Gap junctional remodeling in relation to stabilization of atrial fibrillation in the goat

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Abstract

Objective: It has been postulated that high atrial rate induced changes at the level of the gap junctions (`gap junctional remodeling', i.e. changes in distribution, intercellular orientation and expression of gap junction proteins), could be part of the vicious circle of electrophysiologic and structural changes leading to sustained atrial fibrillation (AF). To obtain experimental evidence in favour of such a postulate the timing of this remodeling process was studied in relation to the development of sustained AF in a goat model.

Methods and Results: Thin sections from the left (LAA) and right atrial appendage (RAA) from goats in sinus rhythm (SR) or AF, induced through programmed endocardial burst pacing for time periods between 0 and 16 weeks, were immunolabeled with antibodies against connexin(Cx)40 and Cx43 and analysed by immunofluorescence and confocal laser scanning microscopy. During SR the distribution pattern for Cx43 was completely homogeneous (LAA and RAA) and for Cx40 mostly homogeneous (LAA: all five goats, RAA: three out of five goats). The distribution pattern for Cx43 remained stable during AF, while the Cx40 distribution pattern became increasingly heterogeneous, both in the LAA and RAA, with increasing duration of pacing. This increase in heterogeneity in Cx40 distribution correlated (Spearman rank order) with an increase in stability of AF and the occurrence of structural changes (myolysis) in atrial myocytes. The Cx40 / Cx43 immuno-fluorescence signal ratio in both the LAA and RAA appeared to be significantly lower in AF (1–16 weeks) as compared to SR (0 weeks); going from 0 to 16 weeks average ratios decreased 54.5% (n=5; P=0.026) in the LAA and 35.8% (n=5; P=0.034) in the RAA. Western blot analyses revealed similar decreases in the total Cx40/Cx43 protein ratio, on average 50.0% (n=5; P=0.008) and 47.8% (n=5; P=0.02) in the LAA and RAA, respectively. No changes were measured in the levels of Cx40 or Cx43 mRNA, as was assessed through RT-PCR.

Conclusion: The time course of changes in the distribution and content of Cx40 gap junctions as observed during endocardial burst pacing of the goat atrium suggests that Cx40 gap junctional remodeling might be involved in the pathogenesis of sustained atrial fibrillation.

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

This article is referred to in the Editorial by R.G. Tieleman and H.J.G.M. Crijns (pages 364–366) in this issue.

1. Introduction

Gap junctions are specialized membrane regions consisting of groups of channels that directly connect the cytoplasmic compartments of two adjacent cells and enable intercellular communication with respect to the exchange of ions and small (<1 kDa) molecules [1–3]. In the mammalian heart gap junctions that connect the cytoplasm of neighbouring cardiomyocytes are composed of connexin (Cx) 40, 43, 45, 46 and 50 (numbers represent molecular mass, in kDa, as predicted from cDNA sequences). Of these Cx43 is the most abundant connexin...
and has been found in all parts of the organ. The other major connexin, Cx40, is specifically present in the atrium and conduction system. Of the less abundant connexins in the heart, Cx45 is preferentially present in the conduction system [4,5], Cx46 seems to be expressed in pacemaker myocytes in the sinoatrial (SA) node [6] while Cx50 has been detected in rat atrioventricular valves [7]. For the coordinated activation of the different cardiac tissues electrical current is transferred from one cardiomyocyte to the next at the gap junctions. Besides the physical characteristics of their structural proteins, the connexins, the number, size and distribution of these gap junctions in the heart play an important role in determining the conduction properties of the different cardiac tissues (reviewed in [8]). Based on the finding that conduction velocity in the ventricular myocardium had markedly slowed in mice heterozygous for a null mutation in the gene encoding Cx43 while intra-atrial conduction velocity remained normal, chamber-specificity of electrical conduction has been suggested [9,10]. Recently, two different groups produced mice with a targeted deletion of the Cx40 gene [11,12]. Both measured a prolongation of the P-wave, the PQ interval and the QRS complex duration, findings consistent with a delayed (up to 30%) conduction of electrical impulses in the atrium [13] and conduction abnormalities characteristic of first-degree atrioventricular block with associated bundle-branch block. In addition, atrial, but no ventricular, tachyarrhythmias occurred spontaneous [12] or could be induced easily (five out of eight [14] or five out of ten [13] Cx40−/− mice). No evidence of spontaneous or inducible arrhythmias was found in the Cx40+/+ (wild-type) or Cx40+/− (heterozygous) mice.

