Enhanced heterogeneity of myocardial conduction and severe cardiac electrical instability in annexin A7-deficient mice


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Abstract

Objectives: Annexin A7 is involved in cardiomyocyte membrane organization and Ca²⁺-dependent signalling processes. We investigated the impact of annexin A7 on cardiac electrophysiological properties using an annexin A7-deficient mouse strain (annexin A7⁻/⁻).

Methods: Nineteen adult annexin A7⁻/⁻ and 14 wild-type mice were examined electrophysiologically in vivo by transvenous catheterization. Hearts were additionally perfused by the Langendorff method and epicardial activation mapping was performed.

Results: The susceptibility to induction of atrial fibrillation was elevated in annexin A7⁻/⁻ mice. Ten deficient animals showed atrial fibrillation (AF) episodes ≥ 1 min and sustained AF ≥ 30 min was observed in 4 annexin A7⁻/⁻ mice, but in none of the wild-type mice. The incidence of ventricular tachycardia (VT) was higher in annexin A7⁻/⁻ mice and VT duration was prolonged. Epicardial mapping showed elevated anisotropy and inhomogeneity of conduction, leading to conduction blocks in the deficient mice. Besides alterations of intracellular calcium homeostasis, electron microscopy showed a homogeneous, electron-dense material that filled the myocardial intercellular compartments and accumulated at the basement membranes. This led to expansion of the extracellular spaces, which was the most probable substrate factor responsible for the disturbances of electrical communication.

Conclusions: Annexin A7 deficiency causes severe electrical instability in the murine heart, including conduction disturbances and anisotropy of impulse propagation, which is accompanied by disturbed calcium handling and intercellular deposits.

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Keywords: Arrhythmia (mechanisms); Cell communication; Conduction; Extracellular matrix; Annexin A7

1. Introduction

Annexin proteins are characterized by Ca²⁺-dependent phospholipid binding to natural and artificial membranes [1]. A variable N-terminal domain confers functional diversity to different annexins, and a conserved core domain is responsible for Ca²⁺- and phospholipid-binding [2]. This protein family is involved in regulation of endo- and exocytotic processes [3], aggregation of chromaffin granules [4], and modulation and formation of ion channels [5]. Annexin A7 (synexin, anxA7) was the first annexin to be identified. It promotes chromaffin granule aggregation and membrane fusion processes [6].
and has been reported to act as a Ca^{2+}-activated GTPase, thereby supporting Ca^{2+}- and GTP-dependent secretory events [7]. AnxA7 influences cellular Ca^{2+} homeostasis and forms voltage-gated Ca^{2+}-channels [8]. It is an abundant protein in murine and human myocardial tissue [9]. Herr et al. created anxA7-deficient mice (anxA7^{−/−}) by homologous recombination in embryonic stem cells [10]. Lack of anxA7 altered shape and osmotic resistance of red blood cells [11] and increased the velocity of Ca^{2+}-waves in cultured primary astroglial cells [10,12]. Frequency-dependent cell shortening and the positive inotropic effect of isoprenaline were blunted in adult anxA7^{−/−} cardiomyocytes, which was attributable to disturbances of the excitation-contraction coupling [10].

Such alterations of cardiomyocyte Ca^{2+}-handling are likely to also influence electrophysiological properties of the heart [13]. We therefore evaluated cardiac electrophysiological characteristics in vivo in anxA7^{−/−} mice and found an elevated susceptibility to atrial fibrillation (AF) and ventricular tachycardia (VT) caused by disturbances of myocardial conductive properties.

2. Methods

The generation of anxA7^{−/−} mice has been previously described [10]. Isogeneic 129SV-wild-type littermates (WT) were used as controls. Mice were handled according to the animal protection law stated in the German civil code and the investigations were approved by the district government of Cologne. The investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health and the Position of the American Heart Association on Research Animal Use (AHA, Nov. 11, 1984).

2.1. Electrophysiological investigation (EPI) and surface-ECG

Thirty-three mice (14 WT, 19 anxA7^{−/−}, 12.7±0.5 weeks, 25–32 g) were electrophysiologically examined using an in vivo single catheter technique as described before [14]. Briefly, preparation, catheterization, and EPI were performed under inhalation anaesthesia (induction period 2.5 vol.% isoflurane in 70%N2O/30%O2). Performing fixed-rate and extrastimulus pacing, sinus node recovery time (SNRT), Wenckebach periodicity (WBP), 2:1 AV conduction, and atrial and AV nodal refractory periods (ARP, AVNRP) were evaluated.

