Ultrastructural and Functional Remodeling of the Coupling Between Ca\textsuperscript{2+} Influx and Sarcoplasmic Reticulum Ca\textsuperscript{2+} Release in Right Atrial Myocytes From Experimental Persistent Atrial Fibrillation

Ilse Lenaerts, Virginie Bito, Frank R. Heinzel, Ronald B. Driesen, Patricia Holemans, Jan D’hooge, Hein Heidbüchel, Karin R. Sipido, Rik Willems

Rationale: Persistent atrial fibrillation (AF) has been associated with structural and electric remodeling and reduced contractile function.

Objective: To unravel mechanisms underlying reduced sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release in persistent AF.

Methods: We studied cell shortening, membrane currents, and [Ca\textsuperscript{2+}]\textsubscript{i}, in right atrial myocytes isolated from sheep with persistent AF (duration 129±39 days, N=16), compared to matched control animals (N=21). T-tubule density, ryanodine receptor (RyR) distribution, and local [Ca\textsuperscript{2+}]\textsubscript{i} transients were examined in confocal imaging.

Results: Myocyte shortening and underlying [Ca\textsuperscript{2+}]\textsubscript{i} transients were profoundly reduced in AF (by 54.8% and 62%, P<0.01). This reduced cell shortening could be corrected by increasing [Ca\textsuperscript{2+}]\textsubscript{i}. SR Ca\textsuperscript{2+} content was not different. Calculated fractional SR Ca\textsuperscript{2+} release was reduced in AF (by 20.6%, P<0.05). Peak Ca\textsuperscript{2+} current density was modestly decreased (by 23.9%, P<0.01). T-tubules were present in the control atrial myocytes at low density and strongly reduced in AF (by 45%, P<0.01), whereas the regular distribution of RyR was unchanged. Synchrony of SR Ca\textsuperscript{2+} release in AF was significantly reduced with increased areas of delayed Ca\textsuperscript{2+} release. Propagation between RyR was unaffected but Ca\textsuperscript{2+} release at subsarcolemmal sites was reduced. Rate of Ca\textsuperscript{2+} extrusion by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was increased.

Conclusions: In persistent AF, reduced SR Ca\textsuperscript{2+} release despite preserved SR Ca\textsuperscript{2+} content is a major factor in contractile dysfunction. Fewer Ca\textsuperscript{2+} channel–RyR couplings and reduced efficiency of the coupling at subsarcolemmal sites, possibly related to increased Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, underlie the reduction in Ca\textsuperscript{2+} release. (Circ Res. 2009;105:00-00.)

Key Words: atrial fibrillation ■ atrial myocytes ■ sarcoplasmic reticulum ■ Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange ■ ryanodine receptor ■ T-tubules

Atrial fibrillation (AF) is the most common arrhythmia with a lifetime risk of developing AF of 1 out of 4 in people older than 40 years.\textsuperscript{1} AF is associated with a significant morbidity. Stroke resulting from embolization of atrial thrombi is the major determinant of this morbidity,\textsuperscript{2} with loss of atrial contractility as major cause of thrombus formation. The atrial mechanical dysfunction can persist for more than one month after cardioversion to sinus rhythm in patients in persistent AF, a phenomenon called “atrial stunning.”\textsuperscript{3} The contractile dysfunction is due to remodeling of the muscle. Contractile force of atrial tissue strips from patients with AF was reduced by 75%.\textsuperscript{4} Exposure to high extracellular Ca\textsuperscript{2+} restored atrial function, implicating a reduced Ca\textsuperscript{2+} availability. Studies on animal models and human myocytes isolated from atrial appendages removed at the time of surgery identified a loss of Ca\textsuperscript{2+} current density as a hallmark of AF.\textsuperscript{5–8} A decrease in mRNA and protein expression of the L-type Ca\textsuperscript{2+} channel, as well as modulation by phosphorylation and redox potential, contributes to the decrease in Ca\textsuperscript{2+} current density.\textsuperscript{9–12} A decrease in the trigger for the Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) can be a major factor in the contractile dysfunction in AF but may not fully explain reduced contractility.\textsuperscript{13} Ca\textsuperscript{2+} release and propagation also depend on properties of the ryanodine receptor (RyR), the extent of a T-tubular system, buffering of Ca\textsuperscript{2+} by mitochondria, sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA), or
myofilaments, and on the SR Ca\(^{2+}\) content. Hyperphosphorylation of RyR and increased SR Ca\(^{2+}\) leak have been implicated in an increased spontaneous Ca\(^{2+}\) release from the SR in AF.\(^{14,15}\) Whether the Ca\(^{2+}\) channel–RyR coupling is intact or whether changes contribute to the contractile dysfunction in chronic AF remains uncertain. Although ideally one would like to study human atrial cells to identify the pathophysiological mechanisms of AF, this approach is limited by the many confounding factors in patient studies, such as sex, age, medication, and concurrent disease. The nature of AF, requiring a substantial mass of cardiac tissue, has therefore early on prompted researchers to use large animals, like dogs or goats for experimental studies.\(^{5,16}\) A prime requirement for these models is that they approach as closely as possible the human condition. Several clinically relevant aspects, such as the time course and nature of the electric remodeling or the link to heart failure, have been successfully implemented in these models, but AF is mostly short-lived. In humans, alterations in gene expression of proteins involved in Ca\(^{2+}\) homeostasis are only observed in patients with persistent AF and not with paroxysmal AF.\(^{17}\) We therefore used a sheep model of persistent AF\(^{18,19}\) to further investigate the mechanisms of atrial contractile remodeling during chronic AF. We focus on the relation between Ca\(^{2+}\) and contraction, on alterations in SR Ca\(^{2+}\) release and the underlying mechanisms.