In a growing number of studies changes in level and distribution of cardiac gap junctions have been linked to various pathological conditions including life-threatening arrhythmias [15–19]. For instance, in ventricular myocardium from hypertrophied and ischemic human hearts Cx43 levels were reduced while in the epicardial border zone of healing canine infarcts a disturbed Cx43 distribution correlated with the location of reentrant circuits [18]. We recently reported an altered distribution of Cx40 containing gap junctions in sustained atrial fibrillation (AF) [20]. In a goat model in which AF was induced through programmed epicardial electrical stimulation [21] and had persisted for at least 2 months, the distribution pattern of Cx43 gap junctions in atrial appendage had remained unchanged and homogeneous. While during sinus rhythm (SR) a similar homogeneous pattern could be observed for Cx40 gap junctions, the Cx40 distribution pattern found during sustained AF showed marked inhomogeneities with areas of low-density Cx40 located next to areas of a higher (normal) Cx40 content. We have suggested that these inhomogeneities in the Cx40 gap junction distribution pattern cause microheterogeneities in conduction by providing small islands of intra-atrial conduction block serving as turning points for the wandering electrical wavelets [20,22]. This remodeling process which we would like to name ‘gap junctional remodeling’ would be part of the vicious circle of electrophysiologic and structural changes leading to sustained AF. In the present communication we will address time course experiments that were initiated to investigate the timing of gap junctional remodeling with respect to the stabilization of AF.

2. Methods

2.1. Goat model of AF and tissue processing

A total of 36 female goats weighing on average 61 kg were used in this study. Of these, six goats were kept in SR and used as controls. The other goats were subjected to, respectively 1, 2, 4, 8 and 16 weeks of AF (six goats per group). Animal handling was carried out according to the Dutch Law on Animal Experimentation (WOD) and the investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997:35:2–3).

Anesthesia in the goats was induced with Nesdonal (15 mg/kg) and maintained by ventilation with halothane (1–2%) and a mixture of O₂ and N₂O. The goats were instrumented with a screw-in electrode with transvenous head (Medtronic®) implanted in a subcutaneous pocket in the neck of each animal. One week after surgery AF was induced by switching on the pacemaker, which produced 50 Hz bursts lasting 2 s at intervals of 1 s at four times the threshold voltage. At the onset of this protocol, which was described by Ausma et al. [49], AF was self-terminating within s whereas over the following days AF episodes became longer and more stable, similar as in the protocol applied by Wijffels et al. [21]. The interburst period was gradually prolonged to eventually 30 min while sustained AF was being maintained. At first, an external ECG was monitored for a few hours. Over the following 2 weeks daily monitoring was performed to check for persistence of AF, while subsequent monitoring was continued on a weekly basis. At the end of the experimental period the goats were anesthetized with Nesdonal (15 mg/kg) and ventilated with halothane (1–2%) and a mixture of O₂ and N₂O. Before sacrifice the pacemakers were switched off and during the following 3 h period the stability of AF was determined. The absence of spontaneous termination of AF during this period was defined as stable AF. Five minutes before the animals were sacrificed 5000 U heparin was injected to avoid blood coagulation and to ensure optimal perfusion fixation of the hearts. Parts of the LAA and RAA were removed and immediately frozen in liquid nitrogen and stored at −80°C until use.

2.2. Morphological evaluation

To perform a morphological evaluation the heart was
fixed within a few seconds by retrograde perfusion with 3% glutaraldehyde, buffered with potassium phosphate to pH 7.4. Subsequently, part of the LAA and RAA were removed and divided into small blocks (4 mm³) and stored in the same fixative until the embedding in Epon. Tissue blocks fixed for at least 24 h were washed with 90 mM potassium phosphate containing 7.5% sucrose, postfixed with 2% osmium tetroxide in 50 mM veronal acetate buffer for 1 h, dehydrated through graded ethanol series, and routinely embedded in the epoxy resin Epon [23]. Light microscopic examination of the morphologic changes was performed on 2-mm thick sections of Epon-embedded blocks from the atrial appendages stained with periodic acid Schiff (PAS) and 0.1% toluidine blue to quantify the loss of myofibrils and glycogen content. To quantify the extent of myolysis in the cardiomyocytes, at least three sections per atrial appendage were analyzed. The extent of myolysis was evaluated only in cells in which the nucleus was present in the plane of the section [23].

