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Effects of Chronic Atrial Fibrillation on Gap Junction Distribution in Human and Rat Atria

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OBJECTIVES To elucidate the structural basis for the electrophysiologic remodeling induced by chronic atrial fibrillation (AF), we investigated connexin40 and connexin43 (Cx40 and Cx43) expression and distribution in atria of patients with and without chronic AF and in an animal model of AF with additional electrophysiologic investigation of anisotropy (ratio of longitudinal and transverse velocities).

BACKGROUND Atrial fibrillation is a common arrhythmia that has a tendency to become persistent. Since gap junctions provide the syncytial properties of the atrium, changes in expression and distribution of intercellular connections may accompany the chronification of AF.

METHODS Atrial tissues isolated from 12 patients in normal sinus rhythm at the time of cardiac surgery and from 12 patients with chronic AF were processed for immunohistology and immunoblotting for the detection of the gap junction proteins. The functional study of the cardiac tissue anisotropy was performed in rat atria in which AF was induced by 24 h of rapid pacing (10 Hz).

RESULTS Immunoblotting revealed that AF did not induce any significant change in Cx43 content in human atria. In contrast, a 2.7-fold increase in expression of Cx40 was observed in AF. Immunohistologic analysis indicated that AF resulted in an increase in the immunostaining of both connexins at the lateral membrane of human atrial cells. A similar spatial redistribution of the Cx43 signal was seen in isolated rat atria with experimentally-induced AF. In addition, AF in rat atria resulted in decreased anisotropy with slightly enhanced transverse conduction velocity.

CONCLUSIONS This experimental study showed that AF is accompanied by spatial remodeling of gap junctions that might induce changes in the biophysical properties of the tissue. (J Am Coll Cardiol 2001;38:883–91) © 2001 by the American College of Cardiology

Atrial fibrillation (AF), a common arrhythmia with an incidence of about 5% in the population aged ≥65 years (1); it has a tendency to become chronic and be more resistant to conversion with increasing duration of the disease (2–6).

Although initiation of AF has been intensively investigated (7–15), theories dealing with determinants of AF do not explain the trend of this arrhythmia to become persistent. In this context, current research has been focused on the electrophysiologic remodeling during chronic AF. Thus, AF leads to shortening of refractory period due to reductions in specific current densities (16,17) (in humans, Refs. 18,19; for review, see Ref. 20), to sinus node dysfunction and to reduced intra-atrial conduction velocity (16). The latter finding could mean a reduced intercellular coupling via gap junctional channels formed by connexins. The major connexins expressed in mammalian myocardium are connexin43 (Cx43) and connexin40 (Cx40) in atrium and conduction system (21,22). Regarding the conduction velocity, the finding of an increased expression of Cx43 but a decreased intra-atrial conduction velocity after 10 to 14 weeks of AF in the dog (23) seems to be somehow paradoxical at first sight. In that study the distribution of Cx40, which is also expressed in the dog atrium, was not determined. Reduced conduction velocity and enhanced Cx43 expression could also mean a change in the distribution pattern of gap junctions. Other investigators (24) showed that, in a goat model of AF, the pattern of Cx40 changed while the Cx43 pattern remained unchanged. The distribution pattern of Cx40 exhibited discontinuities but did not affect conduction velocity. Because data on human AF are still lacking, the present study was undertaken to clarify the clinical significance of gap junction remodeling in AF.

METHODS

Human studies. We investigated patients undergoing cardiac surgery either for correction of a valvular defect or for coronary bypass grafting and who were suffering from chronic AF for at least one year (n = 12; age 70 ± 2 years; body weight 77 ± 5 kg) or exhibiting normal sinus rhythm (SR) (n = 12; age 63 ± 4 years; body weight 63 ± 4 kg).

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Abbreviations and Acronyms

AF	=	atrial fibrillation
anisotropy	=	ratio of longitudinal and transverse velocities (V_L/V_T)
Cx43	=	connexin43
Cx40	=	connexin40
d.U.	=	densitometric units
ECL	=	enhanced chemiluminescence
SDS-PAGE	=	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SR	=	sinus rhythm
V_L	=	longitudinal velocity
V_T	=	transverse velocity

A survey on the heart diseases in both groups is given in Table 1.

Tissue from all patients was excised from the right atrial appendage (with little appertaining atrial tissue) at the time of cardiac surgery, immediately frozen in liquid nitrogen and stored at −80°C. The protocol used for the experiments was approved by the Human Ethical Committee at the University of Cologne.