**Methods**

**Animal Characteristics and Instrumentation**

A detailed description is provided in the Online Data Supplement, available at http://circres.ahajournals.org. Figure 1 summarizes the salient features of the sheep model for persistent AF induced by long-term intraatrial pacing.\(^{18,19}\) At regular time intervals, pacing was interrupted to assess the underlying rhythm (Figure 1A and 1B). The sheep were considered to be in persistent AF if no sinus rhythm could be demonstrated anymore in the follow-up period. Electric remodeling was present, with shortening of the atrial effective refractory period in vivo and shortening of the action potential in vitro (from 111 ± 6 to 84 ± 12 ms at 75% repolarization; stimulation rate, 1 Hz; AF, n = 6; versus control, n = 19; P < 0.05; Figure 1C) with

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**Figure 1.** Sheep model for permanent atrial fibrillation. A and B, Peripheral ECG and intracardial electrograms during rapid atrial pacing at 15 Hz and after interrupting (arrow). At baseline, sinus rhythm reappeared (A), but after 6 weeks of pacing, AF persisted (B). C, Multicellular action potential recordings in CTRL and AF atrial strips at 1 Hz. D and E, Persistent AF results in mild left ventricular dysfunction at 18 weeks.
Results

Decreased Cell Contraction and [Ca^{2+}]\text{i}, Transients in AF During Field Stimulation

Figure 2A (a) shows typical recordings of cell shortening (ΔL/L₀) in control (CTRL) and AF. In AF cells, fractional shortening was significantly decreased, on average by 54.8% (Figure 2A, b). Time to peak of contraction was comparable within the groups (Figure 2A, c), but the rate of relaxation, evaluated from the time constant of the exponential decline, was significantly decreased in AF cells (Figure 2A, d). Figure 2B (a) shows typical examples of [Ca^{2+}]\text{i}, transients. The amplitude was significantly decreased in the AF group (Figure 2B, b). Time to peak was increased in AF (Figure 2B, c), without change in relaxation rate (Figure 2B, d).

The data suggest that the reduced [Ca^{2+}]\text{i}, transient could be responsible for the observed depressed cell contraction. Therefore contraction would potentially normalize with a larger [Ca^{2+}]\text{i}, transient as during maximal SR Ca^{2+} release obtained during a 10 mmol/L caffeine application. The amplitude of the [Ca^{2+}]\text{i}, transient during caffeine application was 2- to 3-fold larger than during a depolarizing step and was not significantly different between AF and CTRL (Figure 2C). With this large [Ca^{2+}]\text{i}, transient, cell shortening was increased 4- to 5-fold and not different between the 2 groups. These data indicate that a defect in Ca^{2+} handling is a major factor in the reduced contraction of atrial myocytes from persistent AF. They also suggest that the amount of Ca^{2+} available in the SR is not decreased in AF myocytes. We therefore further examined the mechanisms underlying the reduced SR Ca^{2+} release during depolarization.

Decreased L-type Ca^{2+} Current Density and Underlying Mechanisms

[Ca^{2+}]\text{i}, transients evoked during voltage clamp to different levels had a similar bell-shaped voltage dependence in AF as in CTRL, indicating that I_{Ca,L} is the main trigger for SR Ca^{2+}...
release (Figure 3A). The amplitude was however profoundly reduced in AF; \( I_{\text{CaL}} \) was also significantly reduced in AF, but the difference was small (Figure 3B).

When we used a pipette solution with high EGTA to record \( I_{\text{CaL}} \), this difference was much larger (Figure 3C) and the voltage dependence of inactivation was shifted to the right (Figure 3D), as was the voltage dependence of activation (Figure 3E). Isoproterenol increased \( I_{\text{CaL}} \) in both groups with a trend to larger increase in AF; using forskolin to bypass possible changes in \( \beta \)-receptor density, the response in AF was much larger than in CTRL (Figure 3F). Protein expression of \( \alpha_{1c} \) subunit of L-type \( \text{Ca}^{2+} \) channel, measured by immunoblotting in left atrium (LA) tissue, was significantly decreased in the AF group by 68% (Online Figure I, A).

**Figure 3.** Decreased L-type \( \text{Ca}^{2+} \) current density and underlying mechanisms. A, Voltage dependence of \([\text{Ca}^{2+}]_i\) transients (CTRL, \( n=24; \) AF, \( n=17 \)). B, Current–voltage relation of \( I_{\text{CaL}} \) without intracellular \( \text{Ca}^{2+} \) buffering (CTRL, \( n=42; \) AF, \( n=37 \)). C, Current–voltage relation of \( I_{\text{CaL}} \), with 10 mmol/L EGTA in the pipette (CTRL, \( n=21; \) AF, \( n=11 \)). D, Voltage dependence of inactivation (CTRL \( n=18; \) AF \( n=7 \)). E, Activation curve in CTRL (\( n=21 \)) and AF (\( n=11 \)) cells. F, Relative increase of peak \( I_{\text{CaL}} \) in response to 3 \( \mu \text{mol/L} \) isoproterenol and 10 \( \mu \text{mol/L} \) forskolin. * \( P<0.05 \).

**Reduced Coupling Between Sarcolemmal \( \text{Ca}^{2+} \) Influx and SR \( \text{Ca}^{2+} \) Release**

The data of Figure 2C suggest that SR \( \text{Ca}^{2+} \) content was not changed in AF. This was further quantified from the integrated \( \text{Na}^{+}/\text{Ca}^{2+} \) exchanger (NCX) current (\( I_{\text{NCX}} \)) during fast application of 10 mmol/L caffeine, as illustrated in Figure 4A (a). The integrated current reflecting SR \( \text{Ca}^{2+} \) content was similar in both
groups. Peak [Ca\(^{2+}\)]\(_i\) of the caffeine-induced transient was also not different (Figure 4A, b), although there was a trend to a decrease, possibly related to slower kinetics of release, as evidenced by the increased time to peak (TtP) (c) in CTRL (n=32) and AF (n=31) cells. B, a, Examples of [Ca\(^{2+}\)]\(_i\) transient measured during a pulse to +10 mV, followed by a fast caffeine application and fractional release of [Ca\(^{2+}\)]\(_i\), (ratio of peak [Ca\(^{2+}\)]\(_i\) of the pulse to peak [Ca\(^{2+}\)]\(_i\) of the caffeine-induced transient) in CTRL (n=32) and AF (n=30) cells. C, a, Tau for exponential fit of the decline of the caffeine-evoked [Ca\(^{2+}\)]\(_i\) transient in CTRL (n=32) and AF (n=31) cells. C, b, Mean peak I\(_{\text{NCX}}\) normalized to cell capacitance in CTRL (n=38) and AF (n=30) cells. D, a, Averaged phase-plane plots for I\(_{\text{NCX}}\) vs [Ca\(^{2+}\)]\(_i\) in CTRL (n=26) and AF (n=25) and slope of the fitted curves. D, b, Mean signal density of the NCX immunoblot in right atrium, normalized to the expression levels of GAPDH and expressed as fraction of CTRL (CTRL n=4, AF n=4). *P<0.05.