2.3. Immunohistochemistry

Unfixed cryostat sections (8 μm) of atrial appendage were stained essentially as described before [24]. Briefly, samples were equilibrated at room temperature and rehydrated in phosphate buffered saline (PBS). They were incubated in PBS containing 0.2% (w/v) Triton X-100 for 1 h, followed by 30 min in a solution of 2% (w/v) bovine serum albumin (BSA) in PBS. All incubations were performed at room temperature, and sections were washed with PBS in-between successive incubation steps. Incubations with primary rabbit anti-Cx40 (Alpha diagnostics; 4 μg/ml, raised against a 19 amino acid peptide sequence from the C-terminal of mouse Cx40) and mouse anti-Cx43 (Transduction Laboratories; 0.5 μg/ml, raised against amino acids 236–382 of the C-terminus of rat Cx43) antibodies were performed overnight at a 1:10 and 1:250 dilution in PBS containing 10% normal goat serum, respectively. An immunohistochemical staining for desmin as a marker for cardiomyocytes was routinely performed on each successive thin section. Mouse monoclonal antibodies against human desmin (1:50) were from Monosan (The Netherlands; clone 33). After washing with PBS and renewed blocking in 2% BSA in PBS, samples were incubated for 2.5 h with secondary antibodies against rabbit or mouse IgG, conjugated with fluorescein isothiocyanate (FITC; Jackson Laboratories) or Texas Red (TR; Jackson Laboratories), respectively. Sections were mounted with Vectashield (Vector Laboratories) and routinely examined for efficiency of staining (desmin staining and Cx40 staining of endothelial cells were used for reference), tissue morphology and cellular composition (>90% myocytes) with a Nikon Optiphot-2 light microscope equipped for epifluorescence. Connexin distribution patterns in each of the samples were classified as either homogeneous, homo/heterogeneous (>90% homogene- nous), heterogeneous or unlabeled (intensity of labeling too faint to judge Cx distribution).

Labeling of Cx40 and Cx43 gap junctions was subjected to a more quantitative analysis by using confocal laser scanning microscopy (Nikon RCM-8000 real time laser confocal microscope). In these analyses thin sections from the RAA and the LAA from one goat at each time period were labeled simultaneously with anti-Cx40 and anti-Cx43 antibodies followed by FITC-conjugated anti-rabbit IgG (Cx40) and TR-conjugated anti-mouse IgG (Cx43). From each thin section ten randomly chosen stacks of optical Z-sections at steps of 1 μm were analysed. Stacks containing blood vessels, expressing Cx40 in their endothelial lining, were omitted. For image analysis the Huygens system 2.0 image processing software package was used (Scientific Volume Imaging, The Netherlands). For each sample average ratios were determined of the relative areas of fluorescence. Mean values were compared between samples using the paired Student’s t-test or when the data did not have a normal distribution by the Mann–Whitney rank sum test. Statistical significance was defined as a value of P<0.05.

2.4. Western blotting and immunoprecipitation

Samples from LAA and RAA, frozen in liquid nitrogen, were homogenized in a RIPA solubilization buffer consisting of 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 mM phenantroline, 1 mM benzamidine, 0.5 mM pefabloc, 5 mM sodium bisulfate, 20 μg/ml pepstatin A). Samples were treated as described by Delorme et al. [25] and 40 μg of total cellular protein was loaded on a 8% SDS–polyacrylamide gel, electrophoresed and transferred onto nitrocellulose membranes according to Bio-Rad’s Mini Trans-Blot instruction manual. Efficiency of transfer was checked by Ponceau Red staining. Membranes were then blocked for 1 h at room temperature in 5% Protifar in 0.1% Tween 20 in PBS (T–PBS). They were rinsed in T–PBS and probed for the presence of Cx40, Cx43 or Cx45 in an overnight incubation at 4°C using the rabbit Cx40 antibody and mouse Cx43 antibody or a rabbit polyclonal antibody (crude serum) against amino acids 259–396 of the C-terminus of mouse Cx45 kindly provided by Dr. T. Steinberg, Institute for Infectious Diseases, St. Louis, MO, USA). After incubation with primary antibody, membranes were washed three times for 5 min in T–PBS and incubated for 1 h at 4°C with a horseradish peroxidase labelled secondary antibody (Bio-Rad; 1:8000). Peroxidase activity was detected on X-ray film using the ECL detection reagents (Amersham) according to the manufacturer’s instructions. Each time different exposures were scanned and analysed using a high resolution scanning system (Microtek ScanMaker 630) in combination with IMAGE QUANT software (Molecular Dynamics).
For immunoprecipitation (IP) 500 μg of total cellular protein in RIPA solubilization buffer was incubated over-night at 4°C on a gently shaking platform with 2 μl Cx43 antibody (0.5 μg/ml). Subsequently, still at 4°C, 50 μl of a 10% gel suspension of protein G–agarose (Boehringer Mannheim) was added for 3 h. The agarose beads were collected through centrifugation (1000 rpm, 5 min) and resuspended in Laemmli sample buffer. A 5- or 15-μl volume was used for gel loading.