SNRT was defined as maximum return cycle length (CL) after 10 s fixed-rate pacing (S1S1: 120 ms, 100 ms and 80 ms). ARP and AVNRP were evaluated by programmed atrial stimulation (8 stimuli fixed rate at S1S1 cycle length: 120 ms, 110 ms, and 100 ms; one short coupled extrastimulus with a 10 ms-stepwise S1S2-reduction). AVNRP was defined as longest S1S2 with loss of AV-nodal conduction, ARP as longest S1S2 with absent atrial response. Ventricular refractory period (VRP) was evaluated similar to ARP.

2.2. Arrhythmia induction

Atrial and ventricular burst stimulations were performed for 5 s (S1S1: 50 ms–10 ms, 10 ms stepwise reduction; stimulus amplitudes 1.0 and 2.0 mA) [14,15]. Ventricular vulnerability was additionally tested by extrastimulus pacing (S1S1: 120 ms, 100 ms, and 80 ms followed by up to 3 extra beats). AF was defined as rapid and fragmented atrial electrograms with irregular AV-nodal conduction for ≥ 1 s [15]. Number of AF episodes, mean ventricular heart-rate during AF and AF duration were analyzed. VT was defined as ≥ 4 ventricular premature ventricular beats. Number of inducible VT episodes/animal, VT duration and tachycardia-CL were determined.

2.3. Langendorff-perfused hearts and epicardial mapping

For analyses of myocardial conduction velocities and characteristics, hearts were Langendorff-perfused, and epicardial activation mapping (EAM) using a 128-electrode...
array was performed. Thirty atrial and ventricular areas of 15
\(\text{anxA7}^{-/-}\) and 8 atrial and ventricular areas of 4 WT mice
were analyzed. Hearts were excorporated and dissected
from surrounding tissue in ice-cold Krebs-Henseleit buffer.
After cannulation of the aorta, hearts were retrogradely
perfused in a Langendorff-apparatus (Radnoti Technologies
Table 1: Baseline surface-ECG and intracardiac EPI parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>anxA7−/− (n = 19)</th>
<th>WT (n = 14)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart-rate (bpm)</td>
<td>370.2 ± 49.7</td>
<td>355.8 ± 36.7</td>
<td>0.40</td>
</tr>
<tr>
<td>P (ms)</td>
<td>14.9 ± 3.7</td>
<td>18.1 ± 2.7</td>
<td>0.01</td>
</tr>
<tr>
<td>PQ (ms)</td>
<td>40.8 ± 6.7</td>
<td>46.4 ± 8.4</td>
<td>0.06</td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>15.3 ± 2.4</td>
<td>17.4 ± 2.7</td>
<td>0.03</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>36.0 ± 4.2</td>
<td>38.9 ± 4.2</td>
<td>0.06</td>
</tr>
<tr>
<td>QTC (ms)†</td>
<td>28.1 ± 2.9</td>
<td>29.9 ± 3.4</td>
<td>0.12</td>
</tr>
<tr>
<td>JTc (ms)++</td>
<td>16.2 ± 3.5</td>
<td>16.6 ± 3.2</td>
<td>0.72</td>
</tr>
<tr>
<td>AH (ms)†</td>
<td>31.8 ± 5.1</td>
<td>30.4 ± 3.2</td>
<td>0.48</td>
</tr>
<tr>
<td>HV (ms)‡</td>
<td>13.0 ± 2.1</td>
<td>11.5 ± 2.0</td>
<td>0.14</td>
</tr>
<tr>
<td>SNRT (ms)§ (SIS1 120 ms)</td>
<td>252.3 ± 92.5</td>
<td>227.9 ± 69.1</td>
<td>0.40</td>
</tr>
<tr>
<td>ARP (ms)†</td>
<td>32.0 ± 5.4</td>
<td>30.0 ± 3.8</td>
<td>0.37</td>
</tr>
<tr>
<td>AVNRP (ms)§ (SIS1 120 ms)</td>
<td>59.7 ± 14.1</td>
<td>48.1 ± 8.4</td>
<td>0.16</td>
</tr>
<tr>
<td>WBP (ms)§</td>
<td>87.4 ± 12.7</td>
<td>84.4 ± 10.1</td>
<td>0.15</td>
</tr>
<tr>
<td>VRP (ms)†+ (SIS1 120 ms)</td>
<td>41.1 ± 14.0</td>
<td>38.2 ± 7.5</td>
<td>0.46</td>
</tr>
</tbody>
</table>

