

Disordered Myocardial Ca²⁺ Homeostasis Results in Substructural Alterations That May Promote Occurrence of Malignant Arrhythmias

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Summary

We aimed to determine the impact of Ca²⁺-related disorders induced in intact animal hearts on ultrastructure of the cardiomyocytes prior to occurrence of severe arrhythmias. Three types of acute experiments were performed that are known to be accompanied by disturbances in Ca²⁺ handling. Langedorff-perfused rat or guinea pig hearts subjected to K⁺-deficient perfusion to induce ventricular fibrillation (VF), burst atrial pacing to induce atrial fibrillation (AF) and open chest pig heart exposed to intramyocardial noradrenaline infusion to induce ventricular tachycardia (VT). Tissue samples for electron microscopic examination were taken during basal condition, prior and during occurrence of malignant arrhythmias. Cardiomyocyte alterations preceding occurrence of arrhythmias consisted of non-uniform sarcomere shortening, disruption of myofilaments and injury of mitochondria that most likely reflected cytosolic Ca²⁺ disturbances and Ca²⁺ overload. These disorders were linked with non-uniform pattern of neighboring cardiomyocytes and dissociation of adhesive junctions suggesting defects in cardiac cell-to-cell coupling. Our findings identified heterogeneously distributed high [Ca²⁺]_i-induced subcellular injury of the cardiomyocytes and their junctions as a common feature prior occurrence of VT, VF or AF. In conclusion, there is a link between Ca²⁺-related disorders in contractility and coupling of the cardiomyocytes pointing out a novel paradigm implicated in development of severe arrhythmias.

Key words

Ca²⁺ overload • Heart ultrastructure • Malignant arrhythmias • Rat • Guinea pig • Pig

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Introduction

General classification of cardiac arrhythmias assumes that all rhythm disturbances result from an abnormality in impulse initiation and impulse propagation, whereby both may coexist. The former is particularly associated with triggered activity and/or abnormal automaticity, whereas the latter with conduction block and re-entry (Hoffman and Rosen 1981, Kléber and Rudy 2004). In both clinical and experimental settings enhanced triggered activity may result from early and delayed after-depolarization (Janse 1999) that are attributed mainly to disturbed Ca²⁺ handling (Janse 1999, Pogwizd and Bers 2004). Dysregulated, spontaneous Ca²⁺ release from the sarcoplasmic reticulum (SR) occurs in the form of waves of self-propagating Ca²⁺-induced Ca²⁺ release (Xie and Weiss 2009). Ca²⁺ leaks, suppression of SERCA, activation of reverse mode of Na/Ca exchanger result in Ca²⁺ overload that has been implicated in both ventricular arrhythmias and contractile dysfunction associated with either chronic or acute heart failure (Lakatta 1992, Hasenfuss and Pieske 2002, ter Keurs and Boyden 2007, Laurita and Rosenbaum 2008).

Importantly, high free Ca²⁺ concentration impairs electrical coupling at the gap junctions *via* inhibition of Cx43 channels that can affect conduction

velocity (Kléber 1992, Shaw and Rudy 1997, De Mello 1999). Consequently, cardiac cell-to-cell uncoupling may result in slowing and blocking of conduction facilitating development of re-entrant arrhythmias such as VF and AF (Peters *et al.* 1997, Janse 1999, Tribulova *et al.* 1999, 2001, 2008, 2009, 2010, Spach 2001, Kléber and Rudy 2004). Evidence suggests that focal areas of uncoupling in the myocardium increase the likelihood of arrhythmic triggers and more widespread uncoupling is required to support sustained arrhythmias (Gutstein *et al.* 2001, 2005).

The mechanisms underlying AF are complex involving increased spontaneous ectopic firing of atrial cells and impulse re-entry through the atrial tissue. Triggered activity as well as impairment of Ca^{2+} handling and cell-to-cell coupling that may lead to altered conduction properties and multiple re-entrant circuits are likely implicated in the development of AF (Tribulova *et al.* 1999, Kostin *et al.* 2002, Wakili *et al.* 2011). AF itself (Kostin *et al.* 2002) likewise rapid atrial pacing (Simor *et al.* 1997, Kondratyev *et al.* 2007) facilitates elevation of cytosolic calcium in part due to the increase of intracellular Na^+ concentration and activation of backward mode of NCX.

