Connexin40 and connexin43 determine gating properties of atrial gap junction channels

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ABSTRACT

While ventricular gap junctions contain only Cx43, atrial gap junctions contain both Cx40 and Cx43; yet the functional consequences of this co-expression remain poorly understood. We quantitated the expression of Cx40 and Cx43 and their contributions to atrial gap junctional conductance (gj). Neonatal murine atrial myocytes showed similar abundances of Cx40 and Cx43 proteins, while ventricular myocytes contained at least 20 times more Cx43 than Cx40. Since Cx40 gap junction channels are blocked by 2 mM spermine while Cx43 channels are unaffected, we used spermine block as a functional dual whole cell patch clamp assay to determine Cx40 contributions to cardiac gj. Slightly more than half of atrial gj and ≤20% of ventricular gj were inhibited. In myocytes from Cx40 null mice, the inhibition of ventricular gj was completely abolished, and the block of atrial gj was reduced to ≤20%. Compared to ventricular gap junctions, the transjunctional voltage (Vj)-dependent inactivation of atrial gj was reduced and kinetically slowed, while the Vj-dependence of fast and slow inactivation was unchanged. We conclude that Cx40 and Cx43 are equally abundant in atrium and make similar contributions to atrial gj. Co-expression of Cx40 accounts for most, but not all, of the differences in the Vj-dependent gating properties between atrium and ventricle that may play a role in the genesis of slow myocardial conduction and arrhythmias.

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1. Introduction

Gap junction channels are critical for the propagation of electrical conduction in the myocardium [1]. The atrial myocardium is distinct from the ventricular myocardium in its morphologic, contractile, and electrophysiological properties. Among the differences between these tissues are their repertoires of gap junction subunit protein (connexin, Cx) expression. While four connexins (mCx30.2/hCx31.9, Cx40, Cx43, and Cx45) have been identified within the heart, immunostaining studies suggest that Cx40 and Cx43 are the most abundant within the areas of working myocardium [2–4]. Cx43 is abundant in gap junctions between both atrial and ventricular myocytes, while Cx40 is found only in atrial gap junctions.

Understanding the behavior of Cx40 as well as Cx43 may be important for elucidating normal atrial conduction and its disturbances. Certain forms of idiopathic atrial fibrillation have been associated with polymorphisms [5] or somatic mutations of Cx40 [6]. The Cx40 polymorphisms (within the promoter region) are also linked to a rare form of atrial standstill when combined with a loss-of-function cardiac sodium channel mutation [7]. Targeted gene deletion of Cx40 in mice produced multiple aberrations: P wave and PQ interval prolongation, prolonged sinus-node-recovery time, prolonged Wenckebach period, burst-pacing induced atrial tachyarrhythmias, reduced atrial, A–V node, and left bundle branch conduction velocity, right bundle branch block, and, paradoxically, reduced interatrial conduction heterogeneity [8–12]. In the ventricle, the heterogeneous loss of Cx43 gap junctions in a murine conditional cardiac Cx43 knockout model best exemplifies how the focal loss of cardiac gap junctions leads to significant dispersion of conduction, increased incidence of spontaneous arrhythmias, and loss of ventricular systolic function with only minor reductions in overall Cx43 expression [13,14].

The gating of Cx43-containing ventricular gap junctions during the action potential is also proposed to promote cardiac arrhythmias via inactivation and recovery that depends on transjunctional voltage (Vj) and contributes to conduction slowing or block and the formation of reentrant arrhythmias [15,16]. Despite the knowledge that reductions in functional connexin expression increase the dispersion of refractoriness and increase myocardial susceptibility to fibrillation, the functional consequences upon gap junction channel gating of Cx40 co-expression with Cx43 have not been examined in detail.

In this study, we quantified Cx43 and Cx40 expression levels and assessed the functional contribution of each connexin to the functional atrial gap junctional conductance (gj) using the dual whole cell patch clamp technique. We used a spermine block assay to assess the functional contribution of Cx40 to cardiac gj by comparing the amount...
of block between wild-type (wt) and Cx40 knockout (Cx40KO) cardiomyocytes. We also investigated the dynamic gating properties of mouse atrial gap junctions in a manner analogous to our previous studies of neonatal murine ventricular gap junctions [15].