Animal studies. For simplification, we chose an animal model, the isolated rat atrium, which is known to be electrically coupled mainly if not solely by Cx43 (25). Rats were euthanized by a sharp blow on the neck and by exsanguination. All experiments were carried out according to the German laws for animal welfare. After excision, the right atrium was placed in an organ bath, fixed at both ends, stretched to its in situ length by a micromanipulator under microscopic control and superfused (7.5 ml/min) with Tyrode's solution (Na^+ 161.02; K^+ 5.36; Ca^{++} 1.8; Mg^{++} 1.05; Cl^- 147.86; HCO_3^- 23.8; $H_2PO_4^-$ 0.42; glucose 11 mmol/l pH 7.4, equilibrated with 5% CO_2/95% O_2, 37°C). Three groups of atria were investigated: 1) atria superfused for 24 h beating at their spontaneous rate (n = 18); 2) atria superfused for 24 h and paced via 2 Pt-electrodes at a rate of 10 Hz (double diastolic threshold) (n = 18); and 3) atria investigated directly or frozen in liquid N_2 after excision (n = 18). Fifty-four rats were investigated, 18 in each group. Six atria of each group were used for immunohistochemistry, six for assessing biophysical properties and the remaining six in each group were used for biochemical analysis. During the experiment, action potentials were recorded from the atria using sharp glass microelectrodes (15 to 20 MΩ, filled with 3 mol/l KCl, HSE-microelectrode amplifier; two-channel digital storage oscilloscope; Hugo Sachs Elektronik, Hugstetten, Germany).

Table 1. Survey on the Diagnosis of Heart Disease in Groups of Patients With SR and AF

Diagnosis	SR Group	AF Group
CHD	8	6
Aortic valve defect	2	2
Mitral valve defect	1	3
Combined CHD + valve defect	1	1
Total	12	12

AF = atrial fibrillation; CHD = coronary heart disease; SR = sinus rhythm.

Western blot analysis. Frozen atrial tissue was quickly powdered and solubilized in Laemmli buffer according to classical protocols (26,27). Lysates were centrifuged (13,000 *g*, 10 min at +4°C) and supernatants were saved. For electrophoresis, 20 μg of total protein was loaded and resolved in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred electrically to nitrocellulose enhanced chemiluminescence (ECL)-membranes (Amersham Pharmacia, Buckinghamshire, United Kingdom) using the tank transfer system (BioRad, Munchen, Germany). After blocking (Roti-block, Roth, Germany), membranes were subsequently incubated with primary antibodies and secondary antibodies coupled to peroxidase (Sigma, Deisenhofen, Germany). The ECL detection was performed using SuperSignal reagent (Pierce, Rockford, Illinois).

Monoclonal anti-Cx43 antibody and monoclonal anti-histone-1 antibody were purchased from Chemicon (Temecula, California). A polyclonal antibody was produced against a polypeptide comprising amino acids 231–330 of rat Cx40. This antibody did not exhibit cross-reactivity with other connexins and recognized a single protein band migrating at 40 kDa in lysates of freshly isolated adult rat ventricular cardiomyocytes, HeLa cells transfected with Cx40 and endothelial cells of vessels (28,29).

Densitometric analysis. Immunoblots were exposed to X-ray film, scanned (Twain, Tualatin, Oregon), and analyzed using TINA software (Isotopenmessgeraete GmbH, Berlin, Germany). The gray scale values of histone-1 signals were compared among patients to determine whether equal amounts of cellular proteins were detected. Histone-1 expression did not differ significantly among patients: SR: 839 ± 15 densitometric units [d.U.]; AF: 850 ± 13 d.U. Subsequently, ratios between gray scale values of the connexin signals and those of the histone-1 signals were calculated to adjust for possible differences in the myocytic protein content between the samples. Data from patients with AF were normalized to those from patients with SR, which were set to 100%. The results are represented as mean ± SEM of n = 6 in every group.

Immunohistochemistry. Indirect immunofluorescence was performed on 10-μm cryosections cut in parallel to the fiber's longitudinal axis. The specimens were fixed with methanol (30 min, 4°C), blocked with phosphate-buffered bovine serum albumin solution (20 min at room temperature); composition: 8 g, NaCl; 0.2 g, KCl; 1.15 g, Na_2HPO_4; 0.2 g, KH_2PO_4; 1 g, BSA in 1 l H_2O; pH 7.5), incubated with the primary antibodies overnight (4°C) and subsequently exposed to fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Sigma). Finally, the slides were washed with phosphate-buffered saline and embedded in Karyon (Merck, Darmstadt, Germany).

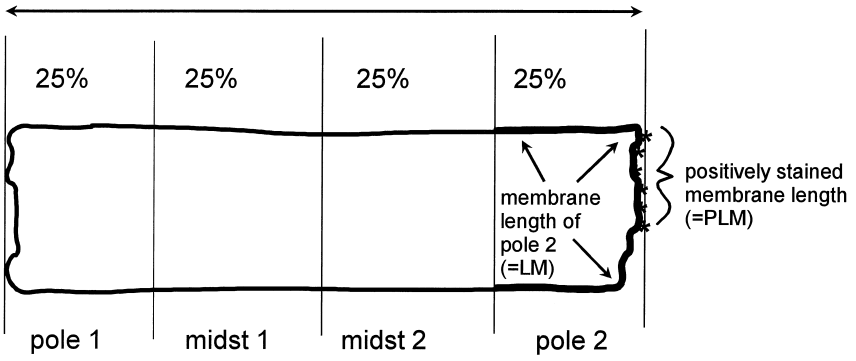

Figure 1. Scheme of the cell sections as used for the analysis. For description, see text.