**Figure 4.** SR Ca\(^{2+}\) content and release. A, a, Examples of NCX current obtained during 10 mmol/L caffeine fast application and calculated SR Ca\(^{2+}\) content in CTRL (n=40) and AF (n=32) cells. A, b and c, Amplitude of the caffeine-evoked [Ca\(^{2+}\)]\(_i\) transient (b) and time to peak (TtP) (c) in CTRL (n=32) and AF (n=31) cells. B, a, Examples of [Ca\(^{2+}\)]\(_i\) transient measured during a pulse to +10 mV, followed by a fast caffeine application and fractional release of [Ca\(^{2+}\)]\(_i\) (ratio of peak [Ca\(^{2+}\)]\(_i\) of the pulse to peak [Ca\(^{2+}\)]\(_i\) of the caffeine-induced transient) in CTRL (n=32) and AF (n=30) cells. C, a, Tau for exponential fit of the decline of the caffeine-evoked [Ca\(^{2+}\)]\(_i\) transient in CTRL (n=32) and AF (n=31) cells. C, b, Mean peak I\(_{\text{NCX}}\) normalized to cell capacitance in CTRL (n=38) and AF (n=30) cells. D, a, Averaged phase-plane plots for I\(_{\text{NCX}}\) vs [Ca\(^{2+}\)]\(_i\) in CTRL (n=26) and AF (n=25) and slope of the fitted curves. D, b, Mean signal density of the NCX immunoblot in right atrium, normalized to the expression levels of GAPDH and expressed as fraction of CTRL (CTRL n=4, AF n=4). *P<0.05.

Increased Activity of the NCX in AF

The relaxation of the caffeine-induced [Ca\(^{2+}\)]\(_i\) transient in myocytes from AF was increased (Figure 4C, a), and peak I\(_{\text{NCX}}\) density was significantly increased as well (Figure 4C, b). To allow for differences in [Ca\(^{2+}\)]\(_i\), we plotted I\(_{\text{NCX}}\) density as a function of [Ca\(^{2+}\)]\(_i\) (Figure 4D, a). With AF, the loop shifted downwards and the slope calculated during the decline of the [Ca\(^{2+}\)]\(_i\) transient was steeper in the AF group, confirming an increased NCX activity. Immunoblot analysis showed a higher protein expression in AF (Figure 4D, b). Protein levels of total SERCA, phospholamban, and Ser16-phosphorylated phospholamban were unchanged (Online Figures I through III).

**Structural Changes in the Coupling Between L-type Ca\(^{2+}\) Channel and RyR**

Atrial cells from small mammals have no or only a rudimentary T-tubular system,\(^{20,21}\) but this may be different in larger mammals.\(^{22}\) The reduction in L-type Ca\(^{2+}\) current density and α1c protein expression could reflect a decrease in T-tubule surface, where L-type Ca\(^{2+}\) channels are mainly located.
After staining the cells with di-8-ANEPPS, confocal images clearly identified tubular structures in a pattern much like that of ventricular myocytes but at lower density (Figure 5A; Online Figure IV). Quantification confirmed that the density was much lower than in ventricular cells obtained from the same sheep (Online Figure V). There was a marked reduction in T-tubule density in the AF group to 44.5% of control levels (Figure 5B). Fourier analysis also indicated a loss of T-tubule organization (Online Figure VI). Decrease in atrial T-tubule density was accompanied, however, by an increase in cell capacitance (AF: 135±9 pF, n=32; versus CTRL: 84±4 pF, n=38; P<0.05), suggesting an increase in cell size. Cell volumes were indeed almost 3-fold larger in AF (Figure 5C, a) with reduced surface-to-volume (S/V) ratio, consistent with loss of T-tubules (Figure 5C, b). Independent measurement of the morphology of isolated atrial myocytes confirmed the cell hypertrophy (cell length and width were, respectively, 188±9 and 26±1 μm in AF versus 140±5 and 18±1 μm in CTRL; P<0.05). Sarcomere length was comparable between both groups (AF: 1.87±0.04 μm; versus CTRL: 1.81±0.08 μm).

Overall RyR distribution with immunofluorescent labeling in isolated myocytes appeared normal (Figure 5D), although density showed a tendency to decrease in AF. An analysis of RyR distribution by measuring the variance of signal density in a grid analysis23 showed no differences between AF and CTRL. Immunoblotting showed a small but significant reduction of RyR expression (Online Figure I, C), but inositol 1,4,5-triphosphate receptor was unchanged (Online Figure III).

Mechanisms of Reduced RyR-Ca Channel Coupling

The data above suggest that loss of T-tubules could reduce the number of functional couplons with more uncoupled or “orphaned” RyR.21,23,24 We therefore examined subcellular patterns of SR Ca^{2+} release in confocal line scan imaging. In the longitudinal scan direction the Ca^{2+} release pattern was somewhat inhomogeneous in CTRL myocytes, but Ca^{2+} release occurred at many sites simultaneously and within a
short time frame, consistent with the presence of a T-tubular system (Figure 6A). We analyzed the time course and distribution of Ca\(^{2+}\) release by defining early and delayed areas.\(^{23}\) In AF, a larger number of areas with delayed Ca\(^{2+}\) release were present and these regions were also increased in width when compared to the control group (Figure 6B), consistent with a decreased T-tubule density. This leads to a slower upstroke of the Ca\(^{2+}\) transient in AF, which was quantified as the fraction of the line that had a fluorescence larger than 50% of the maximal (\(F > F_{50\%}\), Figure 6C). Ca\(^{2+}\) release remained below \(F_{50\%}\) along 32.1±3.7% of the line in AF versus 21.3±3.3% in CTRL (\(P<0.05\)).

In the transversal scan, the Ca\(^{2+}\) release pattern was much more irregular with often a horse-shoe appearance, although the pattern could be interrupted by areas of early release. We always observed early release at the subsarcolemma (Figure 7A). Ca\(^{2+}\) release propagation in the transversal direction thus appeared less dependent of T-tubule presence and mostly driven by propagation between RyR following the SS release, as inferred for human and small animal atrial myocytes.\(^{21,25,26}\) Consistent with this type of propagation, in spatial average, Ca\(^{2+}\) transients from transversal scans had longer time to peak than the longitudinal transients and lower amplitudes.

From these recordings, we can derive information on the coupling efficiency between Ca\(^{2+}\) channels and RyR, and on propagation between RyR. The SS Ca\(^{2+}\) transients can be assumed to result from couplons of Ca\(^{2+}\) channels and RyR. In the SS transients of the transversal line scan, amplitude in AF tended to be smaller than in control and tended to be abbreviated (Figure 7B). Early release transients in the longitudinal scan that most likely also result from couplons at T-tubular junctions also had smaller amplitude in AF (Figure 7C). In the central regions of the transversal scans, we quantified the propagation between RyR (Figure 7D). The average propagation speed was not altered in AF. Propagation within delayed areas of the longitudinal scan should also reflect the same process\(^{23}\) and was indeed comparable and not different between the groups (Figure 7D).

