Review

Cardiac gap junctions and connexins: their role in atrial fibrillation and potential as therapeutic targets

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Received 3 September 2001; accepted 26 November 2001

Abstract

In the heart, changes in velocity and in patterns of conduction of myocardial electrical activity can affect cardiac rhythm and the coordination of contraction. Abnormal electrical coupling between cardiomyocytes through gap junctions is, therefore, considered an important factor in various pathophysiologic conditions. In the present report we summarize the literature on gap junctions and their structural proteins, the connexins, in the normal and fibrillating atrium. Putative implications of the recently reported remodelling of atrial gap junctions for stability of the arrhythmia will be discussed. Also the reversibility of the remodelling process will be addressed in the light of a potentially new therapeutic target for controlling the progression of atrial fibrillation (AF). © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Arrhythmia (mechanisms); Gap junctions; Remodeling; Supraventr. arrhythmia

Gap junctions are clusters of closely packed channels (Fig. 1A and B) that directly connect the cytoplasmic compartments of neighbouring cells and allow the passage of ions and small molecules (<1 kDa). Of each gap junction channel 1 hemichannel or connexon is contributed by each of the two cardiomyocytes (Fig. 1C). Connexons are formed by oligomerization of six transmembrane protein molecules called connexins. These connexins, of which 21 members have been identified, are encoded by a family of closely related genes and consist of four highly conserved α-helical membrane-spanning segments (M1–M4) separated by two extracellular (E1 and E2) and one intracellular loop (CL), with cytoplasmic amino and carboxy termini (Fig. 1D). The transmembrane segments 1, 2 and 4 consist predominantly of hydrophobic amino acids while segment 3 is of a more amphiphatic character which suggests a role in the inner lining of the channel pore [1]. The transmembrane segments and the amino terminus are well conserved, whereas the other cytoplasmic domains are unique both in sequence and in length [1]. In mammalian heart connexins (Cx) 37, 40, 43, 45, 46 and 50 (numbers represent molecular mass, in kDa, as predicted from cDNA sequences) are present.

Cx43 is the most abundant and has been found in almost all parts of the organ, with the exception of the cells of the sinoatrial and atrioventricular nodes. Cx40 on the other hand seems to be present specifically in the atrium and the ventricular conduction system. Of the less abundant connexins in the heart, Cx45 is preferentially present in the conduction system [2–4]. Cx46 has been demonstrated in the rabbit sinoatrial node [5] and Cx50 in the atrioventricular valves of the rat heart [6]. Cx37 seems limited to the endocardial endothelium [7–10]. In atrium Cx43 and Cx40 co-localize to a considerable extent (reviewed in [11]). This is possibly (partly) due to the fact that connexons composed of one kind of connexin (‘homomeric connexin’) can combine with connexons made of another connexin to form a so-called ‘heterotypic gap junction channel’. Alternatively, connexons can consist of more than one connexin (‘heteromeric connexons’). In car-

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Time for primary review 27 days.
diomyocytes, single gap junction channel conductances range from about 20 pS for homotypic Cx45 channels to 45–75 pS for Cx43 channels and to about 200 pS for Cx40 channels. These conductance values depend on pH [12], on extracellular [Ca²⁺] [13–15], on extracellular fatty acid composition [16,17] and last but not least on the phosphorylation state of the connexins (reviewed by Van Veen [18]).

Immediately following their synthesis connexins are inserted in the endoplasmatic reticulum (ER) membranes [19] and subsequently oligomerize into connexons in the Golgi-complex [20]. There is evidence that Cx43 phosphorylation plays a functional role in gap junction activity and/or in their assembly in the membrane [21].

Since it was found that ventricular conduction velocity was significantly affected in heterozygous Cx43 knockout mice while atrial conduction velocity in these same hearts appeared to be normal, it was suggested that the connexins 43 and 40 may be chamber-specific determinants of myocardial conduction, Cx43 in the ventricle and Cx40 in the atrium [22]. In support of the notion that Cx40 is important for conduction of electrical impulses in the atrium two groups observed that mice with a targeted deletion of the Cx40 gene showed a prolongation of the P-wave, the PQ interval and QRS complex duration [23,24], findings consistent with diminished atrial conduc-

tion velocity (up to 30%) [25], impaired left bundle branch conduction and right bundle branch block [26]. In addition, atrial but not ventricular tachyarrhythmias occurred spontaneously [24] or could be induced easily (five out of eight [27] or five out of ten [25] Cx40⁻/⁻ mice). No evidence of spontaneous or inducible arrhythmias was found in the Cx40⁺/+ (wild-type) or Cx40⁻/⁻ (heterozygous) mice.