Immunofluorescent data processing and analysis. The slides were investigated at 1,000× magnification using a commercial image analysis system (JAVA, Jandel Scientific, Erkrath, Germany) and a Zeiss Axiolab fluorescence microscope. For evaluation, only cells cut longitudinally were analyzed: each cell was divided into four areas (Fig. 1); the longitudinal cell axis was determined and divided into four sections of equal length, giving four areas: the left and right cell pole and the two mid-areas as depicted. We measured the length of the plasma membrane of each section and the length of the immunofluorescence-positive membrane of the section, and we calculated the ratio between positively stained membrane length and plasma membrane length.

Twelve human (6 per group) and 18 rat (6 per group) specimens were investigated by immunostaining. Five slide preparations per specimen and about 20 cells per slide were analyzed (i.e., 600 cells in each group).

Biophysical measurements. We also investigated anisotropy (the ratio of longitudinal and transverse velocities $[V_L/V_T]$ in the fiber direction, assessed according to Delmar et al. [30]) of atria beating at their spontaneous rate for 24 h and of atria subjected to stimulation at 10 Hz for 24 h. Atria were transferred to an organ bath and superfused with Tyrode's solution. Only those specimens in which fiber orientation could be clearly identified by microscopical anatomical inspection were used. An electrode array of four platinum electrodes oriented either longitudinally or transversely to the fiber axis was positioned on the atria. For final positioning in longitudinal direction, we searched the electrode position where the fastest conduction velocity could be recorded. Transverse direction was defined as orthogonal to the longitudinal axis. Atria were stimulated at the edge with rectangular pulses (10 Hz, Stimulator T, HSE-Elektronik, Hugstetten, Germany). Monophasic action potentials were recorded 3 mm apart from the stimulation site in both the transverse and longitudinal direction (Signal Memory Recorder, Kontron, Duesseldorf, Germany).

Statistics. Statistical analysis was performed using two-way analysis of variance with subsequent Student *t* test for paired and unpaired observations employing Bonferroni corrections for multiple comparisons on a level of significance of $p < 0.05$. In total, 54 rats and 24 humans were investigated, and all values are given as mean ± SEM of $n = 6$ in every experimental subgroup (or for immunohistology of 600 cells per group; see the preceding text).

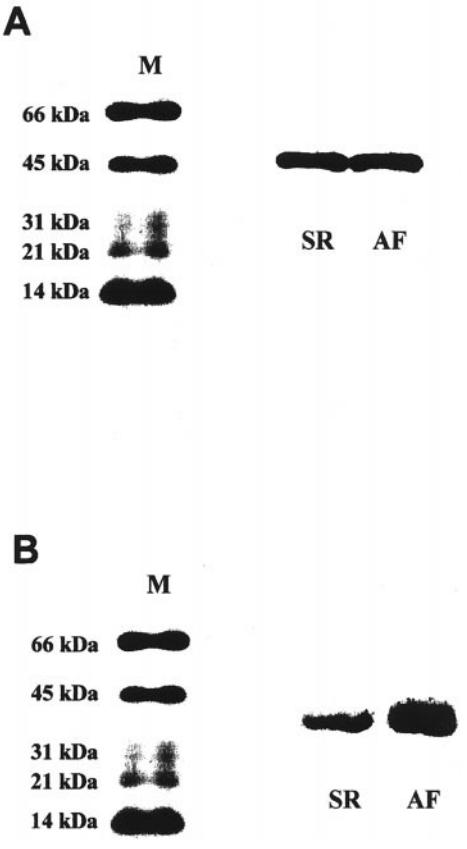

Figure 2. Atrial fibrillation (AF) and the connexin expression. **(A)** Western blots of human atrial tissue lysates with monoclonal antibody raised against residues 252–271 of rat connexin43. A specific band migrating at 45 kDa is detected in the lysates prepared from atria of patients with sinus rhythm (SR) and with atrial fibrillation (AF). **(B)** Western blots of cardiac lysates with anti-connexin40 antibody. The antibody recognized a single band migrating at 40 kDa in lysates prepared from atria of patients with SR and with AF.

