Decreased connexin43 expression in the mouse heart potentiates pacing-induced remodeling of repolarizing currents

Andrianos Kontogeorgis,1,7* Xiaodong Li,1,6* Eunice Y. Kang,1 Jonathan E. Feig,1 Marc Ponzo,1 Guoxin Kang,1 Riyaz A. Kaba,5,7 Andrew L. Wit,5 Edward A. Fisher,1,2 Gregory E. Morley,1,4 Nicholas S. Peters,5,7 William A. Coetzee,3,4 and David E. Gutstein1,2

1Leon H. Charney Division of Cardiology, Department of Medicine and Departments of 2Cell Biology, 3Pediatric Cardiology and 4Physiology & Neuroscience and Pharmacology, New York University School of Medicine; and 5Department of Pharmacology, Columbia University College of Physicians and Surgeons, New York, New York; 6Department of Cardiology, Shandong Provincial Hospital, Jinan, Shandong, China; and 7Department of Cardiology, St Mary’s Hospital, Imperial College London, United Kingdom

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Kontogeorgis A, Li X, Kang EY, Feig JE, Ponzo M, Kang G, Kaba RA, Wit AL, Fisher EA, Morley GE, Peters NS, Coetzee WA, Gutstein DE. Decreased connexin43 expression in the mouse heart potentiates pacing-induced remodeling of repolarizing currents. Am J Physiol Heart Circ Physiol 295: H1905–H1916, 2008. First published August 29, 2008; doi:10.1152/ajpheart.590.2008.—Gap junction redistribution and reduced expression, a phenomenon termed gap junction remodeling (GJ R), is often seen in diseased hearts and may predispose toward arrhythmias. We have recently shown that short-term pacing in the mouse is associated with changes in connexin43 (Cx43) expression and localization but not with increased inducibility into sustained arrhythmias. We hypothesized that short-term pacing, if imposed on murine hearts with decreased Cx43 abundance, could serve as a model for evaluating the electrophysiological effects of GJR. We paced wild-type (normal Cx43 abundance) and heterozygous Cx43 knockout (Cx43+/-; 66% mean reduction in Cx43) mice for 6 h at 10–15% above their average sinus rate. We investigated the electrophysiological effects of pacing on the whole animal using programmed electrical stimulation and in isolated ventricular myocytes with patch-clamp studies. Cx43+/- myocytes had significantly shorter action potential durations (APD) and increased steady-state (I$_{ss}$) and inward rectifier (I$_{K1}$) potassium currents compared with those of wild-type littermate cells. In Cx43+/- hearts, pacing resulted in a significant prolongation of ventricular effective refractory period and APD and significant diminution of I$_{ss}$ compared with unpaced Cx43+/- hearts. However, these changes were not seen in paced wild-type mice. These data suggest that Cx43 abundance plays a critical role in regulating currents involved in myocardial repolarization and their response to pacing. Our study may aid in understanding how dysynchronous activation of diseased, Cx43-deficient myocardial tissue can lead to electrophysiological changes, which may contribute to the worsened prognosis often associated with pacing in the failing heart.

ventricular myocytes; gap junction

DOW NREGULATION OF CONNEXIN43 (Cx43), the major cardiac gap junction protein subtype, has been described in cardiac disease including ischemia, infarction, hibernating myocardium, and dilated cardiomyopathy (20, 21, 24, 30). Altered expression of Cx43 in cardiac disease is a potentially significant contributor to arrhythmogenicity (19, 33). Experimental models have demonstrated the relationship between decreased cardiac Cx43 expression and increased susceptibility to ventricular arrhythmias. In a heart-specific knockout of Cx43, we observed that ventricular expression levels of Cx43 are inversely related to inducibility into malignant ventricular arrhythmias (8). Additionally, gap junction remodeling (GJR), a process marked by decreased expression and intracellular redistribution of Cx43, corresponds spatially to the central common pathway of reentrant circuits in the canine peri-infarct zone (30).

We recently demonstrated that alterations in Cx43 expression in the wild-type mouse heart could be induced by ventricular pacing over a short period of time. However, pacing-induced changes in Cx43 expression were not accompanied by decreased cardiac function, prolonged refractoriness, or increased inducibility into sustained arrhythmias (22). In the present study, we hypothesized that alterations in Cx43 expression induced by pacing might have more profound effects on ventricular function and electrophysiology in the setting of reduced basal levels of Cx43. To test our hypothesis, we paced heterozygous Cx43 knockout mice (Cx43+/-), with Cx43 expression at 34.3 ± 4.4% of wild-type Cx43 levels, and compared them with paced wild-type mice.

Pacing in Cx43+/- hearts was not associated with significant changes in global ventricular function. However, in Cx43+/- hearts, pacing resulted in a significant prolongation of ventricular effective refractory period and APD and significant diminution of I$_{ss}$ compared with unpaced Cx43+/- hearts. To investigate the mechanisms whereby pacing in Cx43+/- hearts influences refractoriness, we studied action potentials from isolated ventricular myocytes and the potassium currents that represent the primary determinants of action potential duration (APD) (28). As our laboratory has previously demonstrated (9), APD was significantly shorter and steady-state and inward rectifier potassium currents (I$_{ss}$ and I$_{K1}$, respectively) were increased in Cx43+/- compared with wild-type myocytes. However, we found that myocytes isolated from paced Cx43+/- hearts had significant prolongation of APD and decreased I$_{ss}$ compared with unpaced Cx43+/- myocytes. These changes were not seen in paced versus unpaced wild-type cardiomyocytes. Thus short-term pacing in Cx43+/- mice, but not in wild-

* A. Kontogeorgis and X. Li contributed equally to this work.