**Increased Glycogen Accumulation but Limited Myolysis**
Quantification of periodic acid–Schiff (PAS) positivity in paraffin sections of atrial tissue revealed a significant in-
crease of glycogen deposition in AF (Figure 8A). PAS staining was also performed on epoxy resin–embedded atrial tissue to obtain detailed information on the specific localization of glycogen (Figure 8B). Longitudinally sectioned cardiac myocytes from CTRL sheep mostly showed regular patterns of myofilaments with parallel rows of mitochondria (Figure 8B, 1). Occasionally, the presence of some distributed PAS-positive glycogen granules around the nuclei was observed (Figure 8B, 2). Longitudinal sectioned cardiac myocytes from sheep in AF often revealed increased depositions of PAS-positive glycogen granules interspersed between the intermyofibrillar spaces (Figure 8B, 3). Only a few myocytes were characterized by abundant accumulation of glycogen granules and a concomitant loss of myofilaments or myolysis (Figure 8B, 4), and these were mostly seen in AF. Semiquantitative analysis of the incidence of these distinctive cellular phenotypes in AF and CTRL confirmed these patterns (Figure 8C), consistent with an increased glycogen deposit in AF, with, however, few myocytes with extensive deposits and myolysis.

Discussion

Cellular Ultrastructural Remodeling in Persistent AF

Persistent AF leads to global remodeling of the atria with dilatation. This dilatation is accompanied by changes in extracellular matrix, which presumably allow cell slippage and rearrangement. Dilatation also results from cellular hypertrophy and increase in cell size, related to a certain extent to ultrastructural changes with glycogen accumulation and cells swelling. The degree of hypertrophy and of glycogen accumulation and myolysis depends on the chronicity.

In the present study of persistent AF cells are hypertrophied with glycogen deposition but limited myolysis; S/V is substantially decreased. The latter could in principle simply be the consequence of the increase in cell volume, as for a brick-like structure with a simple unfolded surface, such a volume increase would result in a reduction of S/V. In the more complex organization of the sarcolemma in ventricular myocytes however, T-tubules are essential in increasing S/V, contributing to synchronization of excitation–contraction coupling. Recent animal studies have demonstrated reduction of T-tubules and reduced S/V in hypertrophy and failure. Our current data show that in atrial myocytes of a larger animal T-tubules are present and subject to remodeling. This is consistent with very recent data of atrial T-tubular remodeling in tachy-pacing induced heart failure. A common feature of the conditions in which this has been observed to date is hypertrophy of the myocytes. It is therefore conceivable that the remodeling of T-tubules is part of the general maladaptive hypertrophy response.

Reduced Efficiency of Coupling Between Ca$^{2+}$ Influx and SR Ca$^{2+}$ Release

A reduction in the [Ca$^{2+}$], transient amplitude appears to be the major change accounting for the reduced contraction of atrial myocytes in persistent AF. Our data identify that multiple changes are involved.

The first is the structural organization, density, and function of the sarcolemmal L-type Ca$^{2+}$ channel. As a general concept, this in line with the earlier data from human studies, as well as animal models of chronic AF which reported reduction in Ca$^{2+}$ current density, with or without reduction in channel expression. Our data are consistent with a reduction in protein expression of the pore unit, but reduced baseline phosphorylation could also contribute. Reduced src kinase activity and enhanced protein phosphatase activity have also been implicated in human AF. In the absence of intracellular Ca$^{2+}$ buffering, however, the difference between AF and control is much less pronounced, presumably because the larger Ca$^{2+}$ transients in control myocytes reduce $I_{Ca}$ to
a much larger extent than in AF. In the present study, we also identified an additional and novel mechanism, i.e., the reduction in the density of the structures where L-type Ca\(^{2+}\) channels are localized, the T-tubules. Taken together, our data indicate that the reduction of Ca\(^{2+}\) influx via L-type channels is a combination of structural and functional alterations.

In ventricular myocytes, loss of T-tubules, even in the absence of any changes in the RyR, leads to a loss of synchrony of SR Ca\(^{2+}\) release and a reduced amplitude of the [Ca\(^{2+}\)]\(_i\) transient.\(^{3,2,4,30}\) A similar mechanism appears to be present in atrial myocytes from chronic AF. In atrial myocytes, Ca\(^{2+}\) release along the long axis, the contraction axis, is remarkably synchronized given the sparse T-tubular system, but this is significantly reduced in AF. Earlier studies on the long axis, the contraction axis, are available. In this study of persistent AF, the removal of Ca\(^{2+}\) from the SR (Figure 4A) and expression of SERCA and phosphorylation. Therefore myofilament function needs to be further investigated.

NCX Remodeling

An increase in NCX function is a common, although not general, observation in ventricular remodeling. In AF either no changes in NCX expression or an increased NCX protein expression has been reported, which may depend on the duration of AF.\(^{9,34,35}\) However, there are few functional data available. In this study of persistent AF, the removal of Ca\(^{2+}\) by NCX during caffeine-induced SR Ca\(^{2+}\) release is faster, a measurement that takes into account differences in S/V. The increased NCX capacity in AF could compensate for the high cellular Ca\(^{2+}\) load as consequence of the high and irregular rates of atrial depolarization. We see no change in relaxation of the [Ca\(^{2+}\)]\(_i\) transient during experiments under membrane voltage control, indicating that despite the higher activity, the contribution of NCX to Ca\(^{2+}\) removal on a beat-to-beat basis is modest and that the dominant mechanism of fast Ca\(^{2+}\) removal after SR Ca\(^{2+}\) release is SERCA. Increased NCX activity in the dyad could, however, reduce the local Ca\(^{2+}\) trigger for activation of RyR.

Limitations

Although we have focused in the present study on the role of Ca\(^{2+}\) handling in the contractile dysfunction, there may also be a role for changes in myofilament Ca\(^{2+}\) response and myofilament organization. It has been reported previously that myocytes from patients with AF show myolysis and glycogen accumulation as observed in hibernating human myocardium.\(^{27}\) In our present model, we saw significant glycogen accumulation but the extent of myolysis was limited. This may well be dependent on the duration of AF.\(^{28}\) It is intriguing that in AF the cell-shortening deficit could be corrected by increasing available Ca\(^{2+}\). The prolonged relaxation despite normal decline of the [Ca\(^{2+}\)]\(_i\) transient may indicate some degree of sensitization, as also suggested by the finding of a lower level of myosin binding protein C phosphorylation.\(^{35}\) Therefore myofilament function needs to be further investigated.