The most life-threatening arrhythmias occur in patients or animals with structural heart disease, where arrhythmogenic substrates play an important role. Individuals without structural heart disease may also suffer from arrhythmias (Priori *et al.* 1999, Jiang *et al.* 2014) due to genetically aberrant ionic currents or acute conditions that modify both Ca^{2+} handling and ion channel function (e.g. drugs, oxidative stress).

Despite progressive current therapies both VF and AF remains a major health problem. Further understanding of the mechanisms and factors responsible for the onset and maintenance of these arrhythmias is essential.

We have previously shown (Tribulova *et al.* 1999, 2001, 2003, 2004, 2009) that acute interventions such as low K^+ perfusion, burst atrial pacing or intramyocardial noradrenaline infusion facilitate occurrence of severe cardiac arrhythmias, whereby alterations in intracellular free calcium and intercellular gap junctions are most likely involved. The purpose of this study was to determine by comprehensive manner the impact of Ca^{2+} disorders on ultrastructure of the cardiomyocytes prior to occurrence and during sustaining of life-threatening arrhythmias in healthy hearts.

Methods

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, Publication No 85-23, revised 1996.

All experiments were approved by the Institutional Animal Care and Use Committees.

There are differences in cardiac Ca^{2+} handling between rats and guinea pigs (Lewartowski and Zdanowski 1990), atria and ventricles (Smyrniak *et al.* 2010), small and large mammals (Trafford *et al.* 2013). Therefore, we aimed to explore ultrastructural alterations of the cardiomyocytes in the heart of rats, guinea pigs and landrace pigs (Tribulova *et al.* 1999, 2001, 2003, 2004) subjected to acute conditions to induce Ca^{2+} disturbances and arrhythmias.

Following experimental protocols were used:

1) Low potassium perfusion of isolated rat heart to induce sustained VF. 2) Low potassium perfusion of isolated guinea pig heart to induce sustained VF. 3) Burst atrial pacing of isolated guinea pig heart to induce prolonged AF. 4) Intramyocardial noradrenaline infusion to open-chest pig to induce VT.

Ad 1) Adult Wistar Kyoto rats were anesthetized and the hearts were quickly excised and perfused *via* cannulated aorta at constant pressure with oxygenated Krebs-Henseleit solution containing (in mmol/l) 118 NaCl, 25 NaHCO_3 , 2.9 KCl, 1.2 MgSO_4 , 1.8 CaCl_2 , 1.3 KH_2PO_4 and 11.5 glucose. Bipolar electrocardiogram was continuously recorded. After 20 min of equilibration period in standard Krebs-Henseleit solution, heart was perfused with a low- K^+ (1.2 mmol/l) solution for a period of 60 min, unless sustained two minutes lasting VF occurred earlier (Tribulova *et al.* 2003). The hearts for electron microscopic examination were taken at the end of the equilibration period (n=6), at 15 min of low- K^+ perfusion (n=6), and at 2 min of VF (n=6).

Ad 2) Adult guinea pigs were sacrificed by stunning and the aorta of the excised heart was immediately cannulated for perfusion at constant pressure with oxygenated Tyrode solution (in mmol/l: 136.9 NaCl, 2.8 KCl, 1.8 CaCl_2 , 1.0 MgCl_2 , 11.9 NaHCO_3 , 0.4 NaH_2PO_4 , 11.5 glucose). Bipolar epicardial electrocardiograms were continuously monitored. After 15 min of stabilization with standard solution the heart was perfused with a low K^+ solution (1.4 mmol/l) until sustained VF occurred (as previously reported Tribulova *et al.* 2001). For electron microscopic examination the

hearts were taken during stabilization (n=6), at 15 min of low K⁺ perfusion (n=6) and during 2 min lasting VF (n=6).