2.5. RT-PCR analyses

Frozen tissue samples of LAA and RAA from four goats at each time period were combined and pulverized. Total RNA was isolated using RNeasy (Qiagen) and chromosomal DNA contaminations were removed during incubation with RNase-free DNase (10 U/μl; Boehringer Mannheim) in the presence of an RNase inhibitor (1 U/μl RNAguard; Pharmacia). For reverse transcription 0.2 μg DNA-free RNA was incubated in a 10-μl volume for 5 min at 65°C in the presence of a degenerate primer T12 VX (V is 3-fold degenerate for A,C,G and T). After adjustment of buffer conditions to 50 mM Tris±HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT and addition of 20 μM dNTPs, 1 U/μl RNAguard and 200 units final concentration of RNA polymerase chain reaction (PCR) in 10 μl aliquots were analysed on 2% agarose gels. Routinely cardiomyocyte-specific staining for Cx43 signals in the LAA and RAA from the goats within each series. Routinely cardiomyocyte-specific staining for desmin was monitored. Representative data are presented in the histogram shown in Fig. 2B. It shows that the Cx40/Cx43 fluorescence signal ratio was signiﬁcantly lower in the RAA as compared to the LAA. This apparent-ly re-ults in lower Cx40 content in the RAA since relative areas of fluorescence differed hardly for TR (Cx43) (Fig. 2A). Fig. 2B further shows that both in the LAA and RAA

3. Results

3.1. Immunofluorescence labeling of Cx40 and Cx43 gap junctions

As we described previously [20], the immunolabeling distribution patterns for both Cx40 and Cx43 gap junctions in goats that had not been paced (0 weeks) were mostly homogeneous (Fig. 1A). A similar gap junction distribution had been found in atrial myocardium from guinea pig [26] and rabbit [27]. Since double labeling was performed it could be observed that Cx40 and Cx43 containing gap junctions colocalized to a large extent in intercalated disks, that appeared either as short transversely orientated lines in the case of longitudinally sectioned myocytes (open arrow) or as ovoid structures (arrowhead) in myocytes that had been cross-sectioned. In samples from goats that had been in AF for 16 weeks (sustained AF) the distribution of Cx43 containing gap junctions was still homogeneous, while the Cx40 distribution pattern was fragmented, showing areas with a significantly lower density or almost devoid of Cx40 gap junctions located next to areas with a SR-like distribution pattern (Fig. 1B). The time course of the observed changes in the distribution of Cx40 gap junctions was investigated. As can be observed in Fig. 1C the distribution of Cx40 gap junctions in goats in SR was completely homogeneous in the LAA while heterogeneity (two out of five) was detected in thin sections of the RAA. Upon induction of AF, heterogeneity could first be observed at the 2 week time point in the LAA while the frequency of occurrence in both the LAA and RAA further increased with time. The Cx43 distribution, on the other hand, remained homogeneous throughout the time course in both the LAA and RAA. A small center of heterogeneity was detected in the RAA from one out of six goats at week 16.

Early in the induction protocol (until about 2 weeks) Cx40 gap junctions merely became redistributed, leading to an increasingly heterogeneous distribution pattern. However, with longer duration of AF it became apparent that in addition overall levels of Cx40 gap junctions decreased (Fig. 2A). In extreme cases Cx40 labeling was hardly detectable anymore, as is indicated in the stack column plots in Fig. 1C by the qualification ‘unlabeled’. No significant changes could be observed with respect to the amount of Cx43 gap junctions: levels looked rather stable both in the LAA and RAA (Fig. 2A). Confocal laser scanning microscopy was used to quantify the levels of Cx40 and Cx43 gap junctional staining. Since shape and size of thin sections often did not exactly match the fixed surface area used for calculation of pixel densities, it was not possible to simply use total pixel densities of FITC- and TR-fluorescence as a measure for the levels of Cx40 and Cx43, respectively. Consequently, we determined ratios of the relative areas of fluorescence for the Cx40 and Cx43 signals in the LAA and RAA from the goats within each series. Routinely cardiomyocyte-specific staining for desmin was monitored. Representative data are presented in the histogram shown in Fig. 2B. It shows that the Cx40/Cx43 fluorescence signal ratio was significantly lower in the RAA as compared to the LAA. This apparent-ly re-ults in lower Cx40 content in the RAA since relative areas of fluorescence differed hardly for TR (Cx43) (Fig. 2A). Fig. 2B further shows that both in the LAA and RAA
Fig. 1. Spatial distribution of Cx40 and Cx43 gap junctions in goat atrial appendage. (A) Composite photograph of a section of LAA from a goat in SR (0 weeks) after staining with anti-Cx40 (upper panel) or anti-Cx43 (lower panel) antibodies; homogeneous staining patterns. (B) Composite photograph of a section of LAA from a goat in sustained AF (16 weeks) after staining with anti-Cx40 (upper panel) or anti-Cx43 (lower panel) antibodies: heterogeneous distribution pattern of Cx40 gap junctions and homogeneous distribution pattern of Cx43 gap junctions. All connexin stainings are from double labeling experiments. Intercalated disks with immunolabeled gap junctions in longitudinally or cross-sectioned myocytes are indicated by open and closed arrowheads, respectively. Bar=50 μm. (C) Stack column plots showing the labeling patterns of Cx40 (upper panels) and Cx43 gap junctions (lower panels) in the LAA and RAA with respect to the duration of AF. Each labeling pattern was qualified as homogeneous, homo-/heterogeneous (>90% homogeneous), heterogeneous or unlabeled (faint gap junction labeling). Percentages of inhomogeneous distributions (heterogeneous+unlabeled) are indicated above the stack columns.