2. Methods

2.1. N2a cell culture

Stable transfectants of mouse Neuro2a (N2a) cells with rat Cx40 or Cx43 have previously been described [17,18]. For transient transfections, communication-deficient N2a cells were transfected with 1 μg of murine Cx43 in pTracer-CMV2 with Lipofectamine2000 according to the manufacturer’s instructions (Invitrogen). Green fluorescent protein (GFP) positive N2a cell pairs were identified under epifluorescence illumination on the stage of an Olympus IMT-2 inverted phase-contrast microscope with 470 nm excitation and >500 nm emission wavelengths and patch clamped [19].

2.2. Myocyte cell culture

Newborn C57Bl/6 mice were anesthetized with 2.5% isoflurane and the hearts were excised in accordance with procedures approved by the institution’s Committee for the Humane Use of Animals. The atria and ventricles were dissociated separately in a Ca2+- and Mg2+-free collagenase dissociation solution and the supernatant was collected as described [15,16]. The primary cell cultures were enriched for cardiomyocytes by differential cell adhesion and plated onto 35 mm culture dishes at low density for electrophysiological examination or higher density for immunoblotting 48–72 h later. Immunofluorescence samples were cultured on 18 mm glass coverslips coated with 10 μg/ml fibronectin in a 12-well plate.

2.3. Electrophysiology

Whole cell gap junction currents were recorded during repeated voltage clamp pulses with ventricular action potential waveforms as previously described [15,20]. Quantitative junctional voltage correction methods were used to correct for series resistance errors resulting from each patch electrode according to the expression [21]:

\[ g_j = \frac{-\Delta I_j}{V_1 - (I_1 \cdot R_{el}) - V_2 + (I_2 \cdot R_{el})}. \]  

Simulated atrial action potential waveforms were also generated using the canine atrial action potential model of Ramirez et al. [22] and used to voltage clamp neonatal atrial cardiomyocyte gap junctions. Steady-state \( V_j \)-dependent inactivation (increasing \( V_j \)) and recovery (decreasing \( V_j \)) normalized junctional conductance–voltage \((G_j - V_j)\) curves were obtained using a 200 ms/mV voltage ramp protocol and fit with a Boltzmann distribution [15,16] (see Supplemental material). Final graphs were prepared using Origin version 7.5 software (OriginLab Corporation, Northampton, MA).

2.4. Immunohistochemistry

The myocyte cell cultures were fixed and indirectly immunolabeled with connexin-specific antibodies according to the procedures of Kwong et al. [23]. Confocal fluorescence micrographs were acquired using the Zeiss LSM 510 META confocal microscope core facility and viewed using the Zeiss LSM Image Browser V3.5 software.

2.5. Immunoblot analysis

Cell and tissue homogenates were prepared using a modification of the methods described by Gong et al. [24]. Aliquots containing 1–30 μg of protein were separated by SDS-PAGE on 10% polyacrylamide gels and blotted onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked in 10% nonfat milk in Tris-buffered saline (TBS), incubated with rabbit polyclonal antibodies directed against carboxy-terminal domains of Cx43 or Cx40, rinsed repeatedly in TBS, and then reacted with peroxidase conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, Inc.). Immunoblots were developed with ECL Plus chemiluminescent reagents (GE Healthcare) and quantified using a STORM Phosphoimager. The abundance of connexin proteins in cell or tissue homogenates was determined by comparison of the intensity of their bands to standard curves obtained by immunoblotting dilutions of bacterially expressed fusion proteins containing the carboxyl terminal tail domains of Cx40 or Cx43 (Supplemental Fig. S1).