†/++: rate corrected QT/JT-intervals; †: interval from atrial to His-signal; ‡: interval from His to first QRS-movement in surface-ECG; §: sinus-node recovery-time; †: atrial refractory period; ‡: AV-nodal refractory period; #: Wenckebach-periodicity; **: †+: ventricular refractory period.

2.4. Electron microscopy

Small pieces of the ventricular myocardium of 4% paraformaldehyde immersion-fixed hearts (5 anxA7−/−, 5 WT) were cut and further fixed with 2% osmium tetroxide in 0.1 M PBS for 2 h at 4 °C. After three-times washing in 0.1 M cacodylate buffer for 10 min, the specimens were dehydrated in a graded ethanol series and infiltrated with and embedded in araldite. Sections of plastic-embedded specimens were cut with glass for thin sections and a diamond knife for ultra thin sections on a Reichert ultramicrotome. The 0.5-μm thin slices were stained with methylene blue and investigated using a light microscope (Axiophot, Zeiss, Oberkochen, Germany). Ultra thin 60 nm sections were examined using an electron microscope (902A, Leo, Oberkochen, Germany) after contrasting with uranyl acetate-lead citrate.

2.5. Measurement of cell shortening

Murine ventricular cardiomyocytes were isolated from anxA7−/− and WT as described previously [10] and placed on laminin-coated glass slides in 12-well dishes covered by cell medium (M199, Gibco, Karlsruhe, Germany, supplemented with vitamins and essential amino acids). Experiments were performed at 32 °C in a pre-warmed Tyrode’s solution (in mM: NaCl 140, KCl 5.8, CaCl2 1, KH2PO4 0.5, glucose 8.3, Na-pyruvate 2 and gassed with carbogen (O2 95%, CO2 5%), pH 7.35–7.45. A perfusate temperature of 37 °C was continuously maintained. The heart was immersed in a water-jacketed chamber and further fixed on a moistened support. Unipolar extracellular electrograms (128) were recorded from the epicardial surface of the ventricles with regard to a reference electrode positioned in the water-bath, using a custom-built electrode array (interelectrode distance: 300±7 μm). Fixed-rate and single extrastimulus stimulation were performed using two adjacent electrodes of the array. Electrograms were recorded using a 128-channel, computer-assisted recording system (Multi Channel Systems, Reutlingen, Germany) with a sampling rate of 25 kHz (25000 samples per second). Data were bandpass filtered (50 Hz), digitized with 12 bit and a signal range of 20 mV.

Activation maps were calculated from these data using custom-programmed software (Labview 7.1, National Instruments, Austin, TX, USA). The first derivative of each unipolar electrogram was evaluated and maximal negative dV/dt activation was defined as timepoint of local activation [16]. With regard to myocardial fibre orientation, longitudinal and transversal conduction velocities (CV) were evaluated by calculating latencies between two electrodes, divided by the interelectrode distance, and anisotropic ratios were calculated as described before [17]. To obtain an index of local conduction slowing for each electrode, the activation time differences to neighbouring points were normalized to interelectrode distance. The largest difference at each site was defined as local phase delay. The variation coefficient of these phase delays was used as inhomogeneity index for inhomogeneity in global conduction, as described before [18,19].
Fig. 3. Fractional shortening (A) and the fura-2 transient (B) measured at increasing stimulation frequencies in murine ventricular cardiomyocytes of anxA7−/− (n=18 from 4 hearts) and WT (n=16 from 6 hearts). C: Fibers were first maximally Ca2+-activated, then the sarcoplasmic reticulum (SR) was loaded under definite conditions and finally caffeine was added to the solution. The ratio of caffeine-induced tension over the maximal Ca2+-activated tension is a parameter characterizing the amount of Ca2+ released out of the SR. D: Caffeine-induced Ca2+ release was significantly reduced in anxA7−/−. This reduction was reversible by PKA-dependent phosphorylation (E). F: The density of the β1-adrenoceptors was significantly down-regulated in anxA7−/−. G: No alterations were observed in the expression of the heteromeric G-proteins. *: P<0.05.
2.6. Determination of intracellular Ca\(^{2+}\) transients