the Cx40/Cx43 fluorescence signal ratio was significantly lower in the AF samples (1–16 weeks) as compared to the SR samples (0 weeks). In all goats analysed this ratio had already significantly decreased within 1 or 2 weeks of burst pacing. Since the intensity of fluorescence that was obtained with either fluorochrome varied between experiments, changes in the Cx40/Cx43 fluorescence signal ratios also varied between different experimental series of stained thin sections. In the histograms in Fig. 2C this variability in the Cx40/Cx43 fluorescence signal ratio is shown for the LAA and RAA from goats in SR (0 weeks) or sustained AF (16 weeks). Nevertheless average ratios from five experiments show a significant change in the Cx40/Cx43 ratio, both in the LAA and RAA, between SR (0 weeks) and sustained AF (16 weeks): LAA, 0.418±0.123 in SR versus 0.190±0.120 in AF (−54.5%; $P=0.026$) and RAA, 0.148±0.040 in SR versus 0.095±0.023 in AF (−35.8%; $P=0.034$).

3.2. Correlation of Cx40 changes with the stability of AF and structural changes

The number of goats with stable AF increased with the time of burst pacing (Table 1; four out of five goats at 4 weeks) and correlated with the observed increase in heterogeneity of the Cx40 gap junction distribution: Spearman rank correlation coefficient LAA 0.54 ($P<0.002$) and RAA 0.40 ($P<0.01$) (Table 2). Besides these changes in the distribution of Cx40 gap junctions also structural changes occur in the atrial myocytes. The time course of these structural changes, with hallmarks the loss of sarcomeres (myolysis) and the accumulation of glycogen, was characterized [28]. This myolysis, measured as the number of cells showing >10% loss of sarcomeres, appears to increase, both at the LAA and RAA, with the duration of AF and the stability of AF (Tables 1 and 2). Higher percentages of atrial myocytes with myolysis also correlate with the observed changes in distribution of Cx40 gap junctions: Spearman rank correlation coefficient 0.48 ($P<0.005$) for LAA and for RAA 0.39 ($P<0.002$) (Table 2).

3.3. Analysis of Cx40 and Cx43 protein levels

Whether overall Cx40 and Cx43 protein levels had changed in AF as compared to SR was investigated by Western blotting. Equal amounts of total cellular protein from the LAA or RAA from in each case four goats in SR or sustained AF were probed with anti-Cx40 (Fig. 3A) or anti-Cx43 (Fig. 3B) specific antibodies. The Cx40 signal consisted of a prominent protein band (asterisk) below the 45-kDa marker and two rather faint bands running at a somewhat higher molecular weight. Only the prominent band appeared to be specific since it disappeared upon preincubation of the antibody with the peptide against
Fig. 2. Confocal analyses on levels of immunolabeled Cx40 and Cx43 containing gap junctions. (A) Micrographs from a representative Cx40 and Cx43 double labeling experiment on a series of thin sections from the LAA and RAA from goats that had been in SR (0 weeks) and AF (1–16 weeks). Arrows indicate Cx40 staining of blood vessel endothelial cells. Bar = 50 μm. (B) Data from image analysis of confocal micrographs from a typical series of stained thin sections. From each thin section ten stacks of optical Z-sections at steps of 1 μm were analysed. Relative areas of FITC (Cx40) and Texas Red (Cx43) fluorescence were determined and Cx40/Cx43 fluorescence signal ratios calculated and expressed as mean ± S.D. Average Cx40/Cx43 fluorescence signal ratios both in the LAA and RAA had significantly (P < 0.05) decreased as a result of AF (1–16 weeks) as compared to ratios in SR (0 weeks). (C) Frequency histograms of Cx40/Cx43 fluorescence signal ratios in thin sections from the LAA or RAA from goats in SR (0 weeks) or sustained AF (16 weeks). Average values ± S.D. are indicated in each panel. P values for differences in ratio between AF and SR are also indicated.