We have restricted our study to the right atrium. Although molecular probing of changes in Ca\(^{2+}\) channel expression and NCX in the LA suggest that similar changes occur in the LA, the functional consequences need to be confirmed, in particular with regard to perspectives for arrhythmogenesis. Several studies have emphasized the predominance of the LA in initiating AF\(^{36}\) and regional differences in electric remodeling.\(^{37}\) Changes in Ca\(^{2+}\) handling and NCX in the LA with focus on a potential link to arrhythmogenesis will be the subject of a future investigation.

Conclusions

In persistent AF, reduced fractional SR Ca\(^{2+}\) release despite preserved SR Ca\(^{2+}\) content is a major factor in the reduced cell contraction. We identified novel mechanisms underlying the reduced SR Ca\(^{2+}\) release, namely a loss of T-tubules with Ca\(^{2+}\), a reduced density and/or the increased number of noncoupled RyR can be expected to prolong the time needed for full propagation of SR Ca\(^{2+}\) release throughout the cell.

There is no apparent decrease in the availability of Ca\(^{2+}\) in the SR (Figure 4A) and expression of SERCA and phospholamban. Yet, taking into account the reduced S/V in AF, this may still result in a reduced increase of cytosolic Ca\(^{2+}\) during release.

Taken together, our data indicate that in chronic AF a reduced number of Ca\(^{2+}\)-channel–RyR couplings due to loss of T-tubules and a decrease in efficiency of the SS couplings both contribute to the reduction in Ca\(^{2+}\) release from the sarcoplasmic reticulum. The larger cell volume increases the time needed for propagation of Ca\(^{2+}\) release and may further reduce the actual availability of Ca\(^{2+}\) to the myofilaments.

Sources of Funding

This work is supported by grants from the Fund for Scientific Research Flanders (to R.W. and K.R.S.); the Fund for Cardio-surgery (to R.W.); the European Union, LSHM-CT-2005-018833, EUGene-Heart (to K.R.S.); and the Belgian Science Program (IAP6/31 to K.R.S.).

Disclosures

None.
References


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_Circ Res._ published online September 17, 2009;

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Expanded Methods

Animal characteristics and instrumentation

Sixteen ewes (body weight: 49.0 ± 2.6 kg) were instrumented with an atrial and ventricular pacemaker using a transvenous approach. A neurostimulator (Itrel, Medtronic, Minneapolis, USA) for rapid pacing of the atria was implanted subcutaneously in the neck of the sheep. The ventricular pacemaker (Kappa, Medtronic, Minneapolis, USA) was inserted at the shoulder. Pacemaker insertion was carried out under general anesthesia with isoflurane (1%) after pre-medication with ketamine (10 mg/kg IM) and xylazine (0.2 mg/kg IM). Anesthesia was induced with 5% isoflurane using a face mask. The sheep were intubated and ventilated with 100 % oxygen. After instrumentation, the sheep recovered for at least 1 week. The atrial pacemaker was then programmed for continuous stimulation at 900 bpm to induce persistent AF. The ventricular pacemaker was programmed in VVI at 40 bpm as back-up. Twenty-one ewes served as controls (CTRL, weight: 48 ± 3.3 kg). At regular time intervals, pacing was interrupted to assess the underlying rhythm (Fig.1A,B). The sheep were considered to be in persistent AF if no sinus rhythm could be demonstrated anymore in the follow up period. As previously described, this animal model leads to prolonged, persistent AF and presence of spontaneous persistent AF for 24 hours was confirmed by Holter recordings in a subset of animals. Electrical remodeling was present, as evident from shortening of
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the atrial effective refractory period and atrial wavelength in vivo. In vitro, in multicellular preparations, correspondingly, a shortening of the action potential was measured using high-resistance microelectrodes (from 111 ± 6 ms to 84 ± 12 ms at 75% repolarization, stimulation rate of 1 Hz, AF: n=6 vs. CTRL: n=19, p<0.05, Fig.1C). Action potentials from AF sheep also lost the normal adaption to rate. Persistent AF also induced hemodynamic and structural changes, like increased atrial pressure, atrial dilatation and atrial fibrosis.

In the current study, sheep were sacrificed after 182 ± 13.9 days of rapid atrial pacing and were in persistent AF for a mean of 129 ± 39 days. The experimental protocol was approved by the ethical committee on animal handling of the University of Leuven.

Atrial cell isolation

Under general anesthesia and after administration of an IV bolus of heparine (100 U/kg) and fentanyl (5 mg), the heart was quickly removed through a thoracotomy. The left atrial tissue was cut and the ventricles were removed just below the AV ring. The right coronary artery was cannulated and mounted into a Langendorff perfusion system; perfusion of the right atrium was checked and leaky atrial branches were sutured. The atrial tissue was briefly rinsed with normal Tyrode solution, followed by a perfusion of Ca^{2+}-free solution for 15 min. Collagenase A (0.8 mg/ml, Roche Diagnostics, Mannheim, Germany) was added to a 0.09 mM Ca^{2+} solution and recirculated for 20 min. The enzymes were washed out with a solution containing 0.18 mM Ca^{2+}. The right atrium was
then removed, cut into small pieces and further dissociated into single cells. Cells were stored in low Ca\(^{2+}\) solution at room temperature and used within 12 h after isolation.

**Measurement of cell shortening and global \([Ca^{2+}]_i\).**

Cells were placed in a perfusion chamber on the stage of an inverted microscope (Nikon Diaphot). Cell shortening (ΔL) was measured with a video edge-detector (Crescent Electronics, USA) during field stimulation and the data were normalized to resting cell length (L\(_0\)). Frequency of frame rate acquisition for the video-edge detector during field stimulation was 60 Hz. Even though contractions of sheep myocytes are rather slow, this may not be the optimal setting for discrimination of kinetics of the contraction. However, since it was set at 60 Hz initially for the first set of experiments, we maintained it during the study to allow comparison of data.

