motion artifact.
Figure 4. Ordinate, action potential duration (APD\textsubscript{50}) values measured at 50\% repolarization in the sinoatrial node (SAN), transitional zone (TZ) and right atrial free wall (RAFW) from microelectrode recordings during sinus rhythm in wildtype hearts. Ordinate, similar values measured using optical mapping data after motion correction. Values in parentheses indicate numbers of microelectrode (n) and optical (pix) recordings at each site. Data are strongly correlated, demonstrating the validity of the motion subtraction procedure.
Figure 5. Right Atrial Ablation. A representative example of right atrial ablation using thermal probe shown in the inset. The black frame highlights the ablated area in the lower portion of the right atrial free wall (RAFW). SAN sinoatrial node region; ST, sulcus terminalis; IVC inferior vena cava.