Address for reprint requests and other correspondence: D. E. Gutstein, New York Univ. School of Medicine, Smilow Bldg., Rm. 804, 522 First Ave., New York, NY 10016 (e-mail: david.gutstein@nyumc.org).

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types, was associated with significant changes in cardiac refractoriness and repolarization. These data suggest that Cx43 plays a critical role in the regulation of repolarizing currents and their response to pacing. In the clinical setting, dyssynchronous activation of diseased myocardium with reduced Cx43 expression may lead to electrophysiological changes, which underlie the worsened prognosis often associated with pacing in the failing heart.

Table 1. Electrocardiographic indexes and electrophysiological data in WT and Cx43+/− unpaced and paced mice

<table>
<thead>
<tr>
<th></th>
<th>WT Unpaced</th>
<th>WT Paced</th>
<th>Cx43+/− Unpaced</th>
<th>Cx43+/− Paced</th>
</tr>
</thead>
<tbody>
<tr>
<td>QRS duration, ms</td>
<td>12.0±0.3 (0.3±0.6)</td>
<td>13.2±1.3 (0.5±1.4)</td>
<td>9.8±0.8* (−0.6±0.7)</td>
<td>10.1±0.7* (0.2±1.4)</td>
</tr>
<tr>
<td>RR interval, ms</td>
<td>136±4.3 (5.7±6.3)</td>
<td>138±6.9 (4.2±7.3)</td>
<td>134±9.8 (−2.9±5.9)</td>
<td>142±13.2 (2.9±14.7)</td>
</tr>
<tr>
<td>QTc, ms</td>
<td>116±3.7 (1.2±6.0)</td>
<td>127±5.3 (1.8±9.2)</td>
<td>130±11.1 (11.4±13.0)</td>
<td>167±23.3 (48.3±34.6)</td>
</tr>
<tr>
<td>VERP100, ms</td>
<td>36.6±3.2</td>
<td>34.3±1.9</td>
<td>30.3±1.8</td>
<td>49.4±5.1†</td>
</tr>
<tr>
<td>VERP80, ms</td>
<td>37.2±3.0</td>
<td>35.7±2.2</td>
<td>29.7±1.0</td>
<td>49.8±5.4†</td>
</tr>
</tbody>
</table>

Postpacing data (and changes from prepacing baseline) are presented as group means ± SE. For electrocardiographic indexes, n = 6 wild-type (WT) unpaced, 6 WT paced, 7 connexin43 (Cx43)+/− unpaced, and 9 Cx43+/− paced; for electrophysiological data [ventricular effective refractory period (VERP)], n = 9 in each WT group and 6 in each Cx43+/− group. Comparisons between groups were performed with ANOVA. *P < 0.05 compared with WT paced; †P < 0.01 compared with all other groups. QTc, rate-corrected QT interval; VERP80 and VERP100, VERP at a pacing cycle lengths of 80 and 100 ms, respectively.

Fig. 1. Prolonged refractoriness in paced connexin43 (Cx43)+/− mice. A: surface electrocardiograms demonstrated a slight but statistically significant shortening of the QRS duration in unpaced and paced Cx43+/− mice compared with the paced wild-type (WT) group and a nonsignificant trend compared with unpaced WT. Other electrocardiographic indexes were unchanged. B: programmed electrical stimulation revealed significantly prolonged refractoriness in paced Cx43+/− mice compared with each of the other groups.
MATERIALS AND METHODS

Procedure for short-term pacing in Cx43-deficient mice. We investigated the effect of reduced Cx43 expression on pacing-induced remodeling of repolarizing currents using Cx43−/− mice (23) and their wild-type littermates, all of which were maintained in a mixed background consisting of C57BL/6J, SV129, and FVB strains. Mice aged 3–6 mo were used for these experiments. All studies were approved by the Institutional Animal Care and Use Committee of the New York University School of Medicine (New York, NY) and performed in accordance with their regulations.

Cx43−/− mice and wild-type littermates were paced for 6 h using the subdiaphragmatic approach as described in detail elsewhere (13). In brief, a stimulating electrode (UE-GM1; Frederick Haer, Bowdoinham, ME) mounted on a micromanipulator (Fine Science Tools, North Vancouver, BC, Canada) was inserted transdiaphragmatically through a 1-cm midline abdominal incision directly into contact with the surface of the right ventricle. Pacing was performed with a Model 2352 Programmable Stimulator (Medtronic, Minneapolis, MN).

Pacing was performed with the output set at twice the stimulating threshold and with a pulse width of 1.0 ms. Unpaced Cx43−/− and wild-type controls were prepared identically to their paced littermates, but the stimulator was left off for the 6-h experimental period. Electrocardiographic recordings were performed at baseline and during and after the pacing protocol as previously described (8). Electrocardiographic signals were monitored to ensure 1:1 capture, and pacing cycle lengths were maintained at 10–15% above the sinus rate for the duration of the experiment.