1. Gap junctions and connexins in healthy atrium

In the atrium, as in ventricle, gap junctions which allow action potentials to be propagated from one cell to the next are located predominantly in the intercalated disks, which are special structures at the end of cardiomyocytes that also function in cell-to-cell attachment through desmosomes (connection of intermediate filaments) and in connecting actin filaments through adherens junctions. In ventricle about 20% of the (smaller) gap junctions are located within so-called plicate (transverse) segments of the intercalated disks while 80% of the (larger) gap junctions are present in so-called interplicate regions, parallel to the long axis of the cells [28,29]. The latter gap junctions can serve conduction of electrical impulses both in the longitudinal and transverse direction. Similar data are not available for atrial myocytes, although it has been
shown in canine crista terminalis that myocytes are inter-connected mainly end-to-end, which suggests a higher percentage of gap junctions in plicate regions, consistent with a higher degree of anisotropy of conduction in the crista compared with the ventricle [30]. Analyses of immunohistochemical stainings of thin sections of healthy atrium from experimental animals has revealed that both the Cx40 and Cx43 distribution patterns are homogeneous [31–33], provided the cardiomyocytes have a uniform orientation, either longitudinal or transverse. In man the situation is not clear; a uniform expression of both connexins was reported in the right atrial appendage of patients undergoing valve replacement or coronary bypass surgery [34], while in a recent study in patients with ischemic heart disease only Cx43 was reported to be distributed homogeneously [35]. It is not clear whether these discrepancies might be due to differences in the evaluation of data from immunofluorescence analyses or are caused by the underlying heart disease.

2. Gap junctions and connexins in the fibrillating atrium

Fig. 2 illustrates that immunolabeling distribution patterns of Cx40 and Cx43 in atrial appendage from goats in sinus rhythm (SR) are mostly homogeneous (upper panels). Since double labeling was performed it can be concluded that both gap junction proteins co-localize to a large extent in intercalated disks, that appear either as short transversely orientated lines in the case of longitudinally sectioned myocytes (arrowhead) or as ovoid structures in cross-sectioned myocytes (arrow). Hardly any immunolabeling activity was found in side-to-side plasmalemmal interactions between atrial myocytes. In atrial appendage from a goat that had been in atrial fibrillation (AF) for at least 2 months (lower panels) the distribution pattern of Cx43 seemed unchanged. It was still homogeneous. The distribution pattern of Cx40 on the other hand was discontinuous, showing small areas (0.15–0.6 mm in diameter) of low Cx40 density located between larger areas with an unchanged density [33]. In time course experiments (Fig. 3A) these changes in Cx40 distribution were seen in the left atrium as of the 2-week time point, while in the right atrium heterogeneity in Cx40 distribution could already be detected before that time (two out of five goats in SR and one out of five goats 1 week in AF) [36]. The frequency of occurrence of this phenomenon increased with time of fibrillation until it was observed in two out of three (67%) left atria and in every right atrium (100%) at 16 weeks in AF. Changes in the distribution of Cx43 were never observed (Fig. 3B); it remained homogeneous throughout the time course. The increase in heterogeneity in the Cx40 distribution correlated (Spearman rank order) with an increase in stability of AF (Fig. 3C and D) and the occurrence of structural changes (‘myolysis’) in atrial myocytes [36]. This suggests that this process of ‘gap junctional remodelling’ is involved in the stabilization of AF. With increasing time of fibrillation it became apparent that next to a redistribution of Cx40, overall levels of this gap junction protein decreased as well. In extreme cases Cx40 could hardly be detected anymore, while Cx43 levels did not seem to change. These conclusions were based both on measurements of the relative areas of fluorescence using the confocal laser scanning microscope and on quantitative data of protein concentrations obtained by western blotting ([36] and Fig. 4B). Going from SR to 16 weeks AF average decreases in the Cx40:Cx43 protein ratio of about 50% were measured both in the left and right atria. These changes at the protein level were not based on changes at the level of transcription. Messenger RNA levels for both Cx40 and Cx43 had remained unchanged as was determined by quantitative competitive PCR analysis (Fig. 4A).