The intracellular Ca\(^{2+}\) transient was measured using the cell permeable Ca\(^{2+}\)-indicator fura-2 acetoxyethyl ester (fura-2 AM, Molecular Probes, Eugene). Isolated cardiomyocytes were incubated at 37 °C for 30 min with fura-2 AM (7 μM) and afterwards incubated without fura-2 AM for 10 min for de-esterification. Fura-2 loaded cells were sited in the inverted microscope, perfused with a Tyrode’s solution at 32 °C and stimulated electrically. Fluorescence was detected by a photomultiplier system, recorded and digitally stored (Scientific Instruments, Heidelberg, Germany). The dual excitation of fura-2 at 380 nm (Ca\(^{2+}\)-free indicator) and 340 nm (Ca\(^{2+}\)-bound indicator) allows determination of the intracellular Ca\(^{2+}\) concentration. Emission was measured at 530 nm. The relative Ca\(^{2+}\) signal was calculated from the ratio 340 nm over 380 nm. Data are presented as ratio of fluorescence intensity at 340 nm and 380 nm excitation.

2.7. Measurement of the caffeine-induced sarcoplasmic reticulum (SR) Ca\(^{2+}\)-release

To prepare the skinned fibers, hearts were placed in ice-cold solution of 50% glycerol and (in mmol/l) imidazole 20, NaCl 10, ATP 5, MgCl\(_2\) 5, EGTA 4, dithiotreitol 2 (pH 7.0). cardiomyocytes were incubated at 37 °C for 30 min with fura-2 AM (7 μM) and afterwards incubated without fura-2 AM for 10 min for de-esterification. Fura-2 loaded cells were sited in the inverted microscope, perfused with a Tyrode’s solution at 32 °C and stimulated electrically. Fluorescence was detected by a photomultiplier system, recorded and digitally stored (Scientific Instruments, Heidelberg, Germany). The dual excitation of fura-2 at 380 nm (Ca\(^{2+}\)-free indicator) and 340 nm (Ca\(^{2+}\)-bound indicator) allows determination of the intracellular Ca\(^{2+}\) concentration. Emission was measured at 530 nm. The relative Ca\(^{2+}\) signal was calculated from the ratio 340 nm over 380 nm. Data are presented as ratio of fluorescence intensity at 340 nm and 380 nm excitation.

2.8. β\(_1\)- and β\(_2\)-adrenoceptor binding studies

Membrane preparations of cardiac tissue were performed as described before [22]. β-adrenoceptors in cardiac tissue homogenates were investigated using \(^{3}H\)-CGP 12.177 (–)-4-(3-t-butyramino-2-hydroxy-propoxy)-(5,7,3-\(^{3}H\) benzimidazol-2-one) as the radio-labelled ligand (specific activity 50 Ci/M). Specific binding was determined as the difference of binding in the absence and presence of 10 μM DL-propranolol. β-adrenoceptor subtypes were determined by competition experiments using the β\(_1\)-selective antagonist CGP 207.12A (0.3 μM) and the β\(_2\)-selective antagonist ICI 118.551 (0.05 μM). β\(_2\) to β\(_1\) ratio was calculated as previously described [23].

2.9. Immunoblotting

After homogenisation of ventricular tissue, protein was determined as outlined before [24]. For western blot analysis, the proteins were separated electrophoretically in a continuous 4% stacking gel and a sodium dodecyl sulfate polyacrylamid (SDS)-12% gel under constant current (70 mA [60 min] and 120 mA [180–240 min]) and transferred onto a polyvinylidiene fluoride membrane (PVDF, Roche, Mannheim, Germany).

