Relationship Between Connexin Expression and Gap-Junction Resistivity in Human Atrial Myocardium

Paramdeep S. Dhillon, MRCP, PhD; Rasheda A. Chowdhury, PhD; Pravina M. Patel, BSc; Rita Jabr, PhD; Aziz U. Momin, FRCS; Joshua Vecht, FRCS; Rosaire Gray, PhD; Alex Shipolini, FRCS; Christopher H. Fry, DSc, PhD; Nicholas S. Peters, FRCP, MD

**Background**—The relative roles of the gap-junctional proteins connexin40 (Cx40) and connexin43 (Cx43) in determining human atrial myocardial resistivity is unknown. In addressing the hypothesis that changing relative expression of Cx40 and Cx43 underlies an increase in human atrial myocardial resistivity with age, this relationship was investigated by direct ex vivo measurement of gap-junctional resistivity and quantitative connexin immunoblotting and immunohistochemistry.

**Methods and Results**—Oil-galp impedance measurements were performed to determine resistivity of the intracellular pathway ($R_i$), which correlated with total Cx40 quantification by Western blotting ($r=0.64, P<0.01, n=20$). Specific gap-junctional resistivity ($R_j$) correlated not only with Western immunoquantification of Cx40 ($r=0.63, P=0.01, n=20$), but also more specifically, with the Cx40 fraction localized to the intercalated disks on immunohistochemical quantification ($r=0.66, P=0.02, n=12$). Although Cx43 expression showed no correlation with resistivity values, the proportional expression of the 2 connexins, (Cx40/Cx40+Cx43) correlated with $R_i$ and $R_j$ ($r\approx0.58, P<0.01$ for $R_i$ and $r\approx0.51, P=0.02$ for $R_j$). Advancing age was associated with a rise in $R_i$ ($r=0.77, P<0.0001$), $R_j$ ($r=0.65, P<0.001, n=23$), Cx40 quantity ($r=0.54, P=0.01, n=20$), and Cx40 gap–junction protein per unit area of en face disk ($r=0.61, P=0.02, n=12$).

**Conclusions**—Cx40 is associated with human right atrial gap-junctional resistivity such that increased total, gap-junctional, and proportional Cx40 expression increases gap-junctional resistivity. Accordingly, advancing age is associated with an increase in Cx40 expression and a corresponding increase in gap-junctional resistivity. These findings are the first to demonstrate this relationship and a mechanistic explanation for changing atrial conduction and age-related arrhythmic tendency. (Circ Arrhythm Electrophysiol. 2014;7:321-329.)

**Key Words:** atrial remodeling • connexins • gap junctions

Gap–junction (GJ) channels are transmembrane proteins that in myocardium mediate cell-to-cell coupling required for ordered action potential propagation.1–4 Each GJ channel is formed from 2 hemichannels (connexons), in-turn composed of 6 connexin (Cx) subunit proteins. In the human atrium, Cx40 and Cx43 are the major connexins, with small amounts of a third, Cx45, also expressed.1–5

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The relative contributions of Cx40 and Cx43 in determining atrial conduction properties have been investigated in murine Cx-gene knockout models, which suggested that reduction of Cx40 slowed conduction, and prolonged P waves, whereas reduction of Cx43 had little effect.6–8 By contrast, in a human open-chested mapping study, conduction velocity (CV) during sinus rhythm and atrial pacing were reduced because the proportion of Cx40 signal increased (and therefore that of Cx43 decreased).9 Consistent with these findings, a study using synthetic strands of neonatal and fetal murine atrial cardiomyocytes showed that Cx40 deletion (Cx40<sup>−/−</sup>) was associated with increased CV, and genetic deletion of Cx43 (Cx43<sup>−/−</sup>) was associated with a decrease of CV.10 In light of these apparently contradictory results, and given the importance of a mechanistic understanding of this relationship specifically in humans, we investigated the relationship between human atrial connexin expression and GJ resistance, the key intermediary and GJ determinant of myocardial CV. Biophysical theory proposes that an increase of GJ resistance ($R_j$) reduces CV.11

We used the validated method of myocardial oil-gap impedance measurements,12 to measure $R_j$, and in the same human myocardial samples used quantitative Cx40 and Cx43 immunoblotting and immunohistochemistry to address the hypotheses that there is a relationship between the relative expression of Cx40 and Cx43 and $R_j$, and that changes in the relative
expression of Cx40 and Cx43 underlie an increasing atrial myocardial resistivity with age.