In atrial tissue samples from patients with frequent episodes of AF that were undergoing a maze operation, Cx40 showed a tendency to be expressed less abundantly and distributed more often in a heterogeneous fashion than in control samples from patients without a history of AF [34]. Slightly different from what has been found in the goat was the observation that in the atria from some of the maze-patients in addition small inhomogeneities in the Cx43 distribution were detected. Both in the goat and in
patients some lateralization of gap junctions had occurred as a result of AF.

In a study addressing the relationship between the complexity of activation and the quantity of connexins in patients with chronic AF (remodelled atria) or sustained induced AF (no remodelling) it appeared that complex chronic AF was associated with about 50% less Cx40 as compared to simple chronic AF [37]. No changes in Cx40 levels were measured between complex and simple sustained induced AF and Cx43 levels were always unchanged [37]. From the same group a paper was published on atrial Cx40 protein levels in relation to susceptibility to post-operative AF [35]. In biopsies of right atrial appendages taken from patients with ischemic heart disease that had undergone coronary bypass surgery the connexin expression was analysed both at the RNA and protein level. It appeared that Cx40 was expressed at significantly higher levels in samples from patients that had developed post-operative AF (incidence approximately 20%). No changes were measured in the levels of Cx43. Its distribution pattern was always homogeneous, while that of Cx40 was said to be markedly heterogeneous, both in the AF and non-AF groups.

In Langendorff-perfused hearts from old (>11 months) versus young (<3 months) guinea pigs that were subjected to burst stimulation, using a protocol similar to the one used in the goat, prolonged (5–15 min) episodes of post-stimulus atrial ‘fibrillofutter’ could be elicited, but only in the older hearts. This phenomenon was accompanied by an overall lower atrial density of Cx43 and its complete absence in certain areas [38]. Densities of Cx40 were not investigated in this particular study.

Contrary to the observations in the studies mentioned above, in which chronic AF was associated with a disturbance in the distribution and/or a decrease in the expression of Cx40 and/or Cx43 gap junction proteins,
Fig. 4. Connexin mRNA and protein levels in goat atrium. (A) Quantitative competitive RT-PCR analyses of Cx40 (upper panels) and Cx43 (lower panels) mRNA levels in RAA from goats in SR or sustained AF. Lanes 1 to 4 of representative agarose gels (left panels) show increasing signal intensities of amplified target (T-upper: Cx40, T-lower: Cx43) DNA obtained in a 20-μl PCR reaction spiked with decreasing amounts of specific competitor (C) DNA (from 7.5 (lane 1) to $1 \times 10^{-15}$ (lane 4) pmol). Cx40 and Cx43 mRNA concentrations were determined from a graph of the logarithmic ratio of amplified T DNA/C DNA versus the logarithm of C DNA [log (cpm T/cpm C)=0; middle panels]. Measured concentrations (amol per liter) from three experiments are given in the right hand panels (bars represent mean±S.E.M. concentrations). No significant differences, either for Cx40 and Cx43, were measured between AF and SR ($P > 0.05$). Based on data from Van der Velden et al. [33] (Cx40) or unpublished (Cx43). (B) Western blotting analyses with protein extracts from the LAA and RAA from goats in SR or sustained AF using anti-Cx40 (upper left hand panels) and anti-Cx43 (lower left hand panels) specific antibodies. The position of the 45 kDa marker (M) is indicated. Preincubation of the Cx40 antibody with peptide (PEP) against which it was raised resulted in the ablation of a band below 45 kDa. The same analysis using a specific peptide for the anti-Cx43 antibody resulted in the ablation of two bands, an upper band running at 46 kDa (asterisk), also obtained in an immunoprecipitation (IP) using protG Sepharose, and one with a higher mobility (about 42 kDa, double asterisk). The upper band shifted towards the lower position upon pre-incubation with calf intestine phosphatase (CIP), indicating that it is a phosphoprotein. Relative Cx40/Cx43 protein ratios as calculated from density scans obtained from five experiments are shown in the right hand panel. Average values obtained in SR and AF were expressed as mean±S.D. Values obtained for the LAA-SR samples were set at 1.0. Both in the LAA and the RAA relative ratios in AF were significantly (*, $P < 0.05$) different from those in SR; (from Van der Velden et al. [36]).