Ad 3) Aged 2-year-old guinea pigs were sacrificed by stunning, and the aorta of the excised heart was immediately cannulated for the perfusion at constant pressure with oxygenated modified Tyrode solution (in mmol/l: 126 NaCl, 2.8 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 24 NaHCO₃, 0.4 NaH₂PO₄, 5.5 glucose). After 20 min of equilibration period left atrium was subjected to programmed stimulation by 1 s lasting burst of electrical rectangular pulses, 50 to 70 pps, 1 ms in duration and 1.5 times the threshold voltage. Right atrial electrocardiogram was recorded for incidence of arrhythmias (as previously reported Tribulova *et al.* 1999). Prolonged AF lasting >1 min was induced after 5-15 min of burst pacing. For the examination the atrial tissue was taken during basic conditions (n=6), during 10 min of pacing (n=6) and during 1 min lasting AF (n=6).

Ad 4) Adult domestic landrace pigs were anesthetized, ventilated and subjected to sternotomy. Electrocardiogram and aortic blood pressure were recorded. Left ventricular intramyocardial infusion of 10 µl/min of noradrenaline in the presence of Ca²⁺ (2.5 mmol/l) was used to induce VT. Its occurrence preceded faster contraction of myocardium around the infusion area and VT precipitated consistently after 1 min lasting noradrenaline infusion. The drill biopsies were randomly taken from the area of infusion during basal conditions (n=6), upon 1 min lasting infusion (n=6) and during VT (n=6) (Podzuweit 1980, Tribulova *et al.* 2004).

The transmural needle biopsies were taken from the free left ventricular wall for transmission electron microscopic examinations. Tissue samples were immediately immersed into ice-cold fixation solution. Its composition was 2.5 % glutaraldehyde in 0.1 mol/l sodium cacodylate, pH 7.4. The tissue was then cut into smaller blocks (approx. 1 mm³) and fixed for 3 h at 4 °C. This step was followed by washout with cacodylate buffer and the tissue blocks were post-fixed with 1 % osmium tetroxide for 1 h at 4 °C. After washout with cacodylate buffer the tissue was dehydrated in graded series of ethanol, infiltrated with propylene oxide and embedded into Epon 812. Semi-thick sections (1 µm) were cut and stained with toluidine blue for light microscopic examination in order to choose a representative area of the tissue sample for ultrathin

sectioning. Areas of mechanical damage, frequently present at the periphery of the tissue blocks could be excluded from electron microscopic examination. *En face* ultrathin sections were cut using LKB Ultratome, mounted on copper grids and stained with uranyl acetate and lead phosphate prior examination in an electron microscope Tesla 500. Five random tissue blocks and five tissue sections per heart were evaluated and time point noted in experimental protocol. Ten images per section were taken using electron microscopic plates that were developed into black and white photography.

Results

Representative electron microscopic images of cardiomyocyte myofibrils and their contractile units, sarcomeres, demonstrated in four distinct patterns are shown in Figure 1. During normal heart conditions the regular contraction is followed by relaxation and these processes are visible according to the relaxed (Fig. 1A) and contracted state of sarcomeres (Fig. 1B). Pathophysiological conditions particularly those accompanied by disturbances in cytosolic [Ca²⁺]_i result in disturbances of contraction-relaxation processes. These are recognized by abnormal patterns of sarcomeres reflecting on over-contraction and/or hyper-contraction of cardiomyocytes (Fig. 1C) and even presence of contracture with contraction bands (Fig. 1D). The latter is considered as severe irreversible change jeopardizing function of the cardiomyocytes. As demonstrated in Figures 3, 4, 5 and 6 an abnormal pattern and nonuniform sarcomere shortening could be seen regardless the pathophysiological model referred in this study.