Patients and Methods

Specimen Source, Collection, and Handling

The use of human tissue conformed to the principles outlined in the Declaration of Helsinki, and the study had local Ethical Committee approval with written informed consent. Atrial appendage biopsies were collected from patients undergoing cardiac surgery at The London Chest Hospital, London, UK. Twenty-three patients (15 male) in sinus rhythm (age 68±8 years, range 53–80 years) undergoing coronary bypass surgery (n=15), aortic valve replacement (n=3), or both (n=5) were included. Patients were in sinus rhythm at the time of surgery as assessed by ECG and did not have any history of atrial arrhythmia. All patients underwent surgery using conventional cardiopulmonary bypass support, and biopsies were collected from the tips of the right atrial appendage immediately after the pericardium was opened and before exposure to cardioplegic agents. Samples were divided immediately on collection; one part was snap-frozen in liquid nitrogen at −70°C for subsequent Cx quantification and immunolabeling, and the remainder was transported to the laboratory in preoxygenated Tyrode solution (4ºC) containing (mM) NaCl 118, KCl 4.0, NaHCO3 24, NaH2PO4 0.4, MgCl2 1.8, glucose 6.1, Na pyruvate 5.0 (pregassed with 95%O2/5%CO2, pH 7.35±0.03). Transportation time to the laboratory was ≈15 minutes. On arrival at the laboratory, the unfrozen specimens were gradually warmed to 37°C, and atrial trabeculae were dissected for physiological experiments. This approach permitted extraction of connexin protein for Western blotting, and parallel determination of GJ resistance in intact myocardium. All chemicals were from Sigma, UK.

Measurements of Longitudinal Impedance and Calculation of Gap Junction Resistance

The method and its validation have previously been described in detail12 and are available in our Data Supplement. Myocardial preparations (≤1 mm diameter, 5–6 mm length) were placed in a 3-chambered bath; the outer chambers were superfused with Tyrode solution at 37°C, while the muscle in the central chamber was coated with mineral-oil gel. Myocardial strips have been shown to remain metabolically and

Table 1. Patient Characteristics

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<th>Operation</th>
<th>History</th>
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ACE indicates ace inhibitor; AR, aortic regurgitation; ARB, angiotensin receptor blocker; AS, aortic stenosis; AVR, aortic valve replacement; BB, β-blocker; CABG, coronary artery bypass grafting; CCB, calcium channel blocker; DM, diabetes mellitus; F, female; HT, hypertension; IHD, ischemic heart disease; LA, left atrium; LVEF, left ventricular ejection fraction; M, male; MI, myocardial infarction; and PWD, P wave duration.
structurally intact during impedance measurements.\textsuperscript{12,13} Alternating current (0.25–150 kHz) was passed between platinum (Pt)-black electrodes in the outer chambers to constrain the current to the intracellular pathway of the muscle within the oil-gap, with a fraction through a parallel extracellular shunt. The frequency-dependent intracellular resistance ($r_i$) of the preparation was recorded with a balanced Wien bridge (Wayne-Kerr, Wetherby, UK). Total preparation impedance, $z$, was modeled as $z = r_i + (r_{ec}/(r_{ec} + r_{GJ}))$, where $r_{ec}$ is the resistance of the extracellular shunt, and $z$ is the impedance of the intracellular pathway. $r_{ec}$ was measured separately as the resistance between 2 Pt-black needle electrodes, a known distance apart, placed in the muscle within the oil-gap to obtain true intracellular resistance, $2r_{ec}$, as $r_{ec} = (r_{r}r_{GJ})(r_{r}+r_{GJ})$. Intracellular resistance itself has 2 components; the sarcoplasm, $r_{s}$, and GJ resistances, $r_{GJ}$, (ie, $r = r_{s} + r_{GJ}$), obtained separately by measuring the frequency-dependent component of $r$ and the limiting value at high frequencies (Figure 1). Preparation length and radius in the oil-gap were measured after measurements. Lower case values of variables ($r, x, \Omega, \text{cm}$) were converted to specific ($R, x, \Omega, \text{cm}^{-1}$) to $\Omega R, \text{cm}$) values by scaling to preparation cross-section area and the proportion of cross-section area occupied by muscle. The nonmuscle fraction of cross-section area was calculated from the value of $r_{ec}$, assuming it was filled with Tyrode (49 $\Omega \text{cm}$).\textsuperscript{12}

### Connexin Protein Quantification by Western Blotting

Tissue homogenates were prepared from frozen tissue samples to give a solution of final concentration 0.5 mg/mL in sample buffer. Total protein (5.0 mg) from each sample was resolved by polyacrylamide gel electrophoresis (BioRad, Hercules) on a 12.5% gel (4.5% acrylamide). Total preparation impedance was modeled as $z = (r_{s}r_{GJ})(r_{s}+r_{GJ})$, where $r_{s}$ is the resistance of the sarcoplasm, $r_{GJ}$, and $z$ is the impedance of the intracellular pathway. $r_{s}$ was measured separately as the resistance between 2 Pt-black needle electrodes, a known distance apart, placed in the muscle within the oil-gap to obtain true intracellular resistance, $2r_{s}$, as $r_{s} = (r_{r}r_{GJ})(r_{r}+r_{GJ})$. Intracellular resistance itself has 2 components; the sarcoplasm, $r_{s}$, and GJ resistances, $r_{GJ}$, (ie, $r = r_{s} + r_{GJ}$), obtained separately by measuring the frequency-dependent component of $r$ and the limiting value at high frequencies (Figure 1). Preparation length and radius in the oil-gap were measured after measurements. Lower case values of variables ($r, x, \Omega, \text{cm}$) were converted to specific ($R, x, \Omega, \text{cm}^{-1}$) to $\Omega R, \text{cm}$) values by scaling to preparation cross-section area and the proportion of cross-section area occupied by muscle. The nonmuscle fraction of cross-section area was calculated from the value of $r_{s}$, assuming it was filled with Tyrode (49 $\Omega \text{cm}$).\textsuperscript{12}

**Table 2. Physiological and Histological Parameters**

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<th>$R_{i}$, $\Omega \text{cm}$</th>
<th>$R_{GJ}$, $\Omega \text{cm}$</th>
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<th>Cx40 Units</th>
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<th>Cx40 Area/Disc, %</th>
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$r_s$ indicates sarcoplasmic resistivity; $R_i$ intracellular resistivity; and $R_{GJ}$ gap-junction resistivity.