2.10. Statistical analysis

Statistical analysis included a two-tailed Student’s t-test or multivariate ANOVA with post-hoc subgroup testing when appropriate (i.e. Tukey’s test). Discrete variables were analyzed by 2-sided Fisher’s exact test. A P-value ≤ 0.05 was regarded as statistically significant.

3. Results

3.1. Surface ECG and EPI

We first carried out ECG recordings (Fig. 1B and Table 1). At equal baseline heart rates, anxA7\(^{-/-}\) exhibited significantly shorter P-wave and QRS duration. Evaluation of standard intracardiac electrophysiological parameters on atrial, AV-nodal and ventricular level showed no significant differences among the groups (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>anxA7(^{-/-})</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5 mice</td>
<td>5 mice</td>
</tr>
<tr>
<td>SERCA2a (density units/mg protein)</td>
<td>174.0±22.0*</td>
<td>381.7±46.5</td>
</tr>
<tr>
<td>Phospholamban (density units/mg protein)</td>
<td>884.6±47.4</td>
<td>912.6±64.0</td>
</tr>
<tr>
<td>SERCA2a/Phospholamban-ratio</td>
<td>0.20±0.01*</td>
<td>0.42±0.02</td>
</tr>
<tr>
<td>Ryanodine receptor (density units/mg protein)</td>
<td>42.5±7.1</td>
<td>48.6±4.5</td>
</tr>
<tr>
<td>NCX (density units/mg protein)</td>
<td>186.7±10.7*</td>
<td>120.5±6.5</td>
</tr>
</tbody>
</table>

*: P<0.05.
anxA7−/−, too, and indicates elevated arrhythmogeneity on atrial level in these mice (Fig. 1E). We defined the probability of AF induction as inducible episodes divided by total testing maneuvers applied in the groups. This surrogate parameter was significantly elevated in the deficient mice (Fig. 1F). Deficient mice were significantly more susceptible to AF induction in the total group of all animals (anxA7−/−: 79 inducible episodes/190 testings; WT: 38 episodes/140 testings) and in the group of inducible animals only (anxA7−/−: 79 episodes/170 testings; WT: 38 episodes/120 testings). These results prove the presence of an elevated electrical instability on atrial level in the anxA7−/− mice.

3.3. Ventricular tachyarrhythmia (VT)

WT were less prone to VT induction and only short lasting, polymorphic VTs were inducible by burst and programmed extra-stimulation protocols (Fig. 2A). The susceptibility towards longer lasting VTs was elevated in the anxA7−/− mice under both the programmed and burst stimulation protocols (Fig. 2B). Moreover, these VT episodes lasted significantly longer in anxA7−/− (0.88±1.3 s versus 0.25±0.07 s; \( P<0.0001 \)). All inducible VTs were self-terminating. Analysis of the number of total inducible VT episodes per inducible animal revealed a significant higher incidence in anxA7−/− animals (total: 8.1±5.3 versus 2.4±1.5 in WT; Fig. 4. At the electron microscopic level, no abnormalities of the cardiomyocyte ultrastructure could be observed in anxA−/− (A, B, C) as compared to WT (D), while a distinct alteration is found in the intercellular spaces of myocardium from anxA−/−. At lower magnification, a homogeneous electron dense material (*) is visible in the enlarged intercellular space between a capillary (CA) and two cardiomyocytes (CM) (A). At higher magnification it is recognizable that the spaces between the cardiomyocytes also are filled by such a homogeneous material (*) (B). In contrast to WT (D), which reveals a normal structured basement membrane (arrows) covering the adjacent cardiomyocytes, such basement membranes cannot be identified in intermyocardial space (*) (material; arrows: missing basement membrane) of anxA−/− myocardium (C). Bar: A=4 μm; B=400 nm, C=200 nm, D=400 nm.