Synchronized myocardial contraction depends on functional adhesion and electrical coupling among cardiomyocytes. Representative electron microscopic images of cardiac cell-to-cell junctions are demonstrated in Figure 2. Adhesive junctions, desmosomes and fascia adherens ensure mechanical contacts for contractile force propagation between neighboring cardiomyocytes. While communicating junctions, gap junctions, ensure electrical contact for action potential propagation. End-to-end type of gap junctions is related to intercalated disc (Fig. 2A) and predominates in ventricles. In addition, side-to-side type is frequent in atria (Fig. 2B). Various cardiac pathologies accompanied by elevation of intracellular free calcium concentration as well as aging promote electrical (Fig. 2C) and mechanical (Fig. 2D) uncoupling.

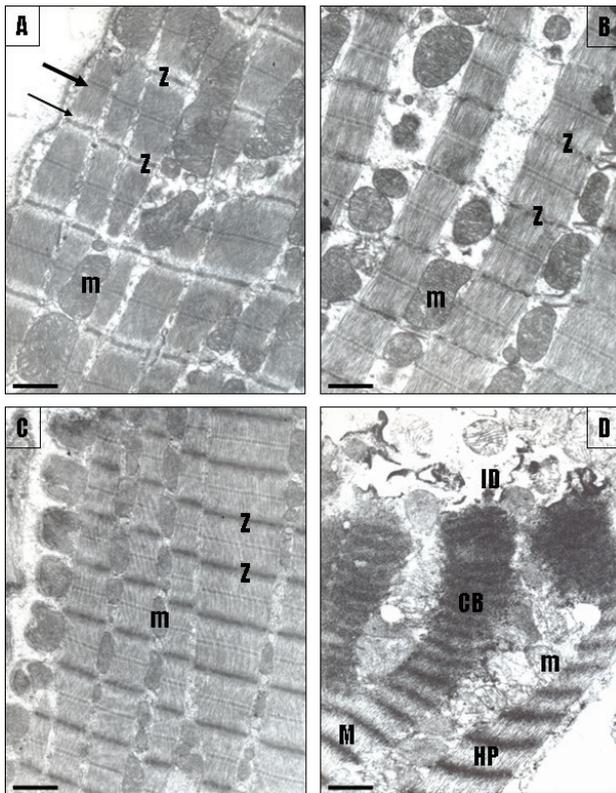


Fig. 1. Electron microscopic appearance of cardiac myofibrils shows distinct patterns of their contractile unit, sarcomere, in baseline conditions (**A,B**) and in conditions of abnormal Ca^{2+} handling and Ca^{2+} overload (**C,D**). **A** – Sarcomeres as delineated by Z lines and composed of myosin (thick arrow) and actin (thin arrow) are in uniform relaxed state. **B** – All sarcomeres are in contracted state evidenced by disappearing of actin areas. **C** – Sarcomeres in over-contracted state can be recognized when Z lines are widened and myosin band is compressed. **D** – Nonuniform sarcomere shortening in cardiomyocyte myofibrils exhibiting hyper-contraction (HP) with moderate disruption of myofilaments (M) or severe disruption and clumping of myofilaments into contraction bands (CB). Normal appearance of mitochondria (m) in A, B, C while swollen with ruptured cristae in D. ID – intercalated disc. Scale bar – 1 μm .

The former is recognized when neighboring cardiomyocytes differ in contractile state, i.e. one is relaxed and another contracted (Fig. 2C). Presence of internalized annular profile of gap junction points out the reduced cell-to-cell coupling impairment of functional electrical communication as well (Fig. 2E,F).

Low potassium perfusion of isolated rat and guinea pig hearts and occurrence of sustained VF

Within 10-20 min of perfusion of rat heart with a K^+ deficient Krebs-Henseleit solution the incidence of premature beats, bigeminy and transient VT and/or VF were registered and these preceded the development of sustained VF. This lethal arrhythmia occurred during 20-40 min of low- K^+ perfusion (as previously reported