### Immunofluorescence Confocal Microscopy

Frozen sections (10 $\mu$m) were mounted onto poly-L-lysine coated slides, fixed in 100% methanol at −20°C and washed with phosphate buffered saline before blocking in 1% BSA for 30 minutes (for Cx43) or 1 hour (for Cx40). The same primary Cx43 and Cx40 antibodies were used for protein quantification. Incubation was at a 1:1000 dilution in BSA for 2 hours for both antibodies. After washing, a secondary antibody tagged with Cy3 fluorescent marker (Chemicon) for Cx43 and fluorescein isothiocyanate (Chemicon) for Cx40 were used at a dilution of 1:500 and 1:50, respectively, in BSA for 45 minutes.
Sections were then washed and mounted with VectaShield mountant (Vector Laboratories, CA). Immunolabeled sections were examined using a Zeiss LSM-780 confocal microscope. To assess cellular connexin distribution, a previously published, blinded, semiquantitative visual method was used. A simple, arbitrary score was derived as follows, based on the degree of clustering of immunolabeled GJ. Longitudinal orientation by standard light microscopy of an adjacent section was first confirmed. Immunolabeled sections were then examined using phase-contrast transmission microscopy, coupled with fluorescence microscope settings that maintained cell outline visualization to further confirm longitudinal cell orientation, to assess the distribution pattern of immunolabeled GJs. Three randomly selected optical fields (≈250,000 μm²) of ≥3 separate tissue sections from each specimen were examined by a single experienced immunofluorescence microscopist blinded to the origin of the specimens. The scoring system assessed Cx40 and Cx43 label distribution, ranging from being confined exclusively to the transversely orientated clusters at cell abutments (score 1), to a distribution of labeling within longitudinal arrays along the length of the myocyte, with markedly diminished labeling at the end-on abutments (score 5). Unlike ventricular myocardium, in which the normal Cx label distribution is highly polarized in the adult ventricle, with mean distribution scores in previous studies of <2, normal atrial myocardium shows much less polarized distribution scores of 2–3. Although only semiquantitative, this technique has proven useful and identifies changes in Cx distribution that are relatively gross and of likely biological significance.

**Measurement of Connexin Expression Per Unit Area Per En Face Intercalated Disc Area**

Transversely sectioned samples labeled with Cx40 were examined using a Zeiss LSM-780 confocal microscope; 15-to-20 en face discs per patient were selected from ≥5 randomly selected fields (230×230 μm). Confocal images were converted into TIFF format using Fiji software and then analyzed with Photoshop CS3 (Adobe) software. Immunolabeled pixels were counted electronically to allow measurement of both intercalated disc area and Cx40 area per en face disk.

**Measurement of Connexin40 Heterogeneity**

For each sample, 6 randomly selected areas (230×230 μm) were imaged on a Zeiss LSM-780 confocal microscope. Using Photoshop CS3 (Adobe), a grid of 7×7 squares was superimposed on the image. The number of squares without label was counted and divided by the total number of squares to give percentage labeled fields as a marker of Cx40 heterogeneity.

**Quantification of Myocardial Fibrosis**

Elastin and collagen autofluorescence were measured with an argon ion laser at 488 nm; 10 μm frozen tissue sections were fixed and mounted at −20°C as above and imaged using a confocal microscope (Zeiss Pascal). Quantification used the ImageJ program (National Institute of Health) to calculate percentage fluorescence in 6 randomly chosen fields per sample.

**Statistics**

Data are presented as mean±SD. Association between variables was analyzed by Pearson correlation tests. A Spearman rank correlation coefficient, rs, expressed the association between variables, with no assumed relationship. The null hypothesis was rejected at P<0.05. Impedance data were fitted to the equation of a circle using the program KaleidaGraph (Synergy Software). All other analysis was performed using GraphPad Prism 2.01 (GraphPad software).

**Results**

**Patient Population**

The relevant clinical, electrocardiographic and echocardiographic characteristics of the patients are shown in Table 1.

There was no association between ECG P wave duration, left atrial diameter, and any physiological or immunohistochemical parameter.

**Specimens**

Biopsy samples were of sufficient size to perform impedance measurements for all patients (n=23). However, connexin quantification by Western blot analysis was only possible in 20/23 samples. Fibrosis quantification and morphometry were performed in 16/23 samples, and intercalated disc area and Cx40 GJ protein per area en face disc were measured in 12/23 patients.