![Activation mapping](image)

Fig. 5. A: Activation mapping of 15 left and 15 right epicardial ventricular areas of anxA−/− and WT-hearts was performed. The figure shows representative examples of conduction properties of these epicardial mappings. In contrast to the homogeneous conduction under spontaneous conditions in WT, irregular breakthrough areas can be seen in anxA−/−, resulting in more heterogeneous transduction in spontaneous sinus rhythm beats (spont). This effect is even more pronounced under epicardial fixed rate stimulation (stim) and extra-stimulation (premature). Distinct blockages of conduction in the mutant mice are present during these stimulation protocols (upper trace, stim, premature). In contrast, WT show homogeneous centrifugal conduction from the stimulation site (white ovals), and no relevant blocking. B: The anisotropic ratio was calculated as described before [17] for all mapped areas (longitudinal conduction velocity (CV) divided by transversal CV with regard to fibre orientation that was determined as described before [17]). This analysis showed significantly enhanced global anisotropy provoked by premature stimulation (*: \( P=0.029 \)). These results indicate preservation of directed conduction under more basal conditions, but loss of physiological and directed global impulse propagation under early extrabeat stimulation performed close to refractoriness, simulating early premature ventricular beats. C: The inhomogeneity index (median of phase delay divided by P5-P95 interval of phase delay calculated for every single electrode compared to all adjacent electrodes of the array) showed highly significantly elevated heterogeneity of conduction in anxA−/− under fixed rate (*: \( P=0.00001 \)) and premature stimulation conditions (**: \( P=0.0004 \)). D: Mean number of adjacent electrodes of the whole array with significant conduction delay defined as CV <0.1 mm/ms was significantly elevated in the deficient mice under fixed rate (*: \( P=0.00001 \)) and premature stimulation (**: \( P=0.0007 \)), but not sinus rhythm.
burst-stimulation: 4.6±2.9 versus 2.0±0.7; programmed stimulation: 4.9±2.6 versus 2.3±1.5; *P<0.05). Significantly more anxA7−/− animals were susceptible to VT induction by programmed extra-stimulation as compared to WT (Fig. 2C). AnxA7−/− also showed a higher number of inducible VT episodes per individual animal (19 testings; Fig. 2D). The probability of VT induction was calculated as described above for AF and was significantly elevated in the deficient mice independent from the stimulation protocol (Fig. 2E). Thus ventricular vulnerability is markedly pronounced in the annexin A7-deficient mice.

3.4. Ca^{2+} homeostasis

As anxA7 is known to influence the Ca^{2+} ionic homeostasis, an analysis of possible Ca^{2+}-dependent mechanisms underlying the incidence of arrhythmias in anxA7−/− mice was performed. Intracellular Ca^{2+} transients and cell shortening were measured in ventricular cardiomyocytes at increasing stimulation frequencies [0.5–5 Hz (=30–300 bpm)]. Under basal conditions, i.e. stimulation frequency of 0.5 Hz, fractional cell shortening was 2.32±0.40% in WT (16 measurements from 6 mice) and 2.97±0.40% in anxA7−/− mice (18 measurements from 4 mice; Fig. 3A). By raising the stimulation frequency to 5 Hz, fractional cell shortening increased in WT (5 Hz: 4.28±0.52%), but declined in anxA7−/− mice (5 Hz: 1.68±0.30%). The diastolic cell length was not changed.

Analyses of the intracellular Ca^{2+} transients at 0.5 Hz determined fura-2 amplitude that were similar in anxA7−/− versus 1.44±0.05; anxA7−/−: 1.33±0.05 versus 1.18±0.06%). AnxA7−/− thus shows a systolic, but not diastolic dysregulation of the cardiomyocatal Ca^{2+} homeostasis homeostasis.

To investigate Ca^{2+} release from the SR during systole, the caffeine-induced tension development was measured in saponin-skinned fibers (Fig. 3C). Under basal conditions, (i.e. absence of PKA-dependent stimulation of the SR Ca^{2+}-release), the caffeine-induced tension was significantly reduced (anxA7−/−: 86.1±6.4%; WT: 101.9±1.2% of maximal tension; Fig. 3D). Incubation of the saponin-skinned fibers with the catalytic subunit of PKA significantly increased the caffeine-induced tension only in anxA7−/− (Fig. 3E). In anxA7−/−, protein expression of SERCA2a was significantly decreased, whereas that of phospholamban was not altered, leading to a significantly decreased SERCA2a/phospholamban ratio (Table 2). This change results in a reduced reuptake of Ca^{2+} into the SR. We detected a compensating upregulation of the protein level of the sodium-calcium antiporter (NCX; Table 2). We furthermore investigated the distribution of the β₁− and β₂-adrenoceptors in cardiac membrane preparations. β₁-adrenoceptors were significantly down-regulated in anxA7−/− (Fig. 3F), without alterations of the β₂-adrenoceptors or the expression of the Gi- and Gs-proteins (Fig. 3G,H). These results indicate relevant changes of Ca^{2+}-handling in the hearts of anxA7−/− that are analogous to human heart failure.