High-rate atrial pacing in the dog unexpectedly revealed an increased expression of Cx43, together with an increased presence of Cx43 in side-to-side junctions [39]. Changes in the distribution of Cx40 were not investigated in this study either.

In patients with chronic AF in combination with left atrial dilatation the organization of gap junctions in intercalated disks was different from that in control patients [40]. The normal organization in which the larger gap junctions can be found at the periphery ( interplicate) and the smaller in the more central parts ( plicate) had changed into one in which the central gap junctions were almost absent. Patterns were identical for both Cx40 and Cx43 containing gap junctions. It seems however, that the observed phenomenon, that might underlie abnormal impulse propagation, is more likely a result of atrial dilatation...
than of AF since it was almost exclusively present in the left atrium [Takeuchi, personal communication].

3. Electrical consequences of AF-induced gap junctional remodelling

Paroxysmal AF, which is characterized by short lasting or and/or infrequent episodes of AF, often develops into sustained AF with episodes of arrhythmic activity lasting more than 24 h. It is generally accepted now through the work of several groups using different animal models (reviewed in [41] and [42]), but also from studies in patients [43], that AF-induced electrical remodelling of the atrium, affecting duration and spatial heterogeneity of the atrial effective refractory period (AERP) and, in some studies, the conduction velocity of electrical impulses, triggers the susceptibility to fibrillation and the generation of sustained AF (“AF begets AF”) [44]. Since it was shown in the goat that the time courses of AERP shortening and the increase in duration of AF episodes did not match, it was concluded that other factors or processes must be involved in rendering the arrhythmia sustained. One candidate is anatomic or structural remodelling, which covers changes due to (micro)fibrosis and changes in atrial myocyte structure [45], including loss of myofibrils (myolysis) and accumulation of glycogen, similar to what has been observed in hibernating myocardium from patients with chronic ischemic heart disease. Another candidate is gap junctional remodelling which describes the high atrial rate-induced changes at the level of the gap junctions, including changes in expression and distribution of connexins and their turnover [36]. It is conceivable that structural remodelling of the atrial myocardium might contribute to inhomogeneities in both conduction and refractoriness. Such changes will favour reentry and stabilize AF. Since the AF-induced decrease in atrial contractility was found to be associated with an increased tendency of the arrhythmia to recur after cardioversion [46], it has been hypothesized to be the clinical equivalent of myolysis seen at the single cell level [47].

However, myolysis has been shown to be of limited importance in this context since an impaired Ca\(^{2+}\) handling and decreased systolic Ca\(^{2+}\) transients [48], possibly due to an altered function of the L-type Ca\(^{2+}\) channels [49], might underlie this phenomenon.

Focusing on gap junctional remodelling, a disturbed Cx43 distribution has been found to correlate with the location of reentrant circuits in the epicardial border-zone of healing canine infarcts [50]. No such data are available for the atrium yet. In the mouse heart the absence of Cx40 significantly increased its susceptibility to atrial arrhythmias [25,27]. In the goat the local absence of Cx40 (heterogeneity in the Cx40 distribution) did not seem to impair impulse propagation nor to have a significant effect on overall intra-atrial conduction velocity [33]. The reason might be the small size (0.15–0.6 mm) of the areas devoid of Cx40. Consequently, the mapping electrodes, that were spaced apart relatively far (2.25 mm) in relation to the size of the Cx40-devoid areas, might not have ‘seen’ these local discontinuities in conduction. Conduction velocity was lower when measured during sustained AF [51], which can be explained to some extent by the higher complexity of activation patterns due to local block. In the study by Kanagaratnam et al. [37] there was no difference in overall conduction velocity between patients with chronic or sustained induced AF. However, complex activation was always associated with a lower conduction velocity. As mentioned before, this correlated with a significantly lower Cx40 expression in patients with chronic AF and remodelled atria [37]. No conduction measurements were performed in the study by Dupont et al. [35] in patients that had developed post-operative AF. It is not known whether the high-rate atrial pacing-induced increase in expression of Cx43 in the dog contributed to the development of sustained AF. There was no evidence in favour of an enhanced atrial conduction, in fact the authors found that the latter might have been depressed [39]. The abnormal organization of gap junctions observed in patients with chronic AF in combination with left atrial dilatation has been suggested to underlie an abnormal impulse propagation in the atrium and contribute to the initiation and/or propagation of AF [40]. However, there are no concrete data available.