In vivo electrophysiology. Programmed electrical stimulation (PES) for the determination of VERP and to assess for inducible arrhythmias was performed as described previously (13).

Echocardiography. Echocardiography was performed according to a previously described protocol (12) on paced and sham-paced C57BL/6J WT mice (n = 6 in each group) before and immediately following 6-h pacing and sham protocols. Follow-up studies were also performed in Cx43−/− mice and wild-type littermates 2 h after cessation of pacing. Mice were imaged using a Philips HDI 5000 echocardiography machine equipped with a 15-MHz linear probe.

Cardiac myocyte isolation. Ventricular myocytes were isolated from Cx43+/+ and wild-type littermate hearts immediately following the pacing or sham-pacing procedures using standard enzymatic techniques. Mice were first anticoagulated with 1,000 U/kg heparin administered intraperitoneally 20 min before removal of the heart. Under isoflurane anesthesia, hearts were rapidly removed and perfused in a constant-pressure Langendorff system with Minimum Essential Medium Eagle (Joklik Modification; Sigma) containing (in mM) 0.44 EGTA, 34.5 NaHCO3, 1 MgSO4, and 10 2,3-butanedione monoxime at 37°C for 5 min. The hearts were then perfused with the same solution containing 0.05% (wt/vol) collagenase (Worthington Type 2), 0.1% bovine serum albumin, and 12.5 μM CaCl2, but without EGTA, at 37°C for 15 min. The heart was removed from the Langendorff system and dispersed mechanically. The dissociated heart tissue was then incubated in medium containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 12.5 μM CaCl2 for 10 min at RT, followed by an additional 10 min incubation in medium containing 5% fetal bovine serum and 12.5 μM CaCl2. Isolated myocytes were then incubated for 30 min at RT in Kraft-Bruhe (KB) solution, which contained (in mM) 20 taurine, 50 glutamine, 10 glucose, 10 HEPES, 0.5 EGTA, 3 MgSO4, 30 KCl, and 85 KOH (pH 7.2 adjusted with KOH). The KB solution was gradually replaced with modified Tyrode’s solution containing (in mM) 140 NaCl, 4 KCl, 10 HEPES, 1.1 MgCl2, 1.8 CaCl2, and 10 glucose (pH 7.4 adjusted with NaOH). For action potential measurements, ventricular myocytes were isolated from both ventricles. For potassium currents, cells were isolated exclusively from the right ventricle. Cells were used for patch-clamp experiments within 6 h of isolation.

Cellular electrophysiology. Isolated myocytes were placed in a bath (~500 μl volume) on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan), and whole-cell patch-clamp recordings (15) were obtained at RT. Experiments were performed using an EPC 9/2 amplifier and Pulse 8.79 software (HEKA Elektronik, Lambrecht, Germany). For recording action potential, modified Tyrode’s solution was used as the bath solution.

Table 2. Postpacing echocardiographic measurements in WT and Cx43−/− mice

<table>
<thead>
<tr>
<th>MATERIALS AND METHODS</th>
<th>WT Unpaced</th>
<th>WT Paced</th>
<th>Cx43−/− Unpaced</th>
<th>Cx43−/− Paced</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDD, mm</td>
<td>3.4±0.10 (−0.05±0.10)</td>
<td>3.2±0.1 (−0.12±0.21)</td>
<td>3.3±0.12 (−0.16±0.21)</td>
<td>3.5±0.15 (0.28±0.14)</td>
</tr>
<tr>
<td>IDSD, mm</td>
<td>1.9±0.09 (0.08±0.06)</td>
<td>1.8±0.14 (0.05±0.18)</td>
<td>1.8±0.11 (−0.04±0.18)</td>
<td>2.2±0.21 (0.41±0.13)</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>43.9±1.4 (−3.2±1.7)</td>
<td>43.6±1.2 (−1.5±2.7)</td>
<td>45.1±1.7 (−1.5±2.7)</td>
<td>38.4±3.5 (−7.0±2.9)</td>
</tr>
<tr>
<td>AWTs, mm</td>
<td>1.0±0.02 (−0.02±0.07)</td>
<td>0.98±0.06 (−0.20±0.12)</td>
<td>1.1±0.08 (−0.03±0.08)</td>
<td>1.1±0.08 (−0.02±0.11)</td>
</tr>
<tr>
<td>AWTd, mm</td>
<td>0.81±0.08 (−0.07±0.08)</td>
<td>0.80±0.08 (0.02±0.12)</td>
<td>0.79±0.06 (−0.03±0.05)</td>
<td>0.88±0.06 (−0.01±0.08)</td>
</tr>
<tr>
<td>PWTs, mm</td>
<td>1.3±0.09 (−0.23±0.13)</td>
<td>1.6±0.10 (0.03±0.08)</td>
<td>1.4±0.10 (−0.14±0.15)</td>
<td>1.4±0.08 (−0.17±0.10)</td>
</tr>
<tr>
<td>PWTd, mm</td>
<td>0.83±0.09 (−0.25±0.17)</td>
<td>1.2±0.14 (0.23±0.18)</td>
<td>0.97±0.08 (−0.19±0.12)</td>
<td>1.1±0.08 (−0.14±0.12)</td>
</tr>
</tbody>
</table>

Postpacing data (and changes from prepacing baseline) are presented as group means ± SE; n = 6 WT unpaced, 6 WT paced, 7 Cx43−/− unpaced, and 9 Cx43−/− paced. Comparisons between groups were performed with ANOVA; no significant differences among the groups were detected. IDD and IDSD, intraventricular dimensions at end diastole and end systole, respectively; AWTs and AWTd, anterior wall thicknesses at end systole and end diastole, respectively; PWTs and PWTd, posterior wall thicknesses at end systole and end diastole, respectively.