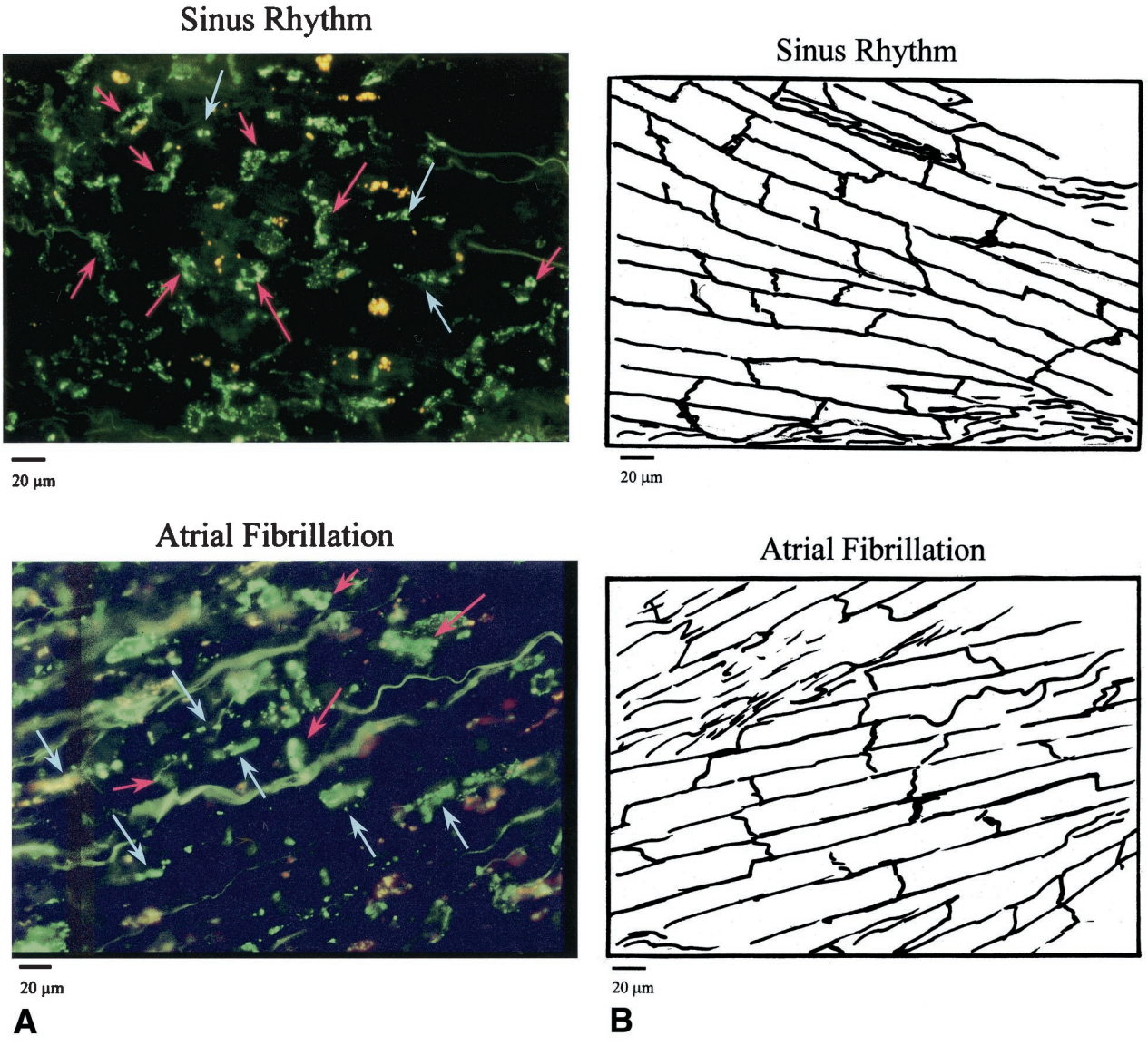

Figure 3. Atrial fibrillation alters the membrane distribution of connexins. Immunostaining for connexin43 (Cx43) **(A,B)** and connexin40 (Cx40) **(C,D)** in atrial tissue from patients with sinus rhythm **(upper parts)** and from patients with chronic atrial fibrillation **(lower parts).** The specific connexin signals either for Cx43 or for Cx40 are shown in **green.** The **red arrows** indicate the immunopositive *cell poles,* whereas the **light blue arrows** point to the *lateral* immunostaining. Diagrammatic representations of the corresponding tissue sections are shown on the **right side.** The **yellowish stainings** indicate deposition of lipofuscin, which is typical for human atrial tissue of adult or aged patients.

RESULTS

Human studies. Western blot analysis for Cx43 and Cx40 expression in atrial tissue of chronic AF patients and patients with SR demonstrated that AF did not evoke significant changes in Cx43 expression level: it was 101 ± 3% of that in atrial tissue of patients with SR. In contrast, Cx40 content did increase in the right atrial appendage of patients with AF up to 277 ± 56% in comparison with those who had SR (Fig. 2A and B).

Morphometric analysis of human atrial cryosections did not reveal significant differences in cardiac cell size among patients with SR (17 ± 8.5 mm width; 81 ± 17 mm length) and AF (20 ± 8 mm width; 85 ± 18.3 mm length).

Immunohistologic analysis of cryosections of atrial tissue immunostained for either Cx40 or Cx43 showed that AF induced changes in the pattern of the Cx43 membrane distribution (Fig. 3A and B). Statistical analysis (600 cells per group) revealed that 48 ± 2% of the *polar* contact membrane and only 5 ± 1% of the *lateral* contact membrane stained positively for Cx43 in cardiomyocytes of patients with SR; in contrast, in patients with AF 53 ± 3% (not significant [NS]/ vs. SR) of the *polar* membrane but 16 ± 3% ($p < 0.05$ vs. SR) of the *lateral* membrane of the cells were found to be immunopositive for Cx43. Thus, *lateral* Cx43 immunostaining was significantly enhanced in AF, whereas *polar* staining was unchanged.