2.6. Real-time PCR analysis

Cellular RNA was extracted with TRIzol®R, quantified by UV absorption, and 0.1 μg of reverse-transcribed cDNA was combined with custom forward and reverse murine Cx40 and Cx43 primers, SuperScript® II enzyme mix, and SYBR® GreenER™ dye in a 200 μl PCR tube (reaction volume = 25 μl). The samples were run for 40 cycles in a 96-well plate Bio-Rad iCycler® located in the qPCR Core facility, Department of Microbiology, SUNY Upstate Medical University. All results are expressed relative to GAPDH. A cellular RNA sample without reverse transcription was run as a negative control to test for genomic DNA. CT (cycle time) values were determined by the apparatus and the quality of the PCR product was confirmed by analyzing the melt-curve. Please note that lower CT values indicate a stronger RNA signal. The difference in the CT values for Cx40 and Cx43 relative to GAPDH are provided as ΔCT (deltaCT) values. Relative connexin RNA expression levels were calculated based on equation: RNA level = \(2^{-(ΔCT\text{sample}−ΔCT\text{Atrial Cx43})}\) which assumes that the product amount doubles with each PCR cycle.

3. Results

3.1. Connexin expression and distribution

To determine the connexin content of the mouse cardiac myocytes, we examined isolated/cultured myocytes by double label immunofluorescence microscopy (Fig. 1). Pairs of neonatal mouse atrial myocytes showed bright staining at appositional membranes using either anti-Cx40 or anti-Cx43 antibodies (Figs. 1A and B), and the immunoreactivity substantially coincided (Fig. 1D). Confocal microscopy confirmed that Cx40 and Cx43 predominantly localized to the same gap junction plaques (Figs. 1E–G). Confocal microscopic examination identified a few plaques that reacted only with the antibodies to one connexin (spots staining red or green only in merged image, Fig. 1G). These results are consistent with previous detection of Cx40 and Cx43 in gap junctions between mouse neonatal atrial myocytes [25–27]. We found that neonatal mouse ventricular myocytes showed intense Cx43 immunoreactivity; occasional cells showed a few spots of Cx40 staining at junctional plaques (data not shown).

The presence of Cx40 and of Cx43 in cell and tissue samples was also examined by immunoblotting (Fig. 2). N2a cells stably transfected with Cx40 or Cx43 (and untransfected cells) were blotted to positive (and negative) controls for detection of Cx40 or Cx43 (Fig. 2A). The levels of Cx40 and Cx43 in the N2a transfectants (determined by comparison to standard curves generated using purified connexin fusion proteins) were Cx40, 128 ± 36 fmol/μg cellular protein, and Cx43, 74 ± 20 fmol/μg cellular protein (Fig. 2B). Cx43 was detected by immunoblotting in cultured atrial and ventricular myocytes and undissociated fresh frozen neonatal murine atrial and ventricular tissues (Fig. 2A, lower panel). In contrast, anti-Cx40 antibodies showed abundant reactivity with homogenates of atrial
tissue and myocytes, weak reactivity with homogenates of ventricle, and no detectable reactivity with homogenates of isolated ventricular myocytes (Fig. 2A, upper panel). The atrial myocytes contained 404 ± 22 fmol/μg of Cx40 and 349 ± 62 fmol/μg of Cx43 (Fig. 2C); these levels did not differ significantly. Atrial tissue also contained similar levels of Cx40 and Cx43. Levels of both connexins were about half as much in the tissue samples as compared to the isolated cells; this difference may be due to the greater content of extracellular matrix proteins in the tissue samples. Limited analysis of quantitative blots showed that the abundances of Cx40 and Cx43 were also very similar to each other in homogenates of adult atrium (not shown).

Fig. 1. Immunofluorescent localization of Cx40 and Cx43 in neonatal mouse atrial cultures. (A–D), atrial myocyte cell pair showing immunolocalization of Cx40 (green in A), Cx43 (red in B). Nuclei were identified by staining with DAPI (blue in C, D). (E–G), confocal images of an atrial myocyte cell culture immunostained for Cx40 (green in E) and Cx43 (red in F). Merged images are shown in the right panel (G) where overlap appears yellow. In atrial myocytes Cx40 and Cx43 localize to gap junction plaques and show substantial overlap, however there are some spots stained with only one color suggesting discrete domains of each connexin. Bar represents 6.25 μm for (A–D), 5 μm for (E–G).