**Figure 2.** The relationship between Cx40 and Cx43 expression, as well as relative Cx ratio (Cx40/Cx40+Cx43), with $R_i$ (open symbols) and $R_j$ (closed symbols). A, Increasing Cx40 expression was significantly associated with both $R_i/R_j$ ($P<0.01$ and $P=0.01$, respectively). B, Cx43 expression did not correlate with $R_i/R_j$. C, The relative Cx ratio (Cx40/Cx40+Cx43) was significantly associated with $R_i/R_j$ ($P<0.01$ and $P>0.02$, respectively).
Connexin Expression and Myocardial Resistivity

Table 2 shows values of physiological and histological parameters for each patient. Figure 2A and 2B show plots of right atrial Cx40 and Cx43 expression against corresponding $R_i$ and $R_j$ values. There were significant positive correlations between Cx40 expression and $R_i$ ($r_s=0.64$, $P<0.01$, $n=20$) as well as $R_j$ ($r_s=0.63$, $P=0.01$, $n=20$). Cx43 expression, however, did not correlate with either $R_i$ or $R_j$, and there was no correlation between either connexin or $R_c$ (not shown). There was also a significant positive correlation between the proportional level of Cx40 expression ($\text{Cx40}/(\text{Cx40}+\text{Cx43})$) and both $R_i$ ($r_s=0.58$, $P<0.01$, $n=20$) and $R_j$ ($r_s=0.51$, $P=0.02$, $n=20$; Figure 2C).

Cellular Connexin Distribution

Figure 3A and 3B shows immunolabeling for Cx40 and Cx43, respectively. Both connexins were located predominantly at the intercalated discs at the poles of the myocytes but as have previously been described in normal atrial myocardium,16 there was also labeled the lateral cell borders of all specimens (more so than in normal ventricular myocardium), with a distribution score (see Methods) of 2.5±1. Figure 3D shows that there was no association between Cx distribution score and $R_j$ for each patient.

Immunolabeled Cx40 Gap Junction Protein Per Unit Area of En Face Disk and Cx40 Heterogeneity

Given the relationship established between Cx40 expression and $R_j$, it was of interest to examine whether $R_j$ was related to Cx40 expression specifically at the intercalated disc. Figure 3C shows enface views of a typical enface intercalated disc. As described in the Methods section, the quantity of Cx40 immunolabeled pixels was measured and expressed as a percentage of the area of the enface disk. The mean intercalated disc area was 96±37.8 $\mu$m$^2$, mean Cx40 area was 39.4±10.2 $\mu$m$^2$, and mean Cx40 GJ area per enface disk was 45±20%. Cx40 expression, measured by Western blot analysis, did not significantly correlate with Cx40 GJ per unit area of enface disc ($r_s=0.19$, $P=0.55$, $n=12$; Figure 4A), but there was a significant positive correlation between Cx40 GJ per unit area of enface disk and $R_j$ ($r_s=0.66$, $P=0.02$, $n=12$; Figure 4B). The heterogeneity of Cx40 labeling was examined by assessing the percentage of labeled cells per field for 12/23 patients, and values are shown in Table 2. There was no significant relationship between Cx40 heterogeneity and junctional resistivity ($r_s=-0.44$, $P=0.15$; Figure 4C).

Relationships Between Age, Connexin Expression, and Myocardial Resistivity

There was a significantly positive correlation between advancing age and both $R_i$ ($r_s=0.77$, $P<0.0001$) and $R_j$ ($r_s=0.65$, $P<0.001$, $n=23$; Figure 5A). There was a corresponding correlation between age and both total Cx40 expression (Western immunoquantification: $r_s=0.54$, $P=0.01$, $n=20$; Figure 5B) and Cx40 GJ protein per unit area enface disk (immunohistochemical quantification: $r_s=0.61$, $P=0.02$, $n=12$; Figure 5C). There was no relationship between age and Cx43 Western immunoquantification, Cx43 immunohistochemistry, or Cx43 redistribution (Figure 5D). There was no relationship between age and $R_c$ (not shown).

Myocardial fibrosis was quantified for 16/23 patient samples to provide average values from 6 randomly selected fields. There was no significant relationship between age and the percentage of myocardial fibrosis (Figure 5E).

Discussion

The main findings of this study are (1) that increased Cx40 expression, and an increase in the relative expression of Cx40
Connexin40 Expression and Atrial Resistivity

Action potential CV in myocardium is determined by several putative factors that include variation in GJ coupling,19 depolarizing currents,20 and tissue architecture.21 Connexin knock-out in mice changes CV in atrial and ventricular myocardium,8 as well as in the specialized conduction system.22 and shows that connexins are a key determinant of impulse propagation. In human studies on patients undergoing cardiac surgery, our previous demonstration of an inverse relationship between human right atrial Cx40 expression and measured CV9 seems counterintuitive because an increase in connexin expression would be expected to increase CV. Better understanding of this relationship, presumed to be mediated by changes in resistivity, is central to both the justification and the findings of the present study, namely that increased Cx40 expression is associated with an increase of $R$ in human atrium,9 and that important both increase with increasing age. The increased resistivity associated with increased Cx40 is an explanation not only for the reduced CV previously measured in open-chested human studies, but also for the increased risk of postoperative AF associated with increased levels of Cx40,23 indicating that the slowing of conduction associated with greater Cx40 expression,10 contributes to the arrhythmogenic atrial substrate and that this may also contribute to the age-related increase in prevalence of AF.