To measure simultaneously \([Ca^{2+}]_i\) transients, myocytes were incubated with 5 µM Fluo-3AM for 10 minutes, followed by several washing steps. The fluorescence signals were calibrated using the approach of Cheng et al.\(^5\) assuming a K\(_d\) of 800 nM and using the resting \([Ca^{2+}]_i\) values determined in the myocytes studied during whole-cell patch clamp recordings. Indeed, in myocytes loaded with K\(_5\)Fluo-3 via the patch pipette, measurement of F\(_{\text{max}}\) at the end of the experiment in each individual cell allows calibration with measurement of resting \([Ca^{2+}]_i\) as described below\(^6,7\). In 32 CTRL cells resting \([Ca^{2+}]_i\) was 134.6 ± 9.4 nM and in 31 AF cells resting \([Ca^{2+}]_i\) was 123 ± 8.1 nM. For the calibration of fluorescence data obtained during field stimulation with the Cheng method we used values of 135 nM for CTRL and 123 nM for AF cells. All experiments were done at 38°C (normal body temperature for sheep).
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*Measurement of membrane currents and \([Ca^{2+}]_i\).*

During whole-cell voltage-clamp experiments, the holding potential was -70 mV. Membrane currents were sampled at 4 kHz and filtered at 1 kHz. For measuring L-type Ca\(^{2+}\) currents (ICa\(_{\text{L}}\)), Na\(^+\) current was inactivated by a depolarizing prepulse to -40 mV. ICa\(_{\text{L}}\) was measured as the difference between the peak inward current and the current at the end of the pulse during depolarizations from -40 mV up to +60 mV. [Ca\(^{2+}\)]\(_i\) transients were measured by including 50 µM K\(_3\)fluo-3 in the pipette solution. The fluorescent signal was calibrated to intracellular Ca\(^{2+}\) values using the method described by Trafford et al., using a background and F\(_{\text{max}}\) value obtained respectively at the beginning and the end of the experiment.\(^6,7\) SR Ca\(^{2+}\) content and Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) properties were measured during a fast application of 10 mM caffeine for 10 s at a holding potential of -70 mV, preceded by a train of 10 conditioning pulses from -70 to +10 mV at 1 Hz. For SR content, the NCX current during the caffeine-induced [Ca\(^{2+}\)]\(_i\) transient was integrated and normalized to cell surface, i.e. cell capacitance. For NCX function, we measured the rate of Ca\(^{2+}\) removal in the continuous presence of caffeine and report the rate constant of an exponential function fitted to the decline of the [Ca\(^{2+}\)]\(_i\) transient.

*Solutions and drugs.*

All experiments were performed in normal Tyrode solution (in mM, NaCl 137, KCl 5.4, MgCl\(_2\) 0.5, CaCl\(_2\) 1.8, Na-HEPES 11.8, and glucose 10; pH 7.40). The Ca\(^{2+}\)-free Tyrode solution for cell isolation contained (in mM: NaCl 130, KCl 5.4, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, Hepes 6, glucose 20, pH 7.2). The pipette solution for whole-cell patch clamp was K-
aspartate based and consisted of (in mM) K-aspartate 120, NaCl 10, KCl 20, K-HEPES 10, MgATP 5, and K<sub>5</sub>fluoro-3 0.05; pH 7.2) In a number of cells, we used different solutions to isolated the Ca<sup>2+</sup> current. For these experiments we used a pipette solution with 10 mM EGTA to buffer most of the [Ca<sup>2+</sup>], transient and a Na<sup>+</sup> free external solution. The pipette solution contained (in mM, N-methyl-D-glutamine (NMDG) 120, TEACl 10, HEPES 10, MgCl<sub>2</sub> 0.5, MgATP 4 and Cs-EGTA 10; pH 7.2) and an external solution of (in mM, NMDG 120, TEACl 20, HEPES 11, MgCl<sub>2</sub> 0.5, CaCl<sub>2</sub> 5.4 and glucose 10; pH 7.35). Forskolin and isoproterenol were purchased from Sigma and prepared as stock solution for a dilution at the time of use of 1:1000 or less.

Confocal [Ca<sup>2+</sup>]<sub>i</sub> imaging
Cardiac myocytes were placed in a perfusion chamber on the stage of an inverted microscope equipped with a confocal line laser scanning head (LSM 510, Zeiss, Jena, Germany) and superfused with Tyrode’s solution at 38°C. Cells were studied in the ruptured whole cell patch clamp mode; the internal solution in the pipette contained the Ca<sup>2+</sup> indicator fluo-3. [Ca<sup>2+</sup>]<sub>i</sub> transients were recorded during steady state stimulation at 1 Hz with depolarizing steps from -70 to +10 mV for 250 ms. The line was positioned in a standard approach for all cells: the central plane in the z-axis was first determined and within this central plane the line was positioned in the center of the XY image, parallel to the long or transversal axis of the cell, but avoiding scans through nuclei. Temporal resolution was 1.54 ms/line and pixel size (x,y) 0.2-0.4 µm. Line scan images were analyzed using custom made algorithms coded in IDL. Images were filtered by a running median of five pixels along
the spatial and temporal axes. Recordings were segmented according to the onset of the whole-line averaged [Ca$^{2+}$]$_i$ transients and 6-10 sequential beats were averaged. The resulting time-averaged line scan image of a [Ca$^{2+}$]$_i$ transient therefore allowed us to identify cell regions with consistently early or delayed Ca$^{2+}$ release upon depolarization, independent of potential beat-to-beat variability in Ca$^{2+}$ release.$^{8,9}$ All temporal data refer to the onset of the whole-line averaged [Ca$^{2+}$]$_i$ transient ($t_0$). Intensity fluorescence values (F) along the scan line were normalized to the fluorescence intensity at diastole ($F_0$). For this, scan lines from the last 50 ms (32 lines) of end-diastole (before $t_0$) were averaged to obtain an averaged $F_0$ line. Each line of the time-averaged line scan image was then divided by the averaged $F_0$ line.

$F_{50}$ was defined as the half-maximum of the normalized overall peak [Ca$^{2+}$]$_i$ dependent fluorescence and served as threshold to discriminate local Ca$^{2+}$ release. Sections of the line where F did not reach $F_{50}$ within 200 ms were excluded from analysis. The fraction of the scan line with delayed release was measured in line scans obtained at 20 ms. All pixels which had Ca$^{2+}$ release below the $F_{50}$ value at 20 ms were considered delayed.

For the transversal line scans a different analysis was performed. Only release under the sarcolemma was analyzed in terms of time to peak and amplitude, with reference to the onset of this local transient. In the central part of the cell the propagation of Ca$^{2+}$ release between sites was measured.

For the analysis of the line scans, the operator was blinded to the groups.

Quantification of the T-tubule network

Atrial and ventricular cardiomyocytes were incubated with the sarcolemmal membrane dye di-8-ANEPPS (Molecular Probes, Carlsbad, USA, 5 µmol/L, incubation for 10 min,
followed by 10 min wash). After establishing the dimensions of the cell in Z-direction, XY planes (1024x1024 pixels) were recorded with a spacing of 1 µm in the Z axis. The middle 10 slices of the stack were used for T-tubule analysis.