Fig. 2. Immunoblot analysis of Cx40 and Cx43 in cultured cells or fresh frozen atrial and ventricular tissues from neonatal mice. Whole cell lysates (for Cx40 detection: 5 μg protein from N2a cells and ventricular samples, 1 μg protein from atrial samples; for Cx43 detection: 2 μg protein for all samples except atrial tissue when 5 μg protein was used) were resolved by SDS-PAGE, transferred to membranes, and blotted with anticonnexin or anti β-tubulin antibodies. (A) Photograph of a representative immunoblot. Migration of molecular weight markers is indicated to the left of the blot. Exposure times differ for the parts of the figure containing N2a cells and heart samples, β-tubulin was used as a loading control for N2a cells. (B) Absolute abundances of Cx40 and Cx43 in N2a transfectants. Bars represent the mean value ± SEM based on 3 experiments. (C) Absolute abundances of Cx40 and Cx43 in mouse neonatal heart (isolated cells and tissue homogenates). Blots also contained serial dilutions of bacterially expressed Cx40 or Cx43 fusion proteins (see Supplementary Fig. S1). Reaction products were quantified using a Phosphoimager and abundances of Cx40 and Cx43 were determined by comparison to these standard curves. Each bar represents the mean value ± SEM based on n number of experiments; for atrial myocytes Cx40 n = 4, Cx43 n = 3, atrial tissue Cx40 n = 4, Cx43 n = 4, ventricular myocytes Cx40 n = 4, Cx43 n = 4, ventricular tissue Cx40 n = 4, Cx43 n = 5. (D) Real-time PCR analysis of Cx40 and Cx43 gene expression in cultured atrial and ventricular myocytes. Atrial Cx40 expression was slightly lower than wild-type (WT) atrial or ventricular Cx43 expression, was barely detectable in WT ventricular myocyte cultures, and was absent from homozygous Cx40 knockout (Cx40KO) cultured atrial myocytes (ND = Not Detectable). Cx40KO atrial Cx43 gene expression was reduced relative to WT samples. Relative expression levels were calculated based on the differences in the cycle times (dCT) required for product amplification (see Methods for details).
The isolated ventricular myocytes contained the highest levels of Cx43 (616 ± 78 fmol/μg), but no detectable Cx40 (<0.25 fmol of Cx40 per μg of cellular protein). Thus, while we cannot conclude that ventricular cells contain absolutely no Cx40 protein, the ratio of Cx40 to Cx43 in the neonatal mouse atrial myocytes and tissue was ∼1:1. The ventricular tissue did contain a small amount of Cx40 (34 ± 4 fmol/μg) which could derive from the neonatal murine ventricular myocytes or from other cell types, such as the His–Purkinje system and the coronary endothelium [2,3,12,28,29]. Cx45 is also present in adult and neonatal myocytes from ventricle and atrium [2,28,29]. Therefore, we performed similar quantitative immunoblots, but we detected not more than 0.25 fmol/μg cellular protein in any sample (not shown) suggesting that Cx45 contributed only a very small fraction of the total connexins in any of these cells.

Real-time PCR analysis of RNA extracts from atrial and ventricular myocyte cultures confirmed high Cx43 atrial and ventricular gene expression levels with respective average delta cycle time (dCT) values of 0.71 ± 0.38 and 0.77 ± 0.26 (± S.D.; N = 3, 2) relative to GAPDH (Fig. 2D). Atrial Cx40 mRNA expression was slightly lower than Cx43, with a dCT value of 1.15 ± 0.25 and was barely detectable in ventricular myocyte culture samples (dCT = 5.4 ± 0.76). In an atrial myocyte culture derived from homozygous Cx40 knockout (Cx40KO) mice, Cx40 mRNA expression was not detectable (ND) and Cx43 expression levels were reduced (dCT = 1.7).

3.2. Functional contributions of Cx40 and Cx43 to atrial gap junctional conductance

One direct way to assess the contribution of each connexin to the functional atrial gap junctional conductance ($g_j$) is to utilize the differential sensitivity of the cardiac connexins to inhibition by intracellular spermine [30]. For this purpose, 2 mM spermine was added unilaterally to one cell patch pipette and the fractional block of $I_j$ was assessed using a Δ ± 10 mV, $V_j$ pulse sequence [30,31].