![Fig. 2. Cx43 expression in Cx43+/− hearts. A: Cx43 abundance as determined by Western blotting followed by densitometry and normalization to GAPDH is significantly lower in Cx43+/− compared with WT hearts. B: no significant differences between unpaced and paced Cx43+/− hearts in expression of Cx43 protein levels were detected by Western blotting.](image-url)
Recording pipettes were fabricated from borosilicate capillary glass (Warner Instruments, Novato, CA) using a Sutter Model P-97 micropipette puller (Novato, CA) and polished using a MF-830 Micro Forge (Narishige, Japan) to resistances of 2–5 MΩ when filled with pipette solution. The pipette solution for recording APs consisted of (in mM) 135 KCl, 4 MgCl₂, 5 EGTA, 10 glucose, 10 HEPES, 5 Na₂-ATP, and 3 Na₂-creatine phosphate (pH 7.2 with LiOH). For recording potassium currents, the bath solution contained (in mM) 135 choline chloride, 5.4 KCl, 1.1 MgCl₂, 1.8 CaCl₂, 0.001 ryanodine, 0.01 atropine sulfate, 0.5 CdCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH) and the pipette solution contained (in mM) 115 potassium aspartate, 5.0 KCl, 7 MgCl₂, 5 EGTA, 10 HEPES, and 4 Na₂-ATP (pH 7.2 with KOH).

After establishing the whole-cell configuration, membrane capacitances ($C_m$) were determined by integrating capacitance transients recorded during a brief 5-mV step from a holding potential (HP) of −70 mV. The cell capacitance was calculated as the ratio of the integral of the capacitance transient divided by the voltage step. Series resistance ($R_s$) was estimated from the decay of the capacitative transients. In each cell, $R_s$ was compensated electronically by 80–90%. Tip potentials were zeroed before membrane-pipette seals were formed. The liquid junction potential was calculated to be −18 mV (Axoscope; Axon Instruments). Membrane potentials were not corrected for the liquid junction potential. Only data obtained from cells with seal resistances >1 GΩ were analyzed.

Action potential and potassium current recordings were performed in the whole-cell configuration. Action potentials were recorded at RT.
in the current-clamp mode by stimulation with a 3-ms pulse at a frequency of 1 Hz. Peak outward potassium current ($I_{\text{Kpeak}}$) was recorded using 1-s depolarizing voltage steps at 10-mV increments between –50 and +50 mV from a HP of –60 mV at 0.01 Hz. The interval between voltage steps was set at 10 s to allow complete recovery of outward potassium current. Steady-state inactivation of the outward potassium current was examined with a standard two-pulse protocol consisting of 1-s prepulses from –80 to +5 mV in 5-mV increments from a HP of –60 mV followed by a 500-ms test pulse to +40 mV. Recovery of transient outward potassium current ($I_o$) from inactivation was measured by using two depolarizing pulses to +40 mV from a HP of –60 mV separated by sequentially increasing intervals of 5 to 185 ms in 10-ms increments (26). Outward potassium current was recorded before and after the application of 2 mM 4-aminopyridine (4-AP) to separate the 4-AP-insensitive current ($I_o$) from the 4-AP-sensitive current ($I_{\text{Ko}}$). $I_{\text{Ko}}$ was recorded in response to voltage steps to potentials between –20 and –140 mV from a HP of –60 mV. Currents were low-pass filtered at 2.9 kHz, digitized at 10 kHz, and stored for subsequent offline analysis.

**Electrophysiological data analysis.** Data were analyzed using Puls-eFit 8.79 (HEKA). $I_o$ was measured 100 ms from the end of each voltage step after the application of 4-AP. The peak $I_o$ was calculated as the maximal current amplitude after the subtraction of the 4-AP-insensitive current from $I_{\text{Kpeak}}$ at each depolarizing voltage step. $I_{\text{Ko}}$ was measured 100 ms from the end of each voltage step. Potassium current densities were obtained by dividing the measured currents by $C_m$.

Inactivation of $I_o$ was fitted using the following equation to current traces after the subtraction of the 4-AP-insensitive current from $I_{\text{Kpeak}}$: $\gamma(t) = a_0 + a_1 \exp(-\tau_1 t) + a_2 \exp(-\tau_2 t)$, where $\gamma$ is time; $\tau_1$ and $\tau_2$ are the time constants of inactivation of $I_o$; $a_1$ and $a_2$ are the amplitudes of the inactivating current components; and $a_0$ is the residual amplitude of the steady-state, noninactivating component of the total outward potassium current after the subtraction of the 4-AP-insensitive component.

Steady-state inactivation curves were obtained by using a two-pulse protocol as described above. Curve fitting after the normalization of the current was performed using the Boltzmann equation, $1/[1 + \exp(-y)]$, where $y$ is the prepulse potential, $y_{50}$ is half-maximal inactivation potential, and $K$ is the slope factor for steady-state inactivation curves.