In a recent review on gap junctions in cardiovascular disease, using a model of human ventricular myocardium, Jongsma and Wilders [52] showed that a significant change in total gap junction content or a shift of gap junctions from the ends of cardiomyocytes to lateral sites might only have a moderate effect on overall conduction velocity; they calculated that a 40% reduction of the gap junctional conductance between cells from 5 to 3 \(\mu S\) results in merely an 11% decrease in longitudinal (\(\Theta_L\)) and a 27% decrease in transverse (\(\Theta_T\)) conduction velocity. In this example the anisotropy ratio changed from 2.7 to 3.3. They further corroborated the conclusion of Spach et al. [53] showing that with respect to overall conduction velocity changes in cell size and geometry might be as important as changes in the levels and positions of intercellular gap junctions. In this respect, a cellular volume increase due to AF-induced remodelling [54] might compensate for changes in overall conduction velocity as a result of local changes in gap junction content. In previous studies based on computer simulations it has been suggested that gap junction conduction has to decrease 100-fold or more until slow conduction occurs [55]. Obviously, conduction velocity is not very sensitive to changes in intercellular levels of gap junctions unless these get extreme. On the other hand, extremely slow conduction due to gap junction uncoupling is safe and velocities as low as 0.26 cm/s can be supported. The increase of the safety factor has been
suggested to be an adaptive mechanism to ensure conduction in situations where membrane excitability has seriously been decreased [56]. Stable slow conduction makes microreentry possible, since it can support wavelengths of excitation that amount up to 1 mm. This is what might occur in the goat atrium. Small clusters of atrial myocytes might become uncoupled, through the absence of Cx40 and perhaps the inactivation of Cx43 gap junctions, which would create a situation that supports microreentry leading to sustained AF. Since it has been shown by Beardslee et al. [57] in a model of acute ischemia that uncoupling of cardiac tissue is associated with dephosphorylation of Cx43, it is tempting to suggest that the partial dephosphorylation of Cx43 that was observed in the fibrillating goat atrium [36] might add to the pro-arrhythmogenic environment that is created by the local absence of Cx40 gap junctions. Since it is known that the curvature of a wavefront may have a clear impact on impulse propagation [58] as might occur around tissue irregularities due to fibrosis, this phenomenon might significantly contribute to the effect of local gap junction uncoupling on impulse conduction and arrhythmogenesis.

In conclusion, on the basis of all available data the authors support the view that local changes (up or down) in the expression of connexins (Cx40 or Cx43) might be at the basis of microheterogeneity (or dispersion) in conduction velocity, creating a situation that supports microreentry, which could lead to sustained AF. Although overall changes in connexin levels might be significant, these changes do not have to influence overall conduction velocities.