To demonstrate the differential sensitivity of the three mammalian myocardial connexins, Cx40, Cx43, and (Cx45), to spermine, the 2 mM spermine block assay was performed on N2a cells stably transfected with Cx40 or Cx43 or transiently transfected with Cx45 (Fig. 3A). The maximum inhibition of $g_j$ was 6 ± 6% for Cx43, 42 ± 10% for Cx45, and 80 ± 6% for Cx40 at $V_j = +50$ mV. Two way ANOVA analysis of $V_j$-dependent spermine inhibition indicated significant differences between these different connexins ($P < 0.05$). These same procedures were applied to paired neonatal primary wild-type (wt) and homozygous Cx40 knockout (Cx40KO) mouse atrial and ventricular myocytes. The spermine block of wt ventricular $g_j$ was significantly less than that of atrial $g_j$ ($P < 0.05$, two way ANOVA), and the maximum inhibitions of $g_j$ at +50 mV were 52 ± 12% and 19 ± 9% respectively for wt atrial and ventricular myocytes (Figs. 3B and C; see also Supplemental material, Fig. S2). The atrial HL-1 cell line exhibited an intermediate degree of spermine block of 37 ± 10%. In contrast, the maximum inhibitions of Cx40KO atrial and ventricular $g_j$ at +50 mV were only 20 ± 14% and 3 ± 7% ($P < 0.05$, two way ANOVA; Figs. 3B and C). There were no significant differences in the steady-state-average $g_j$ values among the connexin-N2a, atrial myocyte, or ventricular myocyte groups (see Supplemental material Table S1). The lack of Cx40 expression in the Cx40KO atrial and ventricular cells and tissues was confirmed by immunoblot analysis (Fig. 3D). Taken together, these data based on the spermine block assay show that the fractional Cx40 contribution to the total cardiomocyte $g_j$ is approximately 40% in wt atrium.
3.3. $V_J$-dependent gating properties

Large myocardial $V_J$ gradients can develop during slow, discontinuous conduction of cardiac action potentials, and $V_J$ gradients equivalent to the amplitude of the ventricular cardiac action potential can induce phasic changes in $g$. Therefore, we examined the dynamic and steady-state gating properties of wt and Cx40KO mouse atrial gap junctions to detect possible functional differences. For the dynamic action potential voltage clamp experiments, cell 2 was continuously voltage clamped to a simulated atrial or ventricular cell resting potential while a sequence of 1/s (basic cycle length, BCL = 1 s) model atrial or ventricular action potentials was applied to cell 1 [22,24]. The atrial action potential waveform results in only minor normalized $g$ (Fig. 4A) inactivation in wt and Cx40KO murine atrial gap junctions owing to a prominent early (phase 1) repolarization from a peak $V_J$ of $>120$ mV (Fig. 4A). To compare the inactivation of atrial gap junctions to their ventricular counterparts, we applied the ventricular model action potential waveform results in only minor normalized $g$ values of the three Boltzmann distributions (see Table S2). (A) Comparison of steady-state recovery phases whether in response to a cardiac action potential or to a slow, steady-state $V_J$ ramp.

3.4. Inactivation kinetics

We further examined the inactivation process by evaluating the first order inactivation kinetics as a function of $V_J$. An example of the biexponential decay time constant determinations ($\tau_{fast}$ and $\tau_{slow}$) for a wt and a Cx40KO atrial $I_J$ recording is displayed in Figs. 5A and B. The $V_J$-dependent closing rate constants ($k(V_J)$) for the fast and slow inactivation gates $k_{fast}$ and $k_{slow}$, were determined using the expression:

$$k_{on} = \left(1 - P_{open}\right) / \tau_{decay}. \tag{2}$$

The wt atrial $V_J$-dependent fast and slow inactivation rates were well described by an exponential function with the values (in ms $^{-1}$) (Figs. 5C and D):

$$k_{fast} = (0.00701) \cdot \exp\left(\left[\left(V_J - 60\right) / (18.6)\right]\right) + (0.00586) \tag{3}$$

and

$$k_{slow} = (0.00665) \cdot \exp\left(\left[\left(V_J - 60\right) / (18.1)\right]\right) + (0.00088). \tag{4}$$

The $V_J$-dependent fast and slow on-rates for the Cx40KO atrial junctions were significantly faster at each examined $V_J$ than their wt