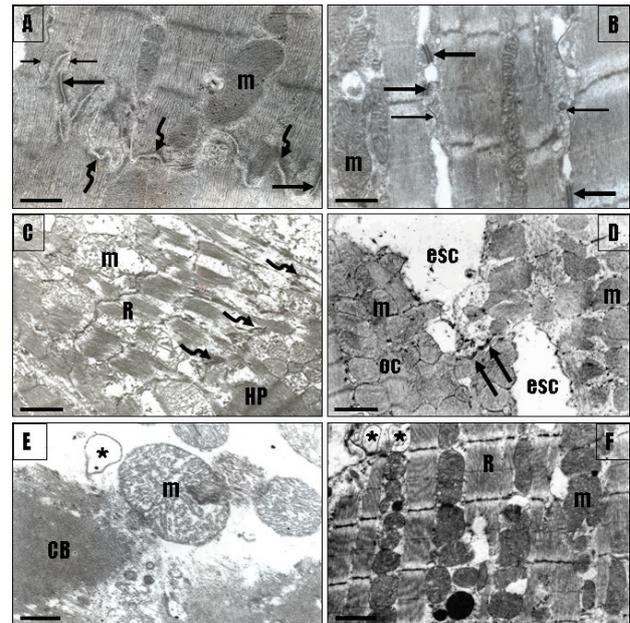


Fig. 2. Typical sub-cellular appearance of two types of cardiomyocyte junctions in normal conditions, i.e. intercalated disc related end-to-end type (**A**) that predominate in ventricles and lateral side-to-side type (**B**) that is frequent in atria. Cardiac cell-to-cell junctions compose adhesive junctions, fascia adherens (curved arrows) and desmosomes (thick arrows) as well as communicating, gap junctions (thin arrows). Note that the aged heart is characterized by the deterioration of intercellular coupling. Moreover, pathological conditions that induce elevation of intracellular free Ca^{2+} promote electrical cell-to-cell uncoupling. This is recognized by asynchrony of contraction between neighboring cardiomyocytes (**C**). Reduced coupling may also result from internalization (asterisks) of gap junctions, seen as annular profiles (**E,F**). m – mitochondria, R – cardiomyocyte in relaxed state, HP – cardiomyocytes in hypercontracted state, OC – overcontracted sarcomeres, CB – contraction bands due to severe injury of integrity of actin and myosin filaments, esc – extracellular space. Scale bar – 1 μm .

Tribulova *et al.* 2003). Continual recordings of ventricular bipolar electrocardiograms during low K^+ perfusion of guinea pig heart revealed changes in R and T configuration, changes in the R vector, incidence of premature beats, bigeminy, sudden VT that usually degenerated into VF. Sustained VF appeared within 15-30 min of low K^+ perfusion (Tribulova *et al.* 2003).

Myocardial ultrastructure alterations. In comparison to the normal subcellular architecture of the cardiomyocyte and preserved intermyocyte junctions (Fig. 1A,B, Fig. 2A,B), myocardial tissue of the heart subjected to K^+ -deficient perfusion was characterized by pronounced heterogeneity of subcellular injury (Fig. 3, Fig. 4). Accordingly, majority of the cardiomyocytes exhibited subcellular changes indicating reversible injury. On the other hand, some cardiomyocytes were

irreversibly injured. As shown on the representative electron microscopic images, perfusion of either rat (Fig. 3) or guinea pig (Fig. 4) hearts with K^+ deficient solution caused: 1) irregular contraction of majority of the cardiomyocytes, whereby over-contraction and hypercontraction of sarcomeres or contraction bands were sporadically observed (Fig. 3A,B, Fig. 4A,B); 2) apparent alterations in intercellular junctions. Accordingly, besides normal connections of neighboring cardiomyocytes with adhesive junctions and gap junctions at the intercalated discs, internalized gap junctions seen as annular profiles were detected (Fig. 3C, Fig. 4C). Furthermore, dehiscence of adhesive junctions was frequently found (Fig. 3B). Impairment of gap junction mediated cell-to-cell coupling was recognized when neighboring cardiomyocytes differ in patterns of sarcomeres, i.e. contracted in one and relaxed in adjacent cardiomyocyte (Fig. 3B, Fig. 4B). Overall, these changes were more pronounced due to transient arrhythmias that preceded occurrence of sustained VF. Marked deterioration of ultrastructure indicating cardiac cell-to-cell uncoupling and dysregulation of synchronized contraction were observed in fibrillating myocardium (Fig. 3D, Fig. 4D).

