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Original article

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 (g_j). 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 g_j. Slightly more than half of atrial g_j and ≤20% of ventricular g_j were inhibited. In myocytes from Cx40 null mice, the inhibition of ventricular g_j was completely abolished, and the block of atrial g_j was reduced to <20%. Compared to ventricular gap junctions, the transjunctional voltage (V_j)-dependent inactivation of atrial g_j was reduced and kinetically slowed, while the V_j-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 g_j. Co-expression of Cx40 accounts for most, but not all, of the differences in the V_j-dependent gating properties between atrium and ventricle that may play a role in the genesis of slow myocardial conduction and arrhythmias.

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 (V_j) 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 (g_j) using the dual whole cell patch clamp technique. We used a spermine block assay to assess the functional contribution of Cx40 to cardiac g_j by comparing the amount

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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 Cx45 in pTracer-CMV2 with Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). Green fluorescent protein (GFP) positive N2a cell pairs were identified under epifluorescent 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 Ca^{2+}- and Mg^{2+}-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]:

$$g_j = \frac{-\Delta I_2}{V_1 - (I_1 \cdot R_{el1}) - V_2 + (I_2 \cdot R_{el2})}. \quad (1)$$

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 chemifluorescent 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®, 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® 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 dCT (deltaCT) values. Relative connexin RNA expression levels were calculated based on equation: RNA level $= 2^{-[\mathrm{dCT(sample)} - \mathrm{dCT(Atrial\ Cx43)}]}$ 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 as 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