Despite this consistency, it remains counterintuitive that resistivity does not reduce with increased Cx40. Indeed, given that the unitary conductance of pure Cx40 channels is higher than that of pure Cx43 and Cx45 channels,24,25 it might be expected that higher levels of Cx40 expression would result in a lower myocardial resistivity. However, when a pair of myocytes expresses ≥2 connexins, the different potential combinations of connexins in the connexon GJ subunits,26,27 result in a corresponding variety of functional properties of the GJ channels. In vitro studies have confirmed that cells that coexpress Cx40 and Cx4328–30 are known to be more susceptible to uncoupling than cells expressing either single connexin type,30 and cell pair studies have shown that GJ channels formed between a cell that expresses only Cx43 and one expressing only Cx40 are nonfunctional.27 We have previously confirmed colocalization of Cx40 and Cx43 within human right atrial GJ plaques,9 and although colocalization does not prove that Cx40 and Cx43 form mixed GJ channels,27 this finding provides a possible explanation for the findings of the present study that when Cx43 and Cx40 are coexpressed, there is an increase in overall GJ resistivity as Cx40 increases. Although we did not demonstrate a direct association between Cx43 expression and $R$, it is possible that a dependency may exist but is undetectable with currently available methods. Furthermore, other studies have suggested interaction between expression of the different connexins, with changes in Cx40 possibly affecting Cx43 expression10 and vice versa.31,32 The evidence is therefore that interactions between coexpressed connexins are not straightforward and predictable, and that the functional consequences of altered Cx expression associated with age, or disease states such as atrial fibrillation, may not be predictable from simple measurement of connexin quantity alone.

Advancing Age, Atrial Connexins, and Resistivity

We,33 and others,34 have shown an age-associated slowing of right atrial CV in human endocardial mapping studies, although the mechanism underlying this phenomenon has never been fully elucidated. Our present study demonstrated that an age-related increase in $R$ associated with an increase in Cx40 expression and intercalated disk-located Cx40, and thus represents the first functional studies of the effects of ageing on cardiac GJs in the human atrium.
Animal studies have confirmed that atrial fibrosis with advancing age is associated with conduction slowing and arrhythmogenesis, and human studies have demonstrated a correlation between levels of atrial fibrosis and advancing age. By contrast, and in keeping with the present study, other studies have refuted an association between atrial fibrosis and advancing age, and these findings also accord with our previous finding that human atrial CV did not directly correlate with atrial connective tissue content.

Among the conflicting studies correlating human atrial connective tissue, the techniques for connective tissue quantification and the age ranges of the subjects varied, but it also remains possible that in the relationship with conduction velocities what is important is not connective tissue quantification, per se, but the extent to which it causes changes in GJ coupling by altering the topology of connectivity of the myocytes. It is conceivable that there may be increased connective tissue with a distribution that does not separate the points of connectivity between the cells, and other distributions that do.

Insights From Correlations of Connexin Expression With Junctional Resistivity

In ventricular myocardium, where there is a single dominant connexin, we and others have previously correlated Cx43 quantity with conduction velocities and, in a recent study, Cx43 quantified by immunofluorescence correlated with direct measurements of intercellular electric conductance (the inverse of resistivity) in engineered rat ventricular myocytes. The present study of atrial myocardium, in which there is the added complexity of 2 coexpressed dominant connexins, Cx40 and Cx43, provides unique insight into human connexin biology, in particular the importance of Cx40 in determining atrial resistivity, and the effects of aging on atrial connexin expression and resistivity. Our results may also explain the finding that increased right atrial Cx40 expression in sinus rhythm is a predictor of the risk of developing postoperative AF, and that an upregulation of right atrial Cx40 has been described in association with atrial fibrillation in man. Although little is currently known about the mechanisms that underlie age-related atrial remodeling, or the control mechanisms...
or modulators of Cx40 expression, in the era of pharmacological,\textsuperscript{37} and genetic modulation,\textsuperscript{48} of connexin expression, human atrial Cx40 expression may be a potential therapeutic target in the prevention of atrial arrhythmias.

**Limitations**

Specimens were of insufficient size to perform immunohistochemical experiments for all patients, and tissue was only collected from the atrial appendages, and it is possible that the GJ remodeling described in this article is limited to the appendage only and is not reflective to changes in other atrial regions. It is possible that the preoperative medication taken by patients in this study could have influenced GJ conductance. The use of impedance methods to analyze properties of myocardium has been used by others, including measurements at a fixed frequency with electrodes on the epicardial surface.\textsuperscript{49} Such methods allow a more rapid assessment of impedance changes than that described here, although our method allows a more quantitative description of the components contributing to overall myocardial impedance. Thus, our method will not permit evaluation of rapid alterations occurring to the myocardial impedance pathway. The connexin redistribution scoring method used in this study is semiquantitative and therefore limited, but we and others have used it previously,\textsuperscript{14–17,42} and have shown excellent interobserver and intraobserver repeatability, and when applied to carefully sectioned tissue to ensure longitudinal sectioning, differences that are gross enough to be distinguished visually by a blinded observer (as well as statistically) are more likely to be of biological significance. However, we accept that confounding factors unavoidable in studies on human myocardium, such as advancing age, hemodynamic changes, etc, are known to be associated with connexin lateralization,\textsuperscript{50} and may have influenced the scores derived. Although our results demonstrate an association between Cx40 expression, GJ resistivity, and advancing age, they do not prove a direct causality between these variables.