The time course of recovery of $I_o$ was described by a first-order exponential function as follows (26): $y = 1 - \exp^{-\alpha x}$, where $y$ is the normalized current, $x$ is time, and $\alpha$ is the time constant of recovery from $I_o$ inactivation.

**Immunoblot analysis.** Immunoblot analysis of ventricular lysates was performed using a custom-manufactured rabbit polyclonal antibody directed against an epitope on the carboxy-terminus of Cx43 (14) and a monoclonal antibody directed against GAPDH (Chemicon/Millpore, Billerica, MA). Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was then applied to immunoblots, followed by HyGlo chemiluminescent processing (Denville Scientific, Metuchen, NJ) and autoradiography. Cx43 band intensities from three separate experiments were quantified by densitometry and normalized to the relative intensity of the corresponding GAPDH band for each sample.

**Statistics.** Data are expressed as means ± SE. Electrophysiographic indexes and echocardiographic measurements obtained after the pacing protocol were compared with baseline measurements with paired two-tailed t-tests (Microsoft Excel). Electrophysiography, echocardiography, PES, and cellular electrophysiology results in the paced and unpaced Cx43$^{+/−}$/mice and their wild-type littermates were compared between groups with ANOVA followed by Fisher’s protected paired least significant difference posttest using StatView 5.0 (SAS Institute, Cary, NC). A post hoc analysis was performed to compare $I_{\text{Ko}}$ in wild-type versus Cx43$^{+/−}$/myocytes. $I_{\text{Ko}}$ in unpaced versus paced subgroups was first compared using ANOVA, followed by pooling and comparison of all data from wild-type versus Cx43$^{+/−}$/myocytes at each voltage using ANOVA. $P < 0.05$ was considered statistically significant.

**RESULTS**

Short-term pacing in Cx43$^{+/−}$/mice results in prolonged ventricular refractoriness. To determine whether short-term pacing in Cx43-deficient mice is associated with altered cardiac electrophysiology in vivo, we first examined surface electrocardiograms and then PES in Cx43$^{+/−}$/mice and matched wild-type littermates. Cx43$^{+/−}$/mice (both paced and unpaced) had a slightly but significantly reduced QRS duration compared with that of paced but not unpaced wild-types (Table 1). This finding contrasts with those of one of the coinvestigators on this study (G. E. Morley), who found no difference in QRS duration between wild-type and Cx43$^{+/−}$/mice (25), and others who observed a prolonged QRS interval in Cx43$^{+/−}$/mice compared with wild-types (11). However, given the poor correlation of the QRS duration with direct epicardial measurements of cardiac conduction (10), the significance of the changes in QRS interval in this model is unclear.

There were no significant differences among the groups in RR interval or rate-corrected QT interval (QTc; Table 1). Interestingly, there was a nonsignificant trend toward increased QTc in the paced Cx43$^{+/−}$/mice compared with baseline recordings and to controls, suggesting increased ventricular refractoriness in the paced Cx43 mutants.

To directly test the effect of pacing in the Cx43$^{+/−}$/mice on refractoriness, we employed PES. After pacing, the VERP at a pacing cycle length of 100 ms (VERP$_{100}$) in Cx43$^{+/−}$/mice was significantly prolonged ($P < 0.01$) at 49.4 ± 5.1 ms compared with that of unpaced Cx43$^{+/−}$/mice (30.3 ± 1.8 ms), unpaced wild-types (36.6 ± 3.2 ms), and paced wild-types (34.3 ± 1.9 ms; Figure 1 and Table 1). Similarly, the VERP at a pacing cycle length of 80 ms (VERP$_{80}$) was significantly prolonged ($P < 0.01$) in the paced Cx43$^{+/−}$/mice (49.8 ± 5.4 ms) compared with unpaced Cx43$^{+/−}$/mice (29.7 ± 1.0 ms), unpaced wild-types (37.2 ± 3.0 ms), and paced wild-types (35.7 ± 2.2 ms; Table 1). Two hours after the cessation of pacing, VERP remained elevated in the Cx43$^{+/−}$/mice (VERP$_{100}$ = 50.0 ± 9.0 ms and VERP$_{80}$ = 51.3 ± 7.4 ms; $n$ = 3). No sustained arrhythmias were induced by either single or double extrastimuli in the wild-type or Cx43$^{+/−}$/groups.

Since short-term pacing in Cx43$^{+/−}$/mice results in significant prolongation of refractoriness, we investigated whether

| Table 3. Comparison of action potential parameters in WT and Cx43$^{+/−}$/unpaced and paced mice |
|---------------------------------|-------------------------------|-------------------------------|
|                                  | WT Unpaced                     | WT Paced                      |
| Amplitude, mV                   | 101.8 ± 3.8                   | 97.2 ± 1.7                    |
| RMP, mV                         | –72.0 ± 0.5                   | –75.2 ± 1.0                   |
| APD$_{20}$, ms                  | 2.7 ± 0.3                     | 2.6 ± 0.2                     |
| APD$_{50}$, ms                  | 9.7 ± 1.0                     | 9.9 ± 0.8                     |
| APD$_{90}$, ms                  | 54.1 ± 4.8                    | 53.5 ± 3.0                    |
| APD$_{50}$, ms                  | 9.0 ms                        | 9.0 ms                        |
| APD$_{90}$, ms                  | 35.0 ± 3.2                    | 35.0 ± 2.2                    |

Data are presented as group means ± SE; $n$ = 6 WT unpaced, 11 WT paced, 7 Cx43$^{+/−}$/unpaced, and 10 Cx43$^{+/−}$/paced. Comparisons between groups were performed with ANOVA. *$P < 0.05$ compared to all other groups; †$P < 0.01$ compared to all other groups. RMP, resting membrane potential; APD$_{20}$, APD$_{50}$, and APD$_{90}$, action potential duration at 20%, 50%, and 90% duration, respectively.