![Fig. 4. $V_J$-dependent gating properties of atrial gap junctions.](image-url)
counterpart \((P<0.05,\ \text{two} \ \text{way} \ \text{ANOVA})\) and were best described by the following equations (in ms\(^{-1}\)):

\[
\begin{align*}
  k_{\text{on}, \text{fast}} &= (0.010523) \cdot \exp \left( \left[ \frac{V_j - 60}{18.8} \right] \right) + (0.00512) \\
  k_{\text{on}, \text{slow}} &= (0.001427) \cdot \exp \left( \left[ \frac{V_j - 60}{18.6} \right] \right) + (0.00078)
\end{align*}
\]

These data provide the first demonstration that the \(V_j\)-dependence of the wt and Cx40KO gap junction atrial fast and slow inactivation rates are essentially the same, increasing e-fold approximately every 18 mV increase in \(V_j\). These voltage constants are similar to those previously reported for the wt fast and slow ventricular inactivation rates [16]. The fast and slow on-rates vary by a factor of 7 (e.g. \(\tau_{\text{fast}} = 35\) or 3 ms and \(\tau_{\text{slow}} = 244\) or 16 ms at 60 or 120 mV) for the Cx40KO and nearly 11 for the wt atrial gap junctions (e.g. \(\tau_{\text{fast}} = 29\) or 4 ms and \(\tau_{\text{slow}} = 308\) or 31 ms at 60 or 120 mV) according to these expressions. Cx40 gap junctions exhibit only slow inactivation that is similar to the slow inactivation component of Cx43 gap junctions [32,33].

We have previously modeled the dynamic gating of cardiac gap junctions based on our experimental description of the time- and \(V_j\)-dependent inactivation kinetics [15,16]. The “dynamic gap junction model” is defined in the Supplemental appendix to this manuscript. The salient features of the model are that there are two (fast and slow) inactivation components, \(G_1^{+ \text{dt}}\) and \(G_2^{+ \text{dt}}\), that possess identical voltage constants (\(\nu\)) for the \(V_j\)-dependent increase in the decay constants. There are also two recovery components, \(R_1^{+ \text{dt}}\) and \(R_2^{+ \text{dt}}\), with different voltage constants (\(V_{R1}\) and \(V_{R2}\)) that are conserved between Cx40 and Cx43. All four components are solved according to the existing \(V_j\) during each time step (dt = 1 ms during patch clamp experiments) and the sum yields the function of \(G_j^{t+1}\) in time (\(= \text{maximum of 1}\)).
This dynamic gap junction model was used to fit the wt atrial G_j during the atrial and ventricular action potential waveforms. Since inactivation and facilitation were negligible during the atrial action potential (Fig. 4A), only the results using the ventricular action potential are presented in Figs. 5E, F. The inactivation kinetics were the same as those described in Figs. 5B and C using a common voltage constant of 18.4 mV for both the fast and slow on-rates. In this example, G_{min1} and G_{min2} were assumed to be equal (0.42/2 = 0.21) and G_{max1} = (0.33) and G_{max2} = (0.22) were adjusted visually to provide a best fit of the time-dependent G_j inactivation curve using the Eqs. 3 and 4 kinetic equations. The time-independent recovery processes were defined by the following parameters: R_{max3} = 0.4, R_{max2} = 1.25, V_{R3} = 47 mV, and V_{R2} = 1.6 mV. The only parameter changes required to fit the Cx40KO atrial G_j curve (Fig. 5F) were substitution of the fast and slow inactivation kinetic equations (Eqs. 5 and 6, Figs. 5B and C) and increasing A_{R2} to 1.54. The major differences between the dynamic gap junction models for the atrial and ventricular gap junctions are the slower atrial inactivation kinetics (ventricular A2 = 0.00148) and the increased early recovery (ventricular V_{R1} = 22 mV) of the atrial G_j model relative to the previously described ventricular G_j model [15]. The time dependence of the wt atrial G_j in response to the canine atrial action potential could be modeled using this approach and required replacing the recovery process (Eq. A5) with a linear function. Determining how action potential morphology affects the magnitude and rate of the recovery process will require additional experimental evaluation, but the inactivation processes are well described by the existing time- and V_j-dependent functions with only minor modifications of the parameter values [15,16].