**Conclusions**

In the human right atrium, Cx40 expression and Cx40 GJ protein per unit area of enface disks are associated with an increase in GJ resistivity. Advancing age is associated with an increase in Cx40 expression and GJ resistivity, and these findings may explain the association of advancing age with atrial arrhythmias.

**Acknowledgments**

Microscopy was performed in the Facility for Imaging by Light Microscopy (FILM) at Imperial College London.

**Sources of Funding**

This study is supported by funding awarded by the British Heart Foundation (FS/03/031/15498, RG/05/009, and RG/10/11/28457).

**Disclosures**

None.

**References**

Atrial arrhythmias are common in humans, and their prevalence increases with advancing age. Modulation of atrial gap–junction coupling is a potential therapeutic target, but the relative roles of the gap-junctional proteins connexin40 (Cx40) and connexin43 (Cx43) in determining human atrial myocardial conduction properties are poorly understood. In the present study, we used ex vivo measurements of gap-junctional resistivity and quantitative connexin immunoblotting and immunohistochemistry to demonstrate that Cx40 is associated with human right atrial gap-junctional resistivity such that increased total, gap-junctional, and proportional Cx40 expression increases gap-junctional resistivity. Accordingly, advancing age is associated with an increase in Cx40 expression and a corresponding increase in gap-junctional resistivity. The present study of human atrial myocardium provides a unique insight into connexin biology, in particular the importance of Cx40 in determining atrial resistivity, and the effects of aging on atrial connexin expression and resistivity. In the era of pharmacological and genetic modulation of connexin expression, human atrial Cx40 expression may be a potential therapeutic target in the prevention of atrial arrhythmias.

**CLINICAL PERSPECTIVE**

Atrial arrhythmias are common in humans, and their prevalence increases with advancing age. Modulation of atrial gap–junction coupling is a potential therapeutic target, but the relative roles of the gap-junctional proteins connexin40 (Cx40) and connexin43 (Cx43) in determining human atrial myocardial conduction properties are poorly understood. In the present study, we used ex vivo measurements of gap-junctional resistivity and quantitative connexin immunoblotting and immunohistochemistry to demonstrate that Cx40 is associated with human right atrial gap-junctional resistivity such that increased total, gap-junctional, and proportional Cx40 expression increases gap-junctional resistivity. Accordingly, advancing age is associated with an increase in Cx40 expression and a corresponding increase in gap-junctional resistivity. The present study of human atrial myocardium provides a unique insight into connexin biology, in particular the importance of Cx40 in determining atrial resistivity, and the effects of aging on atrial connexin expression and resistivity. In the era of pharmacological and genetic modulation of connexin expression, human atrial Cx40 expression may be a potential therapeutic target in the prevention of atrial arrhythmias.
Relationship Between Connexin Expression and Gap–Junction Resistivity in Human Atrial Myocardium
Paramdeep S. Dhillon, Rasheda A. Chowdhury, Pravina M. Patel, Rita Jabr, Aziz U. Momin, Joshua Vecht, Rosaire Gray, Alex Shipolini, Christopher H. Fry and Nicholas S. Peters

Circ Arrhythm Electrophysiol. 2014;7:321-329; originally published online March 7, 2014; doi: 10.1161/CIRCEP.113.000606
Circulation: Arrhythmia and Electrophysiology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3149. Online ISSN: 1941-3084

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SUPPLEMENTAL MATERIAL

Measurements of longitudinal impedance and calculation of gap junction resistance

The longitudinal impedance of atrial preparations (≤1 mm diam. 5-6 mm length) was measured by constraining alternating current to flow along the intracellular pathway. A three-chambered bath (4.5 cm x 2 cm x 1 cm) (Supplemental Figure 1), separated by rubber membranes, was used, and the preparation was pulled through tight holes in the membranes with at least 1 mm protruding into the outer chambers. The length of muscle in the central chamber was 2.5 mm. The central chamber contained mineral oil, and the outer chambers contained Tyrode's solution at 37ºC. Alternating current (0.25-150 kHz) was passed between platinum (Pt)-black electrodes in the outer chambers to constrain current to the intracellular pathway of the muscle within the oil-gap, with a fraction through a parallel extracellular shunt. The frequency-dependent intracellular resistance ($r$) of the preparation was recorded with a balanced Wien bridge (Wayne-Kerr, Wetherby, UK). The bridge maintained a constant 10 mV peak-to-peak signal across the preparation, permitting the system to be analysed as a lumped circuit. We avoided a non-voltage-clamped arrangement as has been used by some others when analysing the impedance properties of skeletal muscle.2,3

It was important to avoid an hypoxic core to the preparation because this would falsely increase $R_i$. Several lines of evidence indicated that this was so: (1) the preparation diameter was <1 mm; (2) impedance measurements were stable for at least 30 minutes and (3) tissue structure, examined by histology and immunohistochemistry, and metabolic integrity, examined by measuring intracellular [ATP], are unaffected by experiments.4
Prior to mounting specimens in the Perspex bath the Pt-black electrode resistance, \( r_p \), and capacitance, \( c_p \), were measured separately in a large volume of Tyrode’s solution. Experimental measurements were performed using alternating current (20 Hz - 300 kHz). Two complete sets of experimental recordings of preparation impedance and phase angle were performed at 10-minute intervals; values always agreed within 10%, and average values were used.