which it was raised (lane PEP). The intensity of this Cx40 band as shown in Fig. 3A was significantly lower in the AF samples than in the SR samples. In Fig. 3B the most prominent Cx43 band which represents the only product from a Cx43 specific immunoprecipitation (lane IP) is also indicated by an asterisk. This band was ablated upon

Table 1
AF stability, Cx40 distribution and myolysis in atrial myocytes subjected to varying episodes of burst pacinga

<table>
<thead>
<tr>
<th>Duration of burst pacing (weeks)</th>
<th>AF stability (% of goats)</th>
<th>Cx40 distribution</th>
<th>Myolysis LAA (% myocytes)</th>
<th>Myolysis RAA (% myocytes)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (n = 5)</td>
<td>LAA: 1.2±0.3 (n = 5) RAA: 1.7±0.6 (n = 5)</td>
<td>10.1±7.6 (n = 6)</td>
<td>8.2±4.7 (n = 6)</td>
</tr>
<tr>
<td>1</td>
<td>40 (n = 5)</td>
<td>LAA: 1.0±0.0 (n = 4) RAA: 2.0±0.4 (n = 5)</td>
<td>11.6±6.2 (n = 6)</td>
<td>16.4±17.2 (n = 6)</td>
</tr>
<tr>
<td>2</td>
<td>40 (n = 5)</td>
<td>LAA: 1.9±0.7 (n = 6) RAA: 3.5±0.5 (n = 6)</td>
<td>15.7±8.5 (n = 6)</td>
<td>20.2±9.3 (n = 6)</td>
</tr>
<tr>
<td>4</td>
<td>80 (n = 5)</td>
<td>LAA: 1.7±0.4 (n = 6) RAA: 3.0±1.1 (n = 6)</td>
<td>26.0±17.1 (n = 6)</td>
<td>23.2±9.8 (n = 6)</td>
</tr>
<tr>
<td>8</td>
<td>83 (n = 6)</td>
<td>LAA: 1.7±0.7 (n = 6) RAA: 2.7±0.6 (n = 6)</td>
<td>37.8±7.0 (n = 6)</td>
<td>45.9±12.4 (n = 6)</td>
</tr>
<tr>
<td>16</td>
<td>100 (n = 6)</td>
<td>LAA: 2.6±1.0 (n = 6) RAA: 3.3±0.5 (n = 6)</td>
<td>45.1±10.5 (n = 6)</td>
<td>51.8±8.3 (n = 6)</td>
</tr>
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</table>

a AF was said to be stable whenever termination failed to occur within the 3-h period preceding sacrifice. For statistical analyses numbers have been ascribed to each Cx40 distribution pattern as follows: homogeneous (=1), homo/heterogeneous (=2), heterogeneous (=3) and unlabeled (=4); n = number of goats.
Table 2
Correlation analysis of the Cx40 distribution, myolysis and stability of AF

<table>
<thead>
<tr>
<th></th>
<th>AF stability</th>
<th>Myolysis LAA</th>
<th>Myolysis RAA</th>
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<tbody>
<tr>
<td>Cx40 distribution</td>
<td>0.54</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>LAA</td>
<td>P&lt;0.002</td>
<td>P&lt;0.006</td>
<td></td>
</tr>
<tr>
<td>Cx40 distribution</td>
<td>0.40</td>
<td>0.44</td>
<td></td>
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<tr>
<td>RAA</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
<td></td>
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<tr>
<td>Myolysis LAA</td>
<td>0.60</td>
<td>P&lt;0.001</td>
<td></td>
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<tr>
<td>Myolysis RAA</td>
<td>0.42</td>
<td>P&lt;0.02</td>
<td></td>
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*Top numbers in each entry denote Spearman rank order correlation coefficients. Bottom numbers give the significance, with P<0.05 denoting a significant correlation.

Finally, since Cx45 is not only co-expressed with Cx40 in mouse and rat hearts’ conduction systems [4,5] but appears to be present also at very low levels in the atrium [29], we performed Western blotting on total goat atrial protein using a specific Cx45 antibody. However, using this approach we were unable to detect this connexin, as it was also undetectable this way in human atrium [29], indicating that its levels are probably extremely low.