4. Discussion

The principal purpose of this investigation was to assess quantitatively the contributions of Cx40 and Cx43 to the total atrial g_j and to determine how connexin co-expression alters the dynamic gating properties of atrial gap junctions relative to ventricular gap junctions. Our data show that atrial myocytes contain nearly equal amounts of Cx40 and Cx43; in contrast, in ventricular myocytes, Cx43 is as abundant, but Cx40 mRNA and protein are barely detectable or below the lower detection limit of our assays. A recent study provides evidence for interatrial differences in Cx40 expression that correlates with heterogeneities in conduction velocity, but the relative Cx40 and Cx43-transfected N2a cells [15]. Thus, we propose that facilitation of atrial gap junctions is reduced in amplitude for both the inactivation and recovery phases whether in response to a cardiac action potential or to a slow, steady-state V_j ramp. This functional gating difference may be due to the nearly equal coexpression of Cx40 and Cx43 in atrial gap junctions as compared to ventricular gap junctions where Cx43 pre-dominates. The Cx40 gene deletion resulted in an increase in the G_j inactivation rates, but did not completely convert the gating properties of atrial gap junctions to match those of ventricular gap junctions (Fig. 4). Thus, we hypothesize that factors in addition to Cx40 (perhaps, the presence of Cx45) alter the function of atrial gap junctions relative to their ventricular counterpart.

The inactivation kinetics for wt and Cx40 KO and atrial gap junctions were revealed to possess similar V_j-dependencies, increasing e-fold for approximately every 18 mV increase in V_j. This V_j-dependence is also similar voltage to what we recently reported for the fast and slow ventricular inactivation rates from wt ventricular gap junctions [16]. This implies that the V_j sensors for both fast and slow gates have the same valence, or that the same voltage sensor is involved in the closing of both fast and slow gates. The V_j sensor of gap junctions is proposed to reside on the connexin NT domain [35,36]. The fast V_j gating particle is thought to reside on the CT domain of Cx43 yet the CT domain interacts with second half of its own cytoplasmic loop (L2) domain, not the NT domain [37,38]. Cumulatively, these findings imply that the molecular bases for fast and slow inactivation components remain to be fully determined.

Action potential morphology will also influence the dynamic gating of cardiac gap junctions. Of particular importance is the magnitude and duration of the action potential derived peak and plateau V_j values. It was previously shown that the vast majority of cardiac g_j inactivation occurs when V_J > 100 mV. Thus, the shorter the duration and the lower the amplitude of the action potential peak and plateau, the less G_j should inactivate. Our data using two different model action potentials, canine atrial and murine ventricular, demonstrate this point. This implies that regional differences in gap junction gating might occur on the basis of alterations in ion channel expression (e.g. ionic transient outward potassium (I_{to}) currents) or function (e.g. ischemia) that affect action potential morphology [39,40]. We are presently investigating how varying action potential delays affect G_j inactivation.

The recovery from inactivation was also slightly altered in atrial gap junctions relative to their ventricular counterparts. The increased G_j during the declining phase of V_j was lower for atrial gap junctions. This facilitation of G_j above initial peak values was absent in HL-1 cells and Cx43-transfected N2a cells [15]. Thus, we propose that facilitation
arises from a post-translational modification of Cx43, and possibly Cx40, protein that occurs in terminally differentiated myocytes but not in continuously dividing mammalian cell lines. Given the slight differences in inactivation and spermine block that were also observed in HL-1 cells, we conclude that primary cardiomyocyte cultures remain a better preparation for examining the possible role of gap junctions in cardiac action potential propagation and arrhythmogenesis. In summary, neonatal murine atrial myocytes express nearly identical amounts of Cx40 and Cx43 protein that contribute equally to functional gap junction coupling, the analysis of unilateral 2 mM spermine block provides a valid functional approach to the assessment of Cx40 contributions to cardiac gap junctional conductance, and quantitation of the \( V_f \)-dependent inactivation rates provides the basis for the development of realistic dynamic gap junction gating models as an investigative tool for further investigation into the role of cardiac \( g_J \) in action potential propagation and arrhythmogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2009.05.014.

References