Sarcolemmal surface membrane was identified in the thresholded XY images as the signal intense margin of the cell and was excluded for T-tubule signal density measurements. T-tubule signal density was then quantified as the number of signal-positive voxels within the cell margins using custom made algorithms coded in IDL. The operator was blinded to the groups during the analysis.

*Spectral analysis: 2D Fourier analysis*

The power spectrum of images of T-tubules staining was calculated using a custom made 2D Fourier analysis software program written in Mathlab. Results are shown as power as a function of spatial frequency (expressed in µm\(^{-1}\)) in the longitudinal direction. Peaks occurred at spatial frequency of 0.55 µm\(^{-1}\) and multiples. To compare between groups, the amplitude of these peaks was calculated as a fraction of the total power amplitude at that spatial frequency; data were pooled for 22 AF cells and 23 CTRL cells.

*Measurements of cell volume.*

To quantify cell volume, myocytes were loaded with 5 µM calcein-AM ester (Molecular Probes, Carlsbad, USA) for 30 minutes, followed by 30 min washout. Quantification was performed as described before. Cell volume was calculated by multiplying the total number of significant pixels from all slices by the pixel size and the between-slice interval (1 µm). All volume measurements were scaled by a correction factor (1.39), which
accounted for overestimation of the cell size in the Z direction due to spherical aberration. This correction factor was calculated using Z-stacks of fluorescent beads with a diameter \( \approx 15 \, \mu m \). Surface to volume ratios (S/V) were evaluated by measuring cell volume and cell capacitance from the same cell.\(^{11}\)

**Immunostaining of RyR and RyR density quantification.**

Myocytes were fixed and permeabilized by adding an equal volume of 4% paraformaldehyde/0.5% Triton-X. Cells were washed in PBS, incubated with PBS/0.1 mol/L glycine and washed again. The cardiomyocytes were then incubated with PBS with 10% goat serum albumin for 10 min, washed and incubated overnight (4°C) with primary antibody (anti-RyR 1/200, ABR, Golden, USA; raised in mouse). The next day, cardiomyocytes were washed in PBS and incubated with FITC-conjugated secondary antibody (anti-mouse IgG, 1:320 in PBS, Sigma) for 2 hrs at room temperature. Following wash with PBS, cells were used for imaging within 4 hrs. Cardiomyocytes incubated with secondary antibody only served as negative control and confirmed the absence of staining. Confocal XY image stacks were recorded from the RyR-stained cardiomyocyte. Density of RyR was quantified using thresholded images similar to the measurement of T-tubules. As a measure for the regularity of RyR distribution we applied a grid analysis as described before\(^9\). Briefly, XY images were superimposed with a grid of 5 \( \mu m \) x 5 \( \mu m \) boxes. In these images, RyR density was calculated individually for each box within the cardiomyocytes; only pixels inside the sarcolemma were analyzed. Homogeneity of RyR distribution within boxes was further assessed by
calculating the variability of the RyR signal as the percentage error of the signal, i.e. the standard deviation divided by the mean value.

**Immunoblotting.**

After sacrifice, left atrial appendage tissue samples were taken for molecular biology; right atrial appendage was collected in animals studied only in vivo without cell isolation at the time of sacrifice.

Tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C. Atrial tissue was homogenized in a buffer containing (in mM) imidazole 10, sucrose 300, dithiotreitol 1, sodium metabisulfite 1, sodium fluoride 25, sodium EDTA 5, sodium pyrophosphate 5, phenylmethylsulfonyl fluoride 0.3 and a protease inhibitor cocktail (“Complete-Mini” Roche Molecular biochemicals, Indianapolis, USA). Equal amounts of proteins were separated on 4–12% Bis–Tris gradient gels (NuPage™ electrophoresis system, Invitrogen, Carlsbad, USA) and blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated overnight with antibodies raised in the rabbit for alpha1c subunit of L-type Ca^{2+} channel (1/300, Alomone Labs, Israel), GAPDH (1/500, Santa Cruz Biotechnology, Santa Cruz, USA) and PS-16 (1/10000, Cyclacel Ltd., Dundee, UK) antibodies and mouse SERCA (1/50000, Affinity BioReagents, Golden, USA), NCX (1/1000, Affinity BioReagents, Golden, USA) and phospholamban (1/10000, Upstate, Billerica, USA) antibodies. Immunoblotting of RyR and of IP3R was performed on microsomes prepared by multiple centrifugation steps following the homogenization protocol. Tris-Acetate gels (3-8%) were used for electrophoresis. After blotting to PVDF membranes, mouse antibody for RyR (1/500, ABR, Golden, USA) was
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used. Detection was performed with alkaline phosphatase-labeled secondary antibodies, using ECF system (Amersham, Buckinghamshire, UK). After scanning the blots, the number of pixels of each positively stained band (antigen–antibody interaction) was calculated. Results were normalized to expression levels of GAPDH (except for data from microsomes) and subsequently to protein expression level of the control group.

Histological analysis

Atrial samples from sinus rhythm and AF sheep hearts were fixed in formalin for 24 hours and routinely embedded in paraffin. After deparaffinization, sections were stained for Periodic Acid Schiff (PAS), counterstained for Harris Haematoxilin and followed by dehydration in graded series of ethanol. An additional series of atrial samples were fixed in 3% glutaraldehyde buffered with 0.1M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.4. After rinsing in the same buffer for 24 hours the samples were postfixated for 1 hour in 2% OsO$_4$ (buffered to pH 7.4 with 0.1M sodium cacodylate). The atrial samples were dehydrated in graded series of ethanol and routinely embedded in Epoxy resin. Semi-thin sections were stained with PAS and toluidin blue for light microscopical evaluation. All histological sections were finally embedded in Depex mounting medium. PAS stained paraffin sections were photographed and the percentage of PAS positivity was determined according to the previously described analysis.\(^1\) Accurate localization of glycogen accumulation was assessed semi-quantitatively in epoxy resin sections. The number of cells showing specific morphological criteria is expressed as percentage of the total number of cells. Only longitudinal sectioned myocytes containing a recognizable nucleus were analyzed for this parameter. All morphometric analyses were performed using a
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Zeiss Axiovert 200N microscope with an Axiocam HrC camera and Axiovision 4.6 morphometry software (Carl Zeiss, Oberkochen, Germany). The operator was blinded during both analyses.