3.4. Analysis of Cx40 and Cx43 mRNA levels

An RT-PCR approach was used for comparative analyses of ratios of Cx40 and Cx43 mRNA. In order to guarantee the specificity of amplification we used primers that were specific for 3’ noncoding regions of goat Cx40 or Cx43 cDNAs. Exponential phase DNA synthesis conditions were chosen, which was checked in time course experiments as shown in Fig. 4A. In Fig. 4B gel patterns are shown from a representative experiment in which amplification was performed on mixtures of cDNA from the LAA and RAA from four goats at each time point. These and other gel patterns were analyzed using a fast imaging system in combination with IMAGEQUANT software, and Cx40/Cx43 mRNA signal ratios were determined. The histogram in Fig. 4C shows average data from independent experiments (n=5); no significant change was measured in the Cx40/Cx43 mRNA ratios, either in LAA or RAA, as a result of AF.

4. Discussion

4.1. Sustained atrial fibrillation

In the conscious goat model as described by Wijffels et al. [21], AF was induced through programmed burst
inconsistent. In dog [35] and rabbit [36] atrial dilatation caused a shortening of the ERP and an increase in the vulnerability to arrhythmias, while in the goat [37] there was hardly any effect upon acute infusion of a blood-expanding fluid. Alternatively, structural or anatomic remodeling, including changes due to microfibrosis and those changes in atrial morphology resembling structural changes that can be seen in ischemic noninfarcted myocardium in a state of functional hibernation [23] might be involved in stabilizing AF. The latter changes include myolysis and disassembly of the contractile apparatus. Some of these changes have been observed already after 1 week of AF, while their frequency of occurrence increased with the time of fibrillation [28 and present communication]. Changes in expression and distribution of connexins and gap junction channels [20, 38] might play an important role in stabilizing AF. We used the expression ‘gap junctional remodeling’ to describe the high atrial rate induced changes at the level of the gap junctions that include their distribution, intercellular orientation and expression of their structural proteins, the connexins.

4.2. Time course of Cx40 gap junctional remodeling

In goats that had been in sustained AF for at least 2 months the distribution pattern of Cx40, but not of Cx43, gap junctions in atrial appendage showed marked inhomogeneities with areas of low-density Cx40 located next to areas of a higher (normal) density Cx40. Both in SR and in AF connexins 40 and 43 showed a high degree of colocalization. There was a small relative increase in lateral connections between myocytes in AF [20]. In order to investigate the involvement of inhomogeneities in Cx40 distribution in the stabilization of AF, time course experiments were started. In goats that were in SR the Cx40 distribution was completely homogeneous in the LAA and mostly homogeneous (three out of five goats) in the RAA. The same analyses of goats that were in AF for 1 week revealed again a completely homogeneous distribution in the LAA and a mostly homogeneous distribution (four out of five goats) in the RAA. At the 2-week time point, which is around or even just before the time where AF became sustained (at about \( t = 4 \) weeks as shown in Table 1) some heterogeneity in the Cx40 distribution was observed in the LAA (one out of six goats) and complete heterogeneity in the RAA (six out of six goats). In those goats that had been in (sustained) AF for longer time periods (4–16 weeks), the frequency of heterogeneity in the Cx40 distribution had increased to 67% (16 weeks; four out of six goats) in the LAA and remained 100% (16 weeks; six out of six goats) in the RAA. During the indicated time period the distribution pattern of Cx43 containing gap junctions remained homogeneous, both in the LAA and RAA of all the goats analysed.

With the observation that the Cx40 fluorescent labeling pattern became heterogeneous as a result of prolonged
periods of AF the overall intensity of labeling also decreased. As we did in our earlier studies we performed measurements with a confocal laser scanning microscope and expressed the intensity of fluorescence due to Cx40 labeling (FITC) relative to that due to Cx43 labeling (TR). The first observation was that the Cx40/Cx43 fluorescence signal ratio was always significantly lower in the RAA as compared to the LAA (Fig. 2B and C). A similar observation has been made in guinea pig [personal communication, Ingrid Ten Velde, Utrecht]. The lower levels of Cx40 in the RAA make it harder to evaluate the Cx40 distribution patterns in the RAA, which favours a heterogeneous qualification. A second observation was a decrease in the Cx40/Cx43 fluorescence signal ratio, both in the LAA and RAA, as a result of AF. In each series of thin sections 1 week in AF resulted already in significantly lower ratios than in SR. Despite the variability in the Cx40/Cx43 fluorescence signal ratios from different experimental series of stained thin sections, average ratios changed 54.5% (n = 5; \( P = 0.026 \)) in the LAA and 35.8% (n = 5; \( P = 0.034 \)) in the RAA due to 16 weeks in AF (Fig. 2C).