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pacing of Cx43+/− mice might also affect systolic performance. To do so, we imaged Cx43+/− mice and matched wild-type littermates with transthoracic echocardiography before and immediately following the pacing and sham-pacing protocol. We found no significant differences in baseline echocardiographic characteristics including ventricular dimensions, fractional shortening, or wall thicknesses in any of the wild-type or Cx43+/− subgroups (supplemental table; all supplementary material can be found with the online version of this article). Similarly, after pacing or sham-pacing in the wild-type and Cx43+/− mice, indexes of ventricular dimensions, fractional shortening, and wall thicknesses were unchanged when compared between groups and compared with baseline values (Table 2).

As expected, Cx43+/− hearts had significantly reduced Cx43 protein levels compared with matched samples from littermate

Fig. 4. Effect of pacing on the inward rectifier potassium current (I_{K1}) in WT and Cx43+/− right ventricular cardiac myocytes. A and B: representative recordings of I_{K1} from WT (A) and Cx43+/− (B) unpaced and paced right ventricular myocytes at a potential of −140 mV. C: voltage-clamp protocol consisted of steps of 10-mV increments between −140 and −20 mV (1-s duration; 0.01 Hz) from a holding potential of −60 mV. D and E: I_{K1} amplitude, measured at the end of a 1-s pulse, was plotted as a function of applied voltage for WT unpaced (n = 5) and paced (n = 5) right ventricular myocytes (D) and Cx43+/− unpaced (n = 5) and paced (n = 6) myocytes (E). The results indicated that there were no significant differences between any of the 4 subgroups. F: post hoc analysis of pooled I_{K1} values comparing results from all WT vs. Cx43+/− cells at each voltage showed significantly increased I_{K1} in the Cx43+/− myocytes compared with WT at voltages of −70 mV. *P < 0.05.
wild-types (34.3 ± 4.4% of wild-type Cx43 levels; \( P < 0.01 \); Fig. 2A). Pacing in Cx43\(^{+/-}\) hearts did not significantly affect overall Cx43 protein levels (Fig. 2B).

Taken together, these data suggest that short-term pacing in the setting of reduced Cx43 expression, but not wild-type levels of Cx43, results in the prolongation of refractoriness without significantly affecting overall ventricular function.

**APD is reduced in unpaced Cx43\(^{+/-}\) myocytes and lengthened by pacing.** To investigate how pacing in the setting of reduced Cx43 expression influences electrophysiological properties, we...
used whole-cell patch-clamp techniques to record action potentials in ventricular myocytes obtained from unpaced and paced wild-type and Cx43<sup>+/−</sup> mice (Fig. 3 and Table 3). Myocytes isolated from unpaced Cx43<sup>+/−</sup> hearts had significantly shorter action potentials than unpaced wild-type cells. In wild-type myocytes, pacing had no effect on APD. Myocytes isolated from unpaced wild-type hearts demonstrated APD measurements that were no different from those of paced wild-type myocytes [APD at 50% duration (APD<sub>50</sub>) = 9.7 ± 1.0 ms in unpaced wild-type vs. 9.9 ± 0.8 ms in paced
wild-type and APD at 90% duration (APD_{90}) = 54.1 ± 4.8 ms in unpaced wild-type vs. 55.3 ± 3.0 ms in paced wild-type; P = not significant (NS) for both comparisons. By contrast, Cx43^{+/−} myocytes demonstrated significant prolongation of APD after pacing compared with unpaced Cx43^{+/−} myocytes (APD_{90} = 5.8 ± 0.9 ms in unpaced Cx43^{+/−} vs. 8.5 ± 0.6 ms in paced Cx43^{+/−}; P < 0.05; and APD_{90} = 35.0 ± 3.2 ms in unpaced Cx43^{+/−} vs. 52.7 ± 4.1 ms in paced Cx43^{+/−}; P < 0.01). Action potential amplitude and resting membrane potential were no different among the unpaced and paced wild-type and Cx43^{+/−} subgroups. Thus, although pacing resulted in no significant change in APD in wild-type cardiac myocytes, the imposition of pacing on Cx43-deficient cells resulted in a significant lengthening of APD compared with its unpaced baseline.

**I_{K1} is increased in Cx43^{+/−} myocytes.** Since APD in cardiac cells is largely determined by voltage-dependent potassium channels (28), we examined whether changes in repolarization in unpaced and paced Cx43^{+/−} myocytes might result from altered potassium currents. We measured I_{K1} in the whole-cell configuration, in which we calculated cell capacitances as 144.6 ± 7.6 pF in wild-type right ventricular myocytes (n = 28) and 145.9 ± 5.5 pF in Cx43^{+/−} right ventricular myocytes (n = 29; P = NS).