Regarding Cx40 distribution, AF provoked similar changes (Fig. 3C and D): 82 ± 2% of the *polar* contact

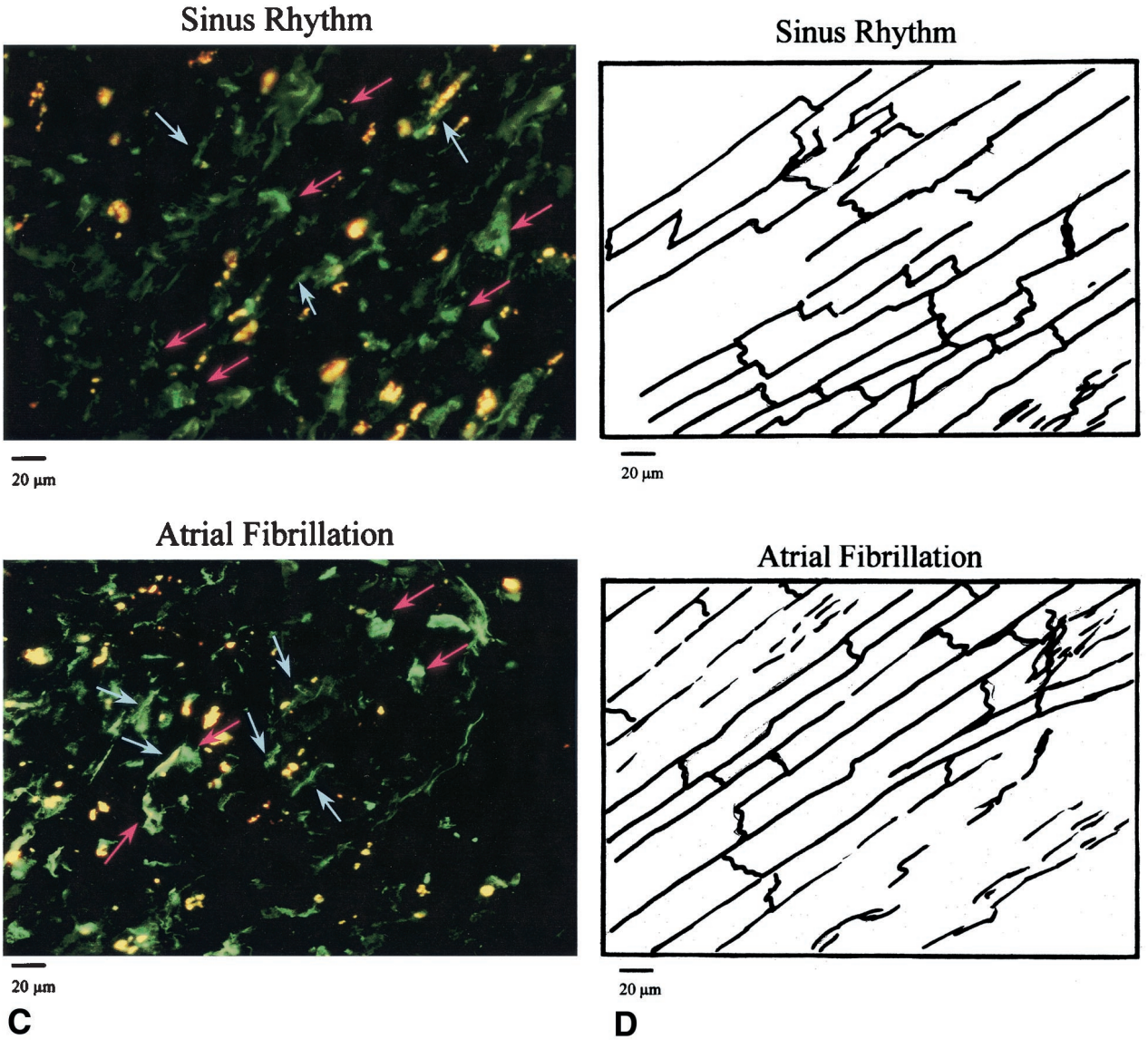

Figure 3. Continued.

membrane and 9 ± 1% of the *lateral* contact membrane were immunopositive for Cx40 in cardiomyocytes of patients with SR, whereas 79 ± 2% (NS vs. SR) of the *polar* membrane and 22 ± 2% ($p < 0.05$ vs. SR) of the *lateral* membrane were positively immunostained for Cx40 in cells of patients with AF. Hence, AF induced changes in the expression and in the spatial organization of Cx40 gap junctions.

In general, we found a significant influence of AF on lateralization of Cx43 and Cx40 ($p < 0.05$), with both connexins exhibiting similar changes. In contrast, in Western blots we found that only Cx40 exhibited significantly enhanced expression while Cx43 remained unchanged. A significant interaction occurred between the factor AF and the connexin-isotype expression ($p < 0.05$), suggesting a differential influence of AF on Cx40 expression.