Statistics

Data are expressed as mean ± standard error of the mean (SEM); statistics were performed on pooled data of n cells or N animals, as indicated. Where cells were pooled, they represent data from at least 3 animals. Differences between group means were tested using the unpaired Student’s t-test for single measurements (Figs. 1, 2, 4, 5, 6, 7, 8); ANOVA for repeated measurements with Bonferroni post-hoc testing was used for multiple measurements at different voltages (Fig. 3). A P-value of less than 0.05 was considered to be statistically significant.

Supplementary Results

$Ca^{2+}$ handling protein expression in left atrial tissue of AF.

NCX protein expression was increased (Online Fig. IA). The mean data in the left panel summarize results normalized to GAPDH for the blot shown on the top, while the right bar graph summarizes normalized data of 3 blots. There was in LA tissue quite some variability as evident from the large error bars. Alpha-1c-subunit of L-type $Ca^{2+}$ channel was significantly reduced in AF (Online Fig. IB). Protein levels of total SERCA, phospholamban and Ser16-phosphorylated phospholamban were unchanged; immunoblotting showed a small but significant reduction of RyR expression in AF compared to CTRL (Online Fig. IC).
Comparison of Ca\(^{2+}\) handling protein expression in RA versus LA.

In CTRL, no differences were observed in protein expression of SERCA, PLB and alpha-1c-subunit of L-type Ca\(^{2+}\) channel of left versus right atrium but NCX expression was significantly lower in right versus left atrium of CTRL (Online Fig. IIA).

In AF, protein expression of SERCA, PLB, and NCX was not different in left versus right atrium (Online Fig. IIB). Protein expression of alpha-1c-subunit was equally low in both atria from AF sheep.

IP3-receptor protein expression in CTRL and AF.

We examined whether the expression of IP3-receptors were affected by AF. Immunoblotting showed no difference in protein expression between both groups (Online Fig. III).

Ventricular T-tubule density in CTRL and AF.

Online Fig.IV recapitulates the manuscript Fig. 4A of atrial cells at larger magnification. Density of ventricular T-tubules was investigated of the same sheep of which atrial myocytes were isolated. There was no difference in T-tubule density of ventricular myocytes in both groups (Online Fig.V).

Fourier analysis of T-tubules

We examined spatial T-tubule periodicity by 2D Fourier analysis, as previously described by Song et al.\(^{13}\) This analysis was performed by a different operator, also blinded to the
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group. This method identifies the occurrence of events (as a power) with a given periodicity (the spatial frequency). Presence of periodic T-tubules will show up as peaks at a given spatial frequency, reflecting distance between T-tubules. A loss of T-tubules is expected to reduce the number of events (power) for the first periodicity as this reflects adjacent T-tubules, normally corresponding to the sarcomere width.

Two typical examples are shown in the top panels of Online Fig.VI. When plotting power versus spatial frequency, atrial myocytes display a clear peak at spatial frequency around 0.55 µm⁻¹, corresponding to T-tubule spacing of 1.8 µm consistent with sarcomere spacing. This spatial frequency of a first peak was similar for AF (1.81 ± 0.02 µm, ncells=22) and CTRL (1.78 ± 0.03 µm, ncells=23). But, the power at this spatial frequency, i.e. the frequency of events of T-tubules at spacing of 1.8 µm was significantly reduced in AF, as illustrated by the examples and supported by the mean data of the height of the peak, measured as the fraction of the total power at this spatial frequency (Online Fig.VI lower panel).

In 13 of 23 CTRL cells, a second harmonic peak could be detected, at double frequency. In AF however, only 8 of 22 cells displayed the second peak and the amplitude is also decreased in AF. This is comparable to what Song et al.¹³ observed and these data indicate a more chaotic organization and loss of T-tubules. The Fourier analysis thus further supports our conclusion of decreased T-tubule density in AF.

To further document the reduced extent of contact between RyR and T-tubule membranes, we have also analyzed the spatial frequency of RyR expression, using 2D Fourier analysis. RyR expression showed a single peak in power at sarcomeric spacing of
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1.66 ± 0.05 µm in CTRL and 1.73 ± 0.04 µm in AF (p=ns; the slightly lower values relate to fixation of the cell). There was no difference in the power amplitude, supporting our previous analysis of unchanged RyR distribution in AF.

Taken together these data show decreased T-tubule density, where L-type Ca channels are located, combined with preserved RyR expression, which must lead to the presence of more “orphaned RyR” in AF.

Legends

*Online Figure I. Immunoblot for Ca\textsuperscript{2+} handling proteins expressed in left atrium.*

A, Immunoblot for NCX and below mean data of this example blot normalized to GAPDH, with in the right panel mean data of 3 blots with AF data normalized to CTRL (n=10 for AF and n=11 for CTRL). B, Immunoblots for alpha-1c subunit of L-type Ca\textsuperscript{2+} channel, normalized to expression levels GAPDH in CTRL (n=11) and AF (n=10) samples. C, Immunoblots for SERCA, PLB, Ser16-phospholamban, with mean data normalized to the expression levels in CTRL (n=11) and AF (n=10) samples; immunoblot for RyR and mean data normalized to the expression levels in CTRL (n=11) and AF (n=10) samples. * P<0.05.

*Online Figure II. Immunoblot for Ca\textsuperscript{2+} handling proteins expressed in left and right atrium of CTRL and AF.*

A, Mean signal density of immunoblots for SERCA, PLB, alpha-1c subunit of L-type Ca\textsuperscript{2+} channel and NCX of left and right atrium of CTRL. Signals have first been,
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normalized to expression levels GAPDH, and subsequently to LA. B, Mean signal density of immunoblots for SERCA, PLB and NCX of left and right atrium of AF sheep (n=4-5). Signals have first been normalized to expression levels GAPDH, and subsequently to LA. * P<0.05.

*Online Figure III. Immunoblot for IP3-receptor protein expression.*

Immunoblot and mean signal density for IP3-receptor in CTRL and AF, normalized to expression levels of CTRL group.

*Online Figure IV. T-tubule density of ventricular myocytes.*

Mean data of signal density for LV myocytes from CTRL (n=15) and AF (n=15).

*Online Figure V. T-tubule staining in atrial myocytes*

Examples as indicated on the figure.

References


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Online Figure I
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Online Figure I - continued
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A  LA:RA comparison in CTRL

Online Figure II

B  LA:RA comparison in AF

Online Figure II
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IP3 receptor

Online Figure III
CTRL, T-tubule signal 9.9%

AF, T-tubule signal 6.1%
Ventricular T-tubule density

Online Figure V
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CTRL

AF

Peak at 0.55 μm\(^{-1}\) (fraction of total)

CTRL

AF

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