3.5. Morphology

Despite typical alterations of Ca^{2+} handling and β-adrenoceptor contribution usually associated to heart failure, a detailed echocardiographical examination showed no significant differences in cardiac contractile function among the groups (analyses included: left ventricular end-systolic volume, left ventricular end-diastolic volume, ejection-volume, diastolic left ventricular mass and left ventricular ejection fraction; data not shown). There were no significant alterations in the heart-to body weight ratio between anxA7−/− and WT (data not shown), indicating an absence of cardiac hypertrophy or dilatation as possible arrhythmogenic factors. Histological examination showed neither enhanced fibrosis nor changes in the number or morphology of cardiomyocytes (data not shown).

3.6. Electron-microscopy

To detect possible ultrastructural alterations at the atrial and ventricular level that may be associated with the induction of arrhythmia, atrial and ventricular tissues were examined by electron microscopy (Fig. 4). In contrast to the WT-group (Fig. 4D), the whole intercellular space of all 5 anxA7−/− myocardi that were analyzed was filled by a homogeneous electron dense material of unknown origin (Fig. 4) that interfered with the basement membranes (Fig. 4C) normally covering the cardiomyocytes and capillaries in WT (Fig. 4D). Moreover, collagen fibres in these intercellular spaces were not detectable (Fig. 4A and B).

3.7. Epicardial mapping

Under conditions of spontaneous sinus beating, epicardial ventricular activation mapping revealed a more heterogeneous conduction in anxA7−/− as compared to WT (Fig. 5A). At comparatively fast conduction velocities, color-coded reconstruction of isochrone lines showed more heterogeneous wave breakthrough in the ventricular myocardium of anxA7−/−. This heterogeneity of conduction successively increased under simulation and extrastimulus administration, resulting in significant conduction blocking in the anxA7−/− mice (Fig. 5A, upper row). This effect was not seen in WT-animals. The anisotropic ratio was higher in anxA7−/− animals after premature beat stimulation (Fig. 5B). The calculation of the inhomogeneity index revealed a significantly enhanced heterogeneity of global conduction (Fig. 5C), and the mean number of adjacent electrodes with significant blockage (conduction delay ≥ 3 ms; CV <0.1 m/s) was higher in the deficient animals (Fig. 5D).
Atrial mapping revealed analogous effects as present in ventricular myocardium with significantly elevated inhomogeneity indices in the mutant mice under fixed rate stimulation (3.9±2.0 versus 2.9±0.8; \( P=0.048 \)) and premature stimulation (5.8±2 versus 3.5±1.7, \( P=0.013 \)), but not basal conditions (4.1±2.2 versus 3.2±1.3; \( P=0.153 \)).

These results demonstrate that disturbances of the conductive properties in the anxA7−/− mice go along with a significantly elevated susceptibility to arrhythmia on the atrial and ventricular levels, particularly under conditions of stimulation and premature beats. These conduction delays are less distinct in sinus rhythm and seem thus in part be compensated under basal conditions.

4. Discussion

Depletion of anxA7 is deleterious to the electrical stability of the murine heart in vivo and leads to an enhanced susceptibility towards induction of long-lasting atrial and ventricular tachyarrhythmias. We have performed a variety of analytical approaches to determine possible molecular and structural alterations underlying this overt pathology. We found a distinct heterogeneity of epicardial conduction properties and relevant blockages under conditions of epicardial stimulation. This is likely due to a severe widening of the intercellular spacing caused by accumulation of a non-collagenous substrate and associated disturbances in direct cell-to-cell communication.