Animal studies. Twenty-four hours of 10-Hz pacing induced fibrillation in all atria subjected to this treatment. Atria first responded in a 1:1 manner to the stimuli (Fig. 4, third line), while within the course of the experiment, the rhythm degenerated into fibrillation. Fibrillation persisted for 2 to 5 min after interruption of the stimulation at the end of the 24 h (Fig. 4, fourth line). Recovery to regular rhythm took 12 ± 1 min (Fig. 4, last line). Fibrillation was not observed in spontaneously beating atria.

Immunohistology revealed that in normal atria (i.e., either directly frozen or kept at their intrinsic beating rate) Cx43 was confined to the cell poles in a highly regular manner, whereas at the lateral cell border only very small expression of Cx43 was found. Quantitative image analysis (600 cells per group) demonstrated that in atria immediately frozen, 41 ± 4% of the membrane at the *cell pole* stained positively for Cx43, whereas only 1.5 ± 0.1% of the *lateral* membrane showed positive immunofluorescence. Very similar findings were obtained for the atria that remained for 24 h in the bath at their spontaneous beating rate.

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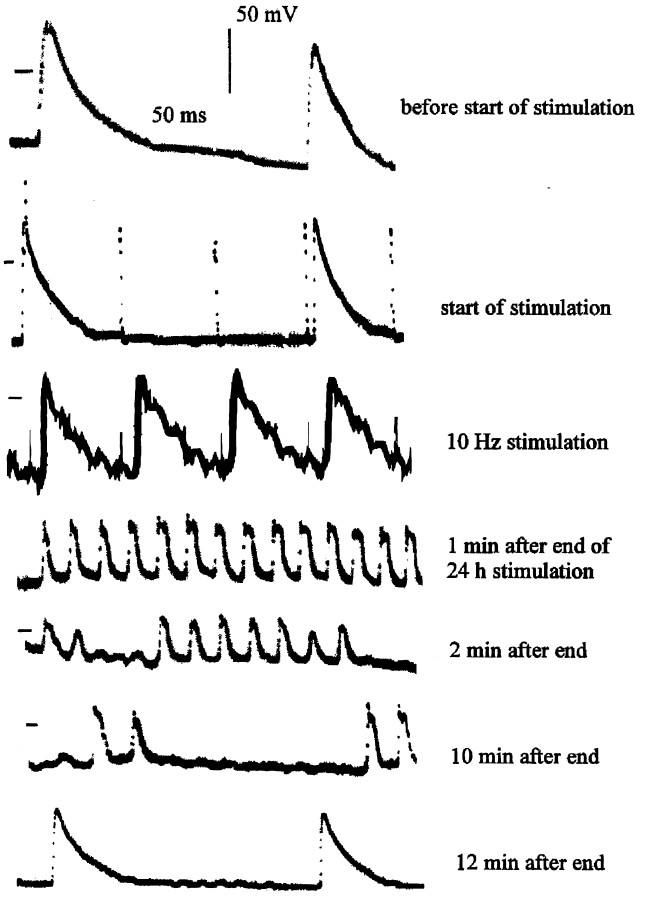

Figure 4. Original recordings of transmembrane action potentials measured with 15-MΩ sharp glass microelectrodes (filled with 3 mol/l KCl) from rat atrium before, during (10 h of rapid pacing) and after the end of 24 h of 10-Hz stimulation. The traces are photographically reproduced from the original oscilloscope signals.

In contrast, in atria rapidly paced for 24 h, the distribution of Cx43 became irregular and the staining was similar at the *polar* membrane and at the *lateral* membrane of the cells: 54 ± 9.5% of the *polar* (NS vs. control) and 61 ± 12% ($p < 0.05$ vs. control) of the *lateral* membrane stained positively for Cx43 (Fig. 5). Rapidly paced atria differed significantly from both other groups (directly frozen and 24 h at intrinsic rate).

Total membrane protein content was not significantly different among the three groups: 398 μg/ml (24 h at intrinsic rate; 345 to 520 μg/ml), 496 μg/ml (10-Hz paced atria; 226 to 570 μg/ml) or 432 μg/ml (directly frozen; 322 to 670 μg/ml). Immunoblotting for Cx43 revealed 54 ± 5 d.U. for atria directly frozen, 37 ± 11 d.U. for atria incubated in the organ bath without pacing and 39 ± 6 d.U. for atria rapidly paced for 24 h. Thus, as in humans, no change occurred in the Cx43 membrane content induced by AF, but did occur in the distribution pattern.