4. Connexins and gap junctions as potential therapeutic targets

In patients with AF long-term maintenance of SR after successful cardioversion is difficult [59]. This is mainly due to a high recurrence rate of AF within the first month after cardioversion, with a peak incidence during the first 5 days [60]. As the reversal of electrical remodelling in man takes about 3 days [61], it is likely that during this period the atrial vulnerability to recurrences of AF is high. Since long duration (>1 year) of AF appears to be the most important factor leading to unsuccessful maintenance of SR [62], and since studies in the goat indicate that the susceptibility of the atrium to AF is still increased after complete reversal of electrical remodelling [44], other factors must play an important role in this respect. Evidence is accumulating in favour of structural [63] and gap junctional remodelling [64]. Since the first theme is being covered by Dr. Allessie’s group in the current issue of Cardiovascular Research and since the focus of the present discussion is on gap junctions as therapeutic targets for AF, the next question that should be asked is whether AF induced gap junctional remodelling can be reversed and whether this process is accompanied by a reduction in atrial vulnerability to AF. Fig. 5A shows typical Cx40 and Cx43 fluorescent gap junction labeling patterns in representative thin sections of right atrial appendage from goats in SR, 16 weeks in AF, and 8 or 16 weeks in SR following (chemical) cardioversion of 16 weeks in AF (8wPCV, 16wPCV). As can be observed in Fig. 5B the distribution of the Cx40 gap junctions, that was mostly homogeneous in SR but heterogeneous in AF, normalized when AF was followed by 8–16 weeks in SR. During this reversal of gap junctional remodelling, induced AF episodes that lasted seconds in SR, on average lasted minutes in the reverse modelling groups. For comparison, reinduction immediately following cardioversion resulted in non-terminating AF [65]. Consequently, these experiments provide evidence that AF induced gap junctional remodelling can be reversed and that this process is accompanied by a significant decrease in the vulnerability of the atrium to AF.

In a 1995 review on altering the topology of gap junctions as a therapeutic target for AF, Spach and Starmer hypothesized that the identification of new molecular and/or genetic targets that alter the distribution of gap junctions will produce better therapeutic results than can be obtained with conventional pharmacological anti-arrhythmic therapy [66]. Six years later we fully endorse the idea of therapeutics for AF based on the control and adaptation of connexin expression and gap junction distribution. However, we still do not quite know which way to go. First of all, there is no evidence that connexin levels during the different stages of AF, ranging from onset to chronic, are controlled at the mRNA level. On the contrary, in the goat model of AF it was shown that mRNA levels, both for Cx40 and Cx43, remained stable [33,36]. This means that Cx40 levels during AF are probably controlled post-translationally or even post-translationally. Through the high frequency of activation intracellular Ca\(^{2+}\) levels in the atrial myocytes increase [67]. There is direct evidence that this AF-induced calcium overload leads to post-tachycardia abnormalities in Ca\(^{2+}\)-handling that produce contractile dysfunction [68]. Consequently, the activity of calpains, calcium dependent neutral proteases, has been reported to go up in human paroxysmal and persistent AF [69]. Since calpains have been detected in the intercalated disk regions of paraformaldehyde fixed sections of atrial myocytes [69], one might speculate about a putative role of calcium induced proteolysis in the regulation of connexin levels in the atrial myocyte. Hypothetically, the specific inhibition of the calpains involved could then affect the time course of events leading to persistent AF, or even promote its reversal. From recent studies in the intact heart it is known that the connexins have relatively short turnover times (Cx43 half-life = 1.3 h [70]). While both the proteasome and the lysosome participate in the degradation of Cx43 [71] there is evidence from experiments using rat aortic A7r5 and mouse atrial HL-1 cell lines expressing Cx40
that this connexin might be a specific substrate for calpain degradation [Van der Velden et al., unpublished data].

Based on the finding that a natural anti-arrhythmic peptide, which was isolated from bovine atria and enhanced synchronization of spontaneously beating embryonic chick heart cells [72], Dhein et al. developed a number of synthetic peptides of which AAP10 (NH$_2$–GLY–ALA–GLY–HYP–PRO–TYR–CONH$_2$) was most effective in reducing dispersion in action potential duration and consequently in suppressing arrhythmias in the late ischemic period in isolated rabbit hearts. The activity of this peptide seems to be based on enhancement of intercellular coupling through gap junctions via a PKC controlled phosphorylation [73]. Since it has been shown that Cx40 is a substrate for protein kinase C mediated phosphorylation [74], although at present it is not known whether this has an effect on the macroscopic conductance of Cx40 gap junctions, one might suggest that the application of the peptide drugs could have an anti-arrhythmic and prophylactic effect on the development of permanent AF. However, so far data from clinical trials are not available yet.

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