AnxA7 is a relevant protein in the investigated murine ventricular tissue (0.04% of total heart protein) and known to play a role in Ca2+-binding and Ca2+-dependent signalling processes. It therefore is proposed to dominantly influence the Ca2+-buffering system. In analogy to human heart failure, anxA7−/− cardiomyocytes show negative force-frequency relation and downregulation of β1-adrenoceptors, but these changes apparently do not result in an impaired left-ventricular contractility under basal in vivo conditions. Abnormal Ca2+ homeostasis is known to contribute to human arrhythmia, i.e. by intracellular Ca2+ overload under conditions of ischemia and reperfusion [25], and sarcoplasmic Ca2+ release influences heterogeneity of cardiac electrical properties [13]. Despite the identified dysregulation of intracellular Ca2+ uptake, no significant diastolic Ca2+ overload was observed. This can be explained with the measured increase of NCX-activity. It is possible, however, that the NCX-dependent prevention of arrhythmogenic diastolic Ca2+ overload is limited at higher heart rates up to 800 bpm (13.3 Hz) or short coupled extras, which were administered during atrial and ventricular stimulation manoeuvres. Thus, altered Ca2+ homeostasis may partly contribute to elevated electric vulnerability, but most likely it is not the predominant arrhythmogenic factor in this mouse model.

Electron microscopy revealed an impaired integrity of the basement membranes and a widening of the intercellular space accompanied by alterations of the structure of the basement membrane of cardiomyocytes. These alterations strongly point towards a disturbed intercellular communication. Although connexin function and distribution were not studied, such deposits are likely to influence cell-to-cell communication resulting in impaired gap-junctional coupling. In analogy to previous studies with connexin43 disorders resulting in strong arrhythmogenic effects [17], we could detect significant arrhythmia-predisposing heterogeneous conduction properties within the ventricular myocardium, most probably related to these ultrastructural alterations. This heterogeneous conduction was even more pronounced under conditions of stress, such as extrastimulus pacing, resulting in significantly more blockage and conduction slowing in the myocardium of anxA7−/− mice. Such distinct proarrhythmic conduction disturbances represent a relevant substrate for the development of arrhythmias [17].

Alterations of the intercellular spacing in the heart have been shown to strongly influence cardiac excitability and myocar-
dial conduction properties [26,27]. Changes of the extracellular matrix and altered collagen distribution also interfere with local conduction in proarrhythmic atrial remodelling models [19]. Proper composition of the extracellular matrix therefore is a major factor in the conservation of a regular conductive function of the myocardium. Thus, we consider these changes as the predominant proarrhythmic substrate factor responsible for increased susceptibility to long-lasting cardiac arrhythmias. It still remains unclear whether the absence of collagen in the deposits has an influence on VT susceptibility and duration as compared to diseases with collagenous aggregations. Furthermore, accompanying arrhythmogenic co-factors such as alterations of ion channel expression and function in cardiomyocytes cannot be ruled out by our experiments and warrant future investigations.

The finding of shorter P and QRS durations is somehow surprising in a mouse strain with elevated arrhythmia suscep-
tibility and conduction blocks under ventricular stimulation, but these findings are not contradictory for elevated proarrhythmic heterogeneous conduction under conditions of stress. Firstly, the surface ECG under basal conditions may not always be a sensitive surrogate for abnormalities in myocardial conductivity. Secondly, no alterations of conduction velocities or conductive blocks were present in the epicardial recordings under basal, i.e. sinus rhythm conditions. This points towards compensatory mechanisms such as alterations of cardiomyocytal ion-channel activation or enhanced adrenergic drive that also might account for the shortening of P and QRS interval in vivo.

Analogously, myocardial ultrastructural alterations associated with enhanced electrical instability are known to occur in the absence of relevant surface ECG changes [28]. We showed a dramatic decrease of conduction velocity, enhancement of heterogeneity of conduction and significantly more blockages under provocation manoeuvres, i.e. fixed rate and in particular extrastimulus pacing. These findings suggest an elevated probability for the co-existence of adjacent fast and slow conducting areas under such conditions. This predisposes the electrical wavefront to hitting refractory tissue,
with an elevated possibility for the development of microreentries, increasing susceptibility and probability of perpetuation of arrhythmias [17,18]. Our electrophysiological findings point towards this pathomechanism as predominant arrhythmogenic factor that might be further pronounced in association with the additional changes found in the in the hearts of anxA7−/− mice, i.e. the disturbed Ca2+ handling. The nature of the accumulated extracellular substrate likely accounting in a relevant part for this electrophysiological phenomenon remains yet unclear and has to be further studied.

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