For investigation of a possible functional relevance of these findings, we measured the biophysical properties of the tissue. The ratio between longitudinal and transverse conduction velocity was 3.6 ± 0.5 for specimens directly

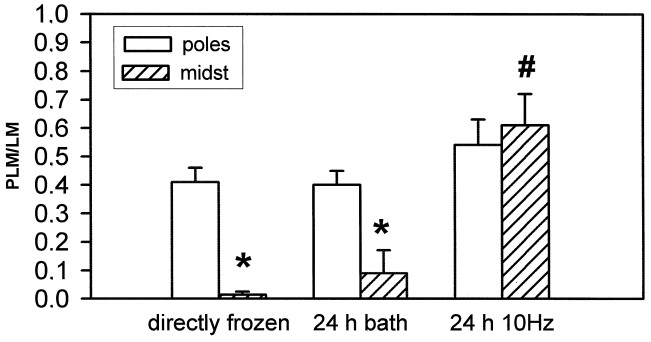

Figure 5. Effect of the induced atrial fibrillation on the connexin43 (Cx43) distribution in the membrane of rat atrial cardiomyocytes. Ratio of the membrane length positively stained (PLM) for Cx43 as referred to the total membrane length (LM) of the *polar* membrane and of the *lateral* membrane. The results are represented as mean ± SEM of 600 cells per experimental group. Significant difference between the staining of the *polar* membrane and the *lateral* membrane is indicated by an **asterisk**; significant difference against the directly frozen control group is indicated by the **pound symbol** (#) (level of significance $p < 0.05$).

investigated or kept for 24 h at their spontaneous rate (longitudinal velocity [V_L]: 0.18 ± 0.03 m/s; transverse velocity [V_T]: 0.05 ± 0.018 m/s), whereas this ratio was significantly diminished to 2.38 ± 0.1 (V_L: 0.19 ± 0.03 m/s was not significantly altered; V_T: 0.08 ± 0.017 m/s was slightly enhanced; $p = 0.1$) in specimens that underwent rapid electrical pacing for 24 h (Fig. 6). Thus, the altered connexin distribution was in parallel to a change in anisotropy.

DISCUSSION

Human study. Connexins usually localize in the end-to-end junctional complexes (as seen in SR in our study) forming low-resistance pathways for propagation of electrical impulses between cardiomyocytes in atria and atrial appendages (31,32). Our main finding in humans is that the pattern of Cx43 distribution markedly changed in chronic AF: enhanced amount of the protein became incorporated into the sites of lateral contacts between cardiomyocytes. Because Western blot analysis revealed no change in the amount of Cx43, this dramatic change in the subcellular distribution does not seem to be attributable to an elevated protein expression.

In contrast to the unchanged Cx43 expression, the amount of Cx40 was elevated in AF. Immunohistology revealed the enhanced Cx40 signal at the lateral membrane of the atrial cardiomyocytes in AF. Therefore, the observed increase in Cx40 may be linked to the development of the additional sites of lateral cell-cell contacts. Contrary to our finding, a decreased content of Cx40 during sustained AF was shown in the goat (24). The discrepancy to our finding in humans could be due to the species difference and to the fact that the study (24) was performed within the first 16 weeks of sustained pacing-induced AF in goats, which might be difficult to compare with changes induced by chronic AF (≥1 year) in humans.

In addition, these investigators (24) also described in-

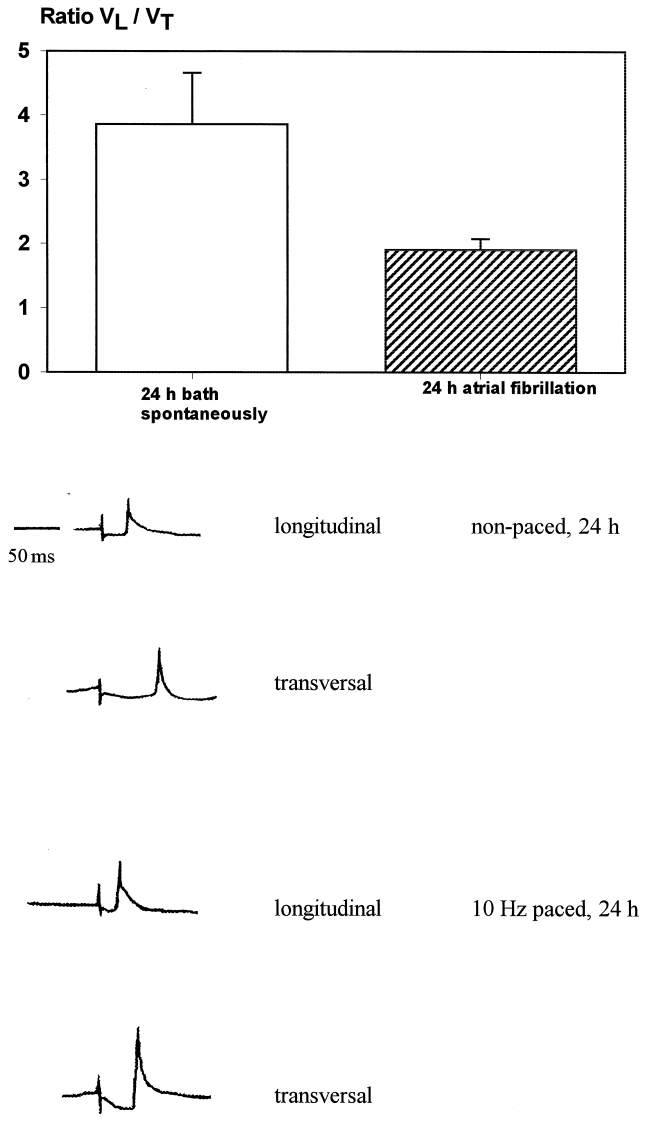

Figure 6. Atrial fibrillation increases the transverse conduction velocity in isolated rat atria. Original traces showing the stimulus and the response for transverse and longitudinal conduction are given. Ratios of the longitudinal and transverse conduction velocities (V_L/V_T) measured after 24 h in the bath (at the spontaneous beating rate) and after 24 h atrial fibrillation are given as mean ± SEM in the **upper panel.** The difference was significant ($p < 0.05$).