We first evaluated the background current I_{K1}, which is responsible for maintaining the resting membrane potential and influences the late phase of repolarization (16). Initially, we did not observe significant differences in a four-way comparison of I_{K1} in unpaced wild-type (peak current = −14.3 ± 1.4 pA/pF), paced wild-type (−13.7 ± 1.3 pA/pF), unpaced Cx43^{+/−} (−17.7 ± 1.2 pA/pF), and paced Cx43^{+/−} (−16.3 ± 1.3 pA/pF) myocytes (Fig. 4, A–E). Because I_{K1} was not statistically different in unpaced versus paced wild-type myocytes across all of the tested voltages, the data from these two groups were pooled at each voltage to increase statistical power. I_{K1} values in unpaced and paced Cx43^{+/−} myocytes were also pooled at each voltage, since they were not significantly different (except at −30 mV; P = 0.028). A post hoc analysis of the pooled values from wild-type versus Cx43^{+/−} myocytes revealed significantly increased I_{K1} in the Cx43^{+/−} myocytes compared with wild-types (Fig. 4F). Thus increased I_{K1} in Cx43^{+/−} myocytes may contribute to APD shortening. However, there was no significant effect of pacing on I_{K1} in either wild-type or Cx43^{+/−} myocytes.

**I_{ss} is increased in unpaced Cx43^{+/−} myocytes but diminished after pacing.** Other currents that are critical in determining APD include two components of the total voltage-dependent outward potassium current (Fig. 5A), which are distinguished by sensitivity to 4-AP. Outward potassium current in cardiac myocytes comprises a 4-AP-insensitive component (I_{ss}; Fig. 5B) and a 4-AP-sensitive component (I_{so}; Fig. 5C) (2, 26, 36). Representative traces in Fig. 5 were recorded from an adult murine right ventricular myocyte. The 4-AP-sensitive current, I_{so}, was no different in amplitude among the unpaced wild-type, paced wild-type, unpaced Cx43^{+/−}, or paced Cx43^{+/−} subgroups (Fig. 6). Furthermore, inactivation kinetics of I_{so} and indexes of the recovery of I_{so} from inactivation were no different in any of the groups tested (Fig. 7 and Table 4).

**Significant elevations in the 4-AP-insensitive current, I_{ss}, have been described in right ventricular myocytes from mice with conditional loss of Cx43 expression in the heart (9).** Similarly, we found that I_{ss} was significantly increased in unpaced Cx43^{+/−} myocytes (peak current = 4.2 ± 0.5 pA/pF) compared with unpaced wild-type myocytes (2.4 ± 0.2 pA/pF; P < 0.01). Pacing did not affect a significant change in I_{ss} current density in wild-type myocytes (2.7 ± 0.1 pA/pF after pacing; Fig. 8, A and C). However, pacing was associated with a significant diminution of I_{ss} current density in the Cx43^{+/−} myocytes (2.9 ± 0.2 pA/pF; P < 0.01 compared with unpaced Cx43^{+/−} myocytes; Fig. 8, B and D) to levels that were not statistically different from those of the wild-type myocytes. Thus changes in the I_{ss} density in unpaced and paced Cx43^{+/−} myocytes most likely represent a major mechanism underlying corresponding alterations in APD.

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### Table 4. Comparison of I_{so} inactivation, steady-state inactivation, and recovery from inactivation in WT and Cx43^{+/−} unpaced and paced mice

<table>
<thead>
<tr>
<th></th>
<th>WT Unpaced</th>
<th>WT Paced</th>
<th>Cx43^{+/−} Unpaced</th>
<th>Cx43^{+/−} Paced</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I_{so} Inactivation Kinetics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_a, \text{ms} )</td>
<td>47.4±6.7</td>
<td>49.2±5.5</td>
<td>45.9±3.8</td>
<td>43.6±1.3</td>
</tr>
<tr>
<td>( \tau_s, \text{ms} )</td>
<td>1,138±188</td>
<td>1,161±182</td>
<td>1,104±126</td>
<td>1,190±144</td>
</tr>
<tr>
<td>( a0, \text{nA} )</td>
<td>0.4±0.06</td>
<td>0.4±0.05</td>
<td>0.4±0.04</td>
<td>0.4±0.04</td>
</tr>
<tr>
<td>( a1, \text{nA} )</td>
<td>1,226±304</td>
<td>1,176±305</td>
<td>1,295±275</td>
<td>1,450±309</td>
</tr>
<tr>
<td>( a2, \text{nA} )</td>
<td>1.7±0.4</td>
<td>1.6±0.3</td>
<td>1.5±0.4</td>
<td>1.6±0.4</td>
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<tr>
<td><strong>Steady-State Inactivation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{1/2}, \text{mV} )</td>
<td>−26.3±2.0</td>
<td>−27.9±2.4</td>
<td>−30.5±3.5</td>
<td>−26.8±2.3</td>
</tr>
<tr>
<td>( K, \text{mV} )</td>
<td>11.3±1.2</td>
<td>11.0±1.3</td>
<td>9.5±1.0</td>
<td>8.3±1.2</td>
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<tr>
<td><strong>Recovery from Inactivation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_{\text{recovery, ms}} )</td>
<td>31.5±4.6</td>
<td>30.3±4.9</td>
<td>44.3±5.5</td>
<td>36.6±3.7</td>
</tr>
</tbody>
</table>