\( r_p \) and \( c_p \) are considered to lie in series but the bridge reads the values as if they are in parallel. In order to obtain their values the following relationships were used.

\[
\begin{align*}
  r_p &= \frac{1}{g(1 + Q^2)} \quad [7] \\
  c_p &= c(1+1/Q^2) \quad [8]
\end{align*}
\]

Where \( r_p \) and \( c_p \) are the polarization resistance and capacitance of the electrodes and \( g \) and \( c \) are the values obtained from the Wein bridge: \( G \) (conductance) is the inverse of \( R \) (resistance). The constant \( Q \) has a value of \( \omega c/g \), where \( \omega \) is the radial frequency of the measurement (i.e. \( \omega = 2\pi f \)).

For impedance measurements of the specimen, the electrode resistance and capacitance were subtracted from the total measured values by use of the network shown in supplemental figure 2. where:

\[
\begin{align*}
  r &= \left[ 1 + (\omega c)^2 \right] \left[ r_p + r_s / \left[ 1 + (r_s \omega c_s)^2 \right] \right] \quad [9] \\
  1/\omega c &= \left[ 1 + 1/(\omega c)^2 \right] \left[ 1/\omega c_p + (1/\omega c_s)/ \left[ 1+(1/r_s \omega c_s)^2 \right] \right] \quad [10]
\end{align*}
\]
the subscript \( p \) refers to polarization (electrode) values and \( s \) refers to sample values. The unsubscripted values are taken directly from the bridge.

If \( r\omega c < 1 \) and \( r_p < r_s \) which was always the case, then equations [9] and [10] can be approximated by

\[
\begin{align*}
    r & = r_s + r_p \\
    r_s & = r - r_p
\end{align*}
\]

[11]

\[
c_s = \frac{c}{1/\omega^2 r^2 c_p}
\]

[12]

Lastly the interelectrode capacitance, \( c_x \), measured between the outer chambers with the preparation removed from the middle chamber, was subtracted from \( c_s \) as the two were assumed to lie in parallel.

The impedance of the system, \( z \), can be expressed by;

\[
z = (r + jx)
\]

[13]

Where \( r \) is the resistance and \( x \) the reactance; \( j \) is the complex operator \( \sqrt{-1} \). The reactance of a capacitor is \(-1/\omega c\) so that the admittance, \( y \), \((=1/z)\) can be expressed as;

\[
y = (g + j\omega c)
\]

[14]

Thus, to obtain the impedance values of myocardium, the measured values of \( c_s \) and \( g_s \) were converted to \( r \) and \(-x\) values by the following relationships;

\[
r = g/(g^2 + (\omega c)^2)
\]

[15]

\[
-x = \omega c/ (g^2 + (\omega c)^2)
\]

[16]

These values could then be expressed in the form of an \( r \) versus \(-x\) plot which provided a locus for every separate time constant.
The results were then manipulated to fit a network where the impedance, \( z \), was modelled as \( z = (z_i r_{ec})/(z_i + r_{ec}) \), where \( r_{ec} \) is the resistance of the extracellular shunt and \( z_i \) is the impedance of the intracellular pathway (Supplemental Figure 3). \( r_{ec} \) was measured separately by measuring the resistance between two Pt-black needle electrodes a known distance apart in the muscle within the oil-gap.

Longitudinal impedance, \( z_i \), was analyzed as two series components; cytoplasmic resistivity, \( r_c \), and junction impedance, \( z_j/z_j + r_c = z_i \). \( z_i \) values were expressed as their resistance, \( r_s \), and reactance, \( x_s \), components, i.e. \( z_i = r_s + j x_s \).

When measurements were completed the length and radius of the preparation in the oil-gap were measured. Lower case values of variables (\( r, x \), expressed as \( \Omega.\text{cm}^{-1} \)) were converted to specific (\( R, X, \Omega.\text{cm} \)) values by scaling to the cross-sectional area (CSA) of each preparation and the proportion of CSA occupied by muscle. The non-muscle fraction of CSA was calculated from the value of \( r_{ec} \), assuming it was filled with Tyrode’s solution (resistivity, 49 \( \Omega.\text{cm} \)). Repeated measures from adjacent preparations from the same heart showed less than 10% variability regardless of the chamber studied.

**Analysis and interpretation of data**

Supplemental Figure 4 shows a plot of resistance, \( R_i \), as a function of frequency between 20 Hz and 300 kHz. \( R_i \) declines with increasing frequency, levelling off toward a constant finite value at higher frequencies. The plot is interpreted as one or more parallel \( r_c \) circuits in series with a resistance. For each \( r_c \) circuit with a different time constant (\( \tau = r_c \)), there will be a specific range of frequencies over which the impedance will decline.