creased heterogeneity of the Cx40 distribution concomitantly with the duration of AF. This emphasizes the importance of our finding of the AF-induced alteration in the spatial organization of gap junctions. However, enhanced immunostaining alone does not mean the presence of functional channels; it is only a detection of protein. Thus, if the lateral Cx43 and Cx40 gap junction channels do participate in the propagation of electrical impulses, the changed pattern of intercellular channels should cause an alteration in the biophysical properties of the tissue. This question of the functional relevance of the connexin reorganization was investigated in the rat model.

Animal study. The rat was chosen to provide a simplified model with only Cx43 as the main (if not the sole) connexin (25). However, this simplification might circumvent possible Cx43/Cx40 interactions in human atria. Conversely, this rat model provided the definite system to observe the direct effect of AF on the connexin distribution irrespective of complex interactions between connexins. The important result is that pacing-induced arrhythmia caused significant changes in the intracellular distribution of Cx43, with enhanced connexin staining at the lateral cell border in fibrillating but not in nonfibrillating rat atria similar to our finding in humans. These changes in Cx43 distribution are reflected by altered biophysical properties of the tissue (altered anisotropy with a somewhat enhanced transversal conduction velocity; Fig. 6). That might indicate that more Cx43 channels are incorporated into the lateral membrane or that the same amount of Cx43 is incorporated in a more dispersive fashion as is consistent with our immunohistological finding.

Immunoblot analysis shows that Cx43 membrane content was not changed in AF in the rat. Recently, Elvan et al. (23) reported on enhanced expression of Cx43 in dog atria. In contrast to our study, these investigators induced AF for 10 to 14 weeks in vivo. Thus, besides species differences, the duration of AF might influence the induction of enhanced gene expression and protein synthesis. In addition, because the atria were kept in saline and not in culture medium (to avoid foaming), enhanced protein synthesis might be difficult to observe. Because of the small amount of Cx43 obtainable from rat atrium, small changes probably cannot be detected. However, other processes such as altered insertion of Cx43 into the membrane or alteration of the hemichannel docking between connecting cells might also contribute to the changes in Cx43 pattern.

Cardiac connexins have a half-life of ~1.6 h (33,34), indicating a considerably rapid turnover (35). Valiunas et al. (36) showed a formation rate of gap junctional channels of 1.3 channels/min. Thus, the contact between cells as formed by gap junctions is not static but dynamic. With regard to the short half-life time of these channels, a period of 24 h appears to be long enough to allow for the observed changes to occur. However, it is uncertain how exactly the cell regulates the location for insertion of new channels and whether this may be affected by pathological conditions. Both N-cadherins and Ca^{++} might play a role in that process (37,38).

According to our results, regulation of this integration process of Cx43 seems to be disturbed by the induced arrhythmia. Because these channels represent low-resistance pathways, this changed pattern of intercellular channels may alter the tissue's biophysical properties. This assumption was verified by our finding that anisotropy was reduced by AF. The values found for longitudinal and transverse conduction velocity accord well with those reported (39,40).

Anisotropy is a characteristic feature of cardiac tissue. Generally, conduction is faster along the fiber axis than transverse to it. This is considered to be the consequence of the length-to-width ratio of the fibers and of the distribution of the gap junction channels (31,41). These channels

are mainly found at the cell poles—that is, at the intercalated disks interconnecting the ends of the myocytes—and only sparsely at the lateral borders, which has been interpreted as the basis of the tissue's anisotropy (41–44).

If anisotropy is decreased, pathways of activation may change. Thus, a more uniform distribution of the gap junctional channels around the cell (as seen here) should decrease the difference in propagation velocity both parallel and transverse to the fiber, which became evident from our experiments, so that the propagation pattern of the activation wave will be changed. This situation would allow multiple wavelets to occur simultaneously and to propagate with continuously changing pathways as described (7,45,46). Even increased expression of Cx43 as observed in dogs (23) could result in decreased conduction velocity (as described by Elvan et al. [23]), provided that similar changes in the intracellular distribution of Cx43 with a concomitant change in anisotropy would occur.

Because 24 h of rapid pacing alters the biophysical structure of the tissue with regard to low-resistance pathways, this subacute change in the intercellular network may stabilize the arrhythmia by forming an arrhythmogenic substrate and may contribute to the chronicity of AF in addition to other factors such as reduced refractory period, changed expression of potassium channels (47) and structural remodeling.

In summary, our study shows that arrhythmia can induce a change in the electrical network, and thus may contribute to the formation of the arrhythmogenic substrate together with electrophysiologic remodeling described by others (16–18,23,48,49).

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