Quantitative analyses of band intensities in Western blots revealed significant reductions in the Cx40/Cx43 protein ratios, measuring on average 50.0% (n = 5; \( P = 0.008 \)) in the LAA and 47.8% (n = 5; \( P = 0.02 \)) in the RAA (Fig. 4C). In sustained AF total Cx40 protein levels were reduced whereas overall levels of the Cx43 protein apparently had not changed. In addition, it was observed that, while Cx43 was mainly in the lower electrophoretic mobility (phosphorylated) form during SR, up to 50% of Cx43, both in the LAA and RAA, appeared to be present in the higher mobility (non-phosphorylated) form during sustained AF. Discrete phosphorylation events have been correlated with oligomerization and insertion of connexons into gap junctions (reviewed in [39]). In addition there is a report on cellular uncoupling and gap junction disassembly through dephosphorylation of Cx43 [40]. This suggests that partial disassembly of Cx43 gap junctions could occur as a gap junctional remodeling event during AF.

The data on Cx40 and Cx43 mRNA levels were in agreement with those obtained from quantitative competitive PCR analyses that we previously performed on atrial tissue samples from goats in sustained AF (>2 months) as compared to SR [20]; Cx40/Cx43 mRNA ratios, either from the LAA or RAA, did not change as a result of AF and both Cx40 and Cx43 mRNA levels seemed stable (Fig. 3C).

4.3. Electrophysiological consequences

As mentioned by Spach and Starmer in a recent review [41], until about 20 years ago the myocardium was considered to behave as an electrically continuous syncytium. Consequently, conduction disturbances leading to reentrant arrhythmias were considered to be due solely to changes in the properties of sarcolemmal ion channels. At present cardiac muscle is more and more seen as discontinuous in nature. In this structure conduction disturbances and block, which are required for reentry, are created by region-to-region variation in the duration of action potentials and by a variation in the electrical load of cells, determined by the myocardial architecture [42–45] and the efficiency of intercellular coupling through gap junctions. In the ventricles, an aberrant Cx43 distribution and content has been described to occur in ischaemic and hypertrophic myocardial disease [15–17,46]. In the epicardial border zone of 4-day-old healing canine infaracts a disturbed Cx43 gap junction distribution has been correlated with the location of reentrant circuits, making gap junctional remodeling an early event in the remodeling of the myocardium after an infarction [18]. In the atrium of the dog an increase in Cx43 gap junctions has been found after prolonged rapid pacing [38]. On the other hand, the distribution pattern of Cx40, which has been shown to be an important connexin in the conduction of electrical impulses in the (mouse) atrium [11–14], changed from homogeneous to heterogeneous during AF in the goat [20] and in man [47]. Until recently one could only speculate about the role of connexins in AF. However, new information became available with the finding that mice with a targeted deletion of the Cx40 gene showed conduction disturbances and an increased susceptibility to atrial tachyarrhythmias [11,12]. In these Cx40 deficient mice an intra-atrial conduction velocity was measured that was reduced up to 30% as compared to wild-type mice [13].

The present communication shows that AF-induced changes at the level and in the distribution of Cx40 gap junctions in the goat atrium already became visible immediately following the initiation of atrial burst pacing. By the time AF was sustained, the Cx40/Cx43 fluorescent signal ratio had reduced significantly in all goats analysed, both in the LAA and RAA (Fig. 2). In addition, the distribution pattern of Cx40 gap junctions had become heterogeneous in the RAA and, to a lesser extent, also in the LAA (Fig. 1C). Consequently, Cx40 gap junctional remodeling might be involved in the pathogenesis of sustained AF, possibly already at an early stage. Using Spearman’s rank order correlation analysis we found a positive correlation between the increased heterogeneity in Cx40 distribution, either in the LAA or the RAA, and AF stability (Table 2). At the same time a positive correlation was found between percentages of myocytes showing myolysis and AF stability ([28] and Table 2).

The Cx40 gap junctional remodeling did not seem to be accompanied by changes in the overall atrial conduction velocity, as measured over Bachmann’s bundle [21] or over the right atrial free wall [20] in atria defibrillated after sustained AF. On the other hand, conduction velocity was significantly lower when measured during sustained AF. This might be explained to some extent by an increase in the number of sites with a slower or blocked conduction...
due to an increase in the number of complex action potentials [48]. It suggests that instead of the occurrence of changes in the macroscopic conduction velocity the generation of small foci of intra-atrial conduction block due to inhomogeneities in the Cx40 distribution pattern might be sufficient for the stabilization of AF.

4.4. Limitations of the study

A limitation of the present study is that only samples from LAA and RAA were analyzed for their expression and distribution of Cx40 and Cx43. However, preliminary analyses revealed a grossly similar Cx40 and Cx43 expression pattern in other parts of the goat atrium. Although we cannot exclude small differences in Cx distribution at different sites in the atrium, changes in the morphology of the atrial myocytes as a result of sustained AF appeared to be the same in the atrial free wall, appendage, trabeculae, interatrial septum and Bachmann’s bundle [23].

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References