More accurate analysis was obtained by plotting, at each frequency, the resistive (\( R_s \)) and reactive (\( -X_s \)) components of \( Z_s \) as a function of each other. Supplemental Figure 5 shows
such plots using the data of Supplemental Figure 4; points for lowest frequencies are on the right side, and each time constant shows as a separate semicircular locus. Semicircles were fitted to the left (higher frequencies) loci, and the intercepts with the $R_s$ axis are shown as $R_1$ and $R_2$. The plots were analyzed in terms of circuit elements in the longitudinal pathway of the muscle preparations, in parallel with a resistive shunt, $R_{ec}$, which is present in the extracellular space of the preparation and in the thin layer of Tyrode's solution adhering to the muscle beneath the oil in the central chamber.

Such plots do not specifically display frequency information, but the values of some frequencies are shown on the plots. The time constant, $\tau$, of the parallel $Rc$ circuit generating a particular dispersion is obtained from the relationship $2\pi f^* \tau = 1$, where $f^*$ is the frequency generating the maximum value of $-X_s$ in the locus. The low-frequency dispersion exhibited a maximum reactance at $\approx$40 Hz, equivalent to a time constant of $\approx$4 ms and similar to that of the myocardial membrane time constant. Thus, the low-frequency dispersion was interpreted as resulting from the surface membrane of the preparation in the outer chambers, $R_m$ and $C_m$ in Supplemental Figure 3.

The high-frequency dispersions (maximum reactance at 10 to 40 kHz) have been interpreted as a junction impedance between cells in the longitudinal pathway. Agents such as heptanol leave the $R_1$ unchanged whilst increasing the $R_2$ value in Supplemental Figure 5. The residual resistance at the higher frequencies was considered to result from the resistance of the sarcoplasm.

Supplemental Figure 3 shows an equivalent circuit that was used to analyze the $-X_s/R_s$ plots of Supplemental Figure 5. Included in the circuit is a shunt resistance, $R_{ec}$, representing current flow through the extracellular compartment of the preparation in the oil gap. The low-frequency intercept of the left dispersion with the resistance axis, $R_2$, is a parallel combination
of $R_{ec}$ and the total intracellular resistivity, $R_i$; where $R_i$ is the sum of $R_c$ and $R_j$. The high-frequency intercept, $R_1$, is a parallel combination of $R_{ec}$ and $R_c$ alone. The difference, $R_2-R_1$, will therefore be a function of $R_j$ and $R_{ec}$. The values of the intercepts $R_1$ and $R_2$ were determined in all preparations, along with the preparation length in the oil gap, total CSA (including the adherent layer of Tyrode's solution), and the proportion of CSA occupied by muscle for calculation of the specific resistances in units of $\Omega$.cm. There were no significant differences in preparation dimensions between each of the four experimental groups.

References


Figure Legends

Supplemental Figure 1. Diagram of the three-chambered impedance bath (not to scale). The outer chambers contained oxygenated Tyrode’s solution (37°C) and the middle chamber contained mineral oil gel. Preparations were pulled through holes in the rubber membranes. Alternating current was passed across preparations via Pt-black electrodes, one in each outer chamber.

Supplemental Figure 2. Model of network used for impedance measurement. \(c = \) capacitance, \(r = \) resistance, \(c_p = \) polarisation capacitance, \(r_p = \) polarisation resistance, \(r_s = \) sample resistance, \(c_s = \) sample capacitance.

Supplemental Figure 3. Equivalent circuit of the muscle preparation used to analyze experimental data. \(r_m\) indicates membrane resistance; \(c_j\), junctional capacitance; and \(c_m\), membrane capacitance.

Supplemental Figure 4. A plot of intracellular resistance, \(R_i\), as a function of measuring frequency. The dotted lines represent the frequency-independent values of \(R_i\) at high and low frequencies. The smaller value at high frequency corresponds to cytoplasmic resistivity, \(R_c\); the difference between the two values is a function of gap junction resistance, \(R_j\).

Supplemental Figure 5. Analysis of intracellular impedance in terms of resistive, \(R_s\) and reactive, \(X_s\). The left semicircular dispersion was fitted to a plot of \(R_s\) against \(-X_s\) at frequencies between 0.02 to 100 kHz using the equation \(X_s = a^2 - (R_c + b) - c\), where \(a, b\) and \(c\) are constants. Intercepts on the abscissa are measures of \(R_c\) \((R_1)\) and \(R_i\) \((R_2)\).
Supplemental Figure 1

Supplemental Figure 1. Diagram of the three-chambered impedance bath (not to scale). The outer chambers contained oxygenated Tyrode’s solution (37°C) and the middle chamber contained mineral oil gel. Preparations were pulled through holes in the rubber membranes. Alternating current was passed across preparations via Pt-black electrodes, one in each outer chamber.
Supplemental Figure 2

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Supplemental Figure 5

Analysis of intracellular impedance in terms of resistive, $R_s$ and reactive, $X_s$. The left semicircular dispersion was fitted to a plot of $R_s$ against -$X_s$ at frequencies between 0.02 to 100 kHz using the equation $X_s = a^2 - R_s + b - c$, where $a$, $b$ and $c$ are constants. Intercepts on the abscissa are measures of $R_c$ ($R_1$) and $R_i$ ($R_2$).