Data are presented as group means ± SE; n = 6 per group. I_{so}, transient outward K\(^+\) current; \( \tau_a \) and \( \tau_s \), time constants of the fast and slow component of inactivation, respectively; \( a0 \), residual amplitude of the steady-state, non-inactivating component of the total outward K\(^+\) current after subtraction of the 4-aminopyridine-insensitive component; \( a1 \) and \( a2 \), amplitudes of the inactivating current components; \( V_{1/2} \), half-maximal inactivation potential; \( K \), slope factor for steady-state inactivation curves at \( V_{1/2} \); \( \tau_{\text{recovery}} \), time constant of recovery from inactivation. Comparisons between groups were performed with ANOVA. P = not significant for all comparisons.
In this paper, we describe an interaction between reduced basal levels of Cx43 and pacing that influences ventricular electrophysiology. Pacing in the setting of heterozygous loss of Cx43, but not in wild-type mice, is associated with prolonged refractoriness. To investigate the prolongation of refractoriness associated with pacing in the setting of reduced Cx43 expression, we studied action potentials and potassium currents from myocytes isolated from paced and unpaced Cx43 mutant and wild-type hearts. Isolated myocytes from Cx43-deficient murine hearts have shortened APDs compared with those measured in wild-type cells, most likely due to increased \( I_{ss} \) and \( I_{K1} \) density. APD is significantly lengthened with a corresponding reduction in \( I_{ss} \) (but not \( I_{K1} \)) in paced Cx43\(^{+/−}\) cells compared with unpaced myocytes, whereas these parameters are unchanged in wild-type myocytes with pacing. Thus, although short-term pacing in the wild-type murine heart is not associated with measurable electrophysiological effects, pacing in the setting of reduced baseline Cx43 expression results in significant prolongation of refractoriness and altered repolarization.

Our results are consistent with prior data showing shortening of the APD\(_{90}\) in cultured strands of neonatal ventricular myocytes isolated from heterozygous Cx43 knockout hearts (35). In another model of loss of Cx43 expression, the heart-specific conditional knockout of Cx43, we observed a reduction in APD with correspondingly increased outward potassium currents (9). However, this is the first study to demonstrate that pacing a Cx43-deficient mouse heart alters indexes of repolarization and refractoriness, whereas no such changes occur with pacing in the wild-type mouse.

An alteration in repolarization after short- or long-term pacing is termed cardiac memory, a phenomenon described in larger animals such as dogs and humans (29, 31, 32). Cardiac memory has not been reported previously in the mouse model and in our study was not evident in wild-type mice after short-term pacing. The mouse is not an ideal species to study repolarization, however, since repolarizing currents in the mouse heart differ substantially from those of larger mammals (27). Nonetheless, because pacing-induced alterations in repolarization consistent with cardiac memory were seen only in the Cx43-deficient mice, it appears that Cx43 may influence processes that are responsible for memory.

A potential link between Cx43 expression and the remodeling of active ion channels has been suggested by experiments involving the infarct border zone in the canine model. GJR in this region was found to correspond spatially to the central common pathway of figure-of-eight reentrant ventricular tachycardia circuits (30). Furthermore, significant alterations in sodium, L-type calcium and potassium currents, and kinetics, as well as associated protein subunits, were observed in the same region of the infarct border zone (4). Thus, in combina-
tion with the data presented in this study, it is reasonable to conclude that reduced Cx43 expression, as observed in the infarct border zone and in paced Cx43+/− mice, may potentiate remodeling of repolarizing currents.

Dysynchrony of cardiac activation is a predictor of poor outcome in patients with systolic dysfunction (5, 17, 34, 37). Patients with heart failure and dysynchrony have been shown to benefit from cardiac resynchronization therapy (CRT) with a biventricular pacemaker (1, 3, 6, 7, 38). However, a substantial proportion of treated patients fail to respond to CRT (1, 3).

Prior studies have indicated that in the diseased heart Cx43 abundance can be variable, with focal areas of reduced expression corresponding to hibernating and ischemic zones (20). Our results suggest that reduced Cx43 expression, as observed in the infarct border zone and in paced Cx43+/− mice, may potentiate remodeling of repolarizing currents in regions with focal areas of reduced expression corresponding to hibernating and ischemic zones (20). Our results suggest that in the diseased heart Cx43 abundance can be variable, with focal areas of reduced expression corresponding to hibernating and ischemic zones (20). Our results suggest that in the diseased heart Cx43 abundance can be variable, with focal areas of reduced expression corresponding to hibernating and ischemic zones (20).

We are currently investigating calcium channel kinetics in myocytes isolated from Cx43+/− hearts to determine whether alterations may contribute to prolonged refractoriness.

In conclusion, the present findings suggest that myocardial tissue with reduced baseline expression of Cx43 responds quite differently to pacing than does myocardium with wild-type levels of Cx43. In the setting of decreased Cx43 expression, short-term pacing results in significant prolongation of refractoriness, as well as changes in APD and \( I_{Na} \). Prior studies have indicated that in the diseased heart Cx43 abundance can be variable, with focal areas of reduced expression corresponding to hibernating and ischemic zones (20). Our results suggest that the imposition of pacing in such a clinical scenario could lead to the remodeling of repolarization currents in regions with reduced Cx43 expression, thus greatly enhancing dispersion of refractoriness around hibernating or ischemic zones and potentially creating a substrate for reentry.

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