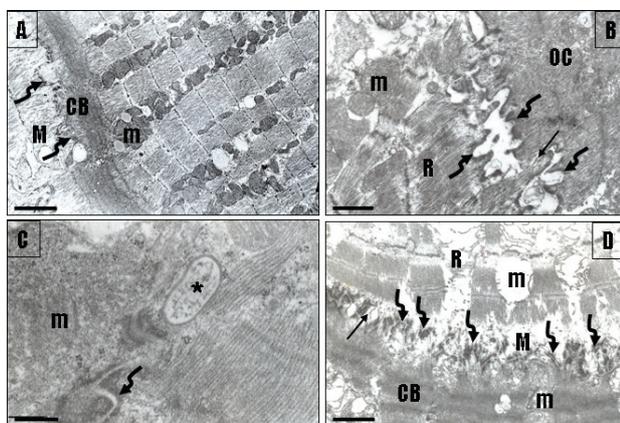


Fig. 3. Representative electron microscopic images show nonuniform sarcomere shortening of myofibrils and abnormalities of intercellular junctions in the rat heart submitted to low K^+ perfusion. **A** – Note contraction bands (CB) in the vicinity of intercalated disc related junctions and disruption of myofilaments (M) in neighboring cardiomyocytes due to low K^+ -induced $[Ca^{2+}]_i$ disturbances. **B** – Cell-to-cell electrical uncoupling is recognized when cardiomyocyte in relaxed state is connected with contracted ones, while dehiscence of adhesive junctions indicates mechanical uncoupling. **C** – Reduced coupling may also result from internalization (asterisk) of gap junctions, seen as annular profiles. **D** – Occurrence of VF aggravates of Ca^{2+} overload-related injury. **B,D** – Lost of integrity of cardiac cell-to-cell junctions, fascia adherens (curved arrows) and gap junctions (thin arrow). R – cardiomyocytes in relaxed state, OC – cardiomyocyte in overcontracted state. Scale bar – 1 μ m.

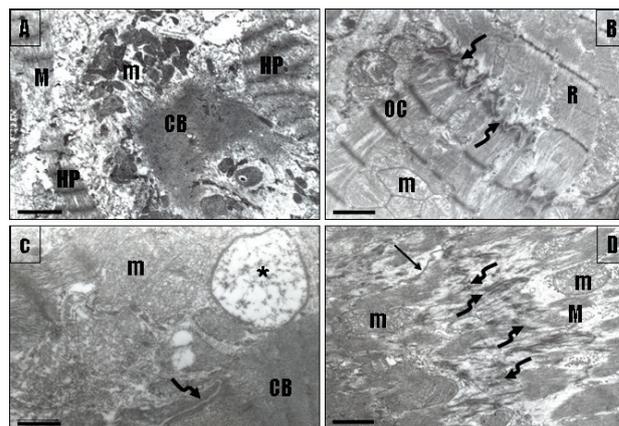


Fig. 4. Representative electron microscopic images show nonuniform sarcomere shortening of myofibrils and abnormalities of intercellular junctions in a guinea pig heart submitted to low K^+ perfusion. **A** – Note hypercontraction (HP) and contraction bands of myofibrils (CB) due to low K^+ -induced $[Ca^{2+}]_i$ disturbances. **B** – Cell-to-cell electrical uncoupling at the gap junctions is recognized when cardiomyocyte in relaxed state (R) is connected with overcontracted (OC) ones. **C** – Internalization of gap junctions (asterisk) is frequently found that may indicate reduced coupling. **D** – Occurrence of VF aggravates Ca^{2+} overload-related injury of the mitochondria (m), myofibrils (M) and intercellular adhesive junctions (curved arrows) and gap junction (thin arrow). Scale bar – 1 μ m.

Burst atrial pacing of isolated guinea pig heart and occurrence of AF

During the first 5 min of repetitive stimulation, a few brief post-stimulus arrhythmias, atrial tachycardias and fibrillo-flutters were recorded. Within 5-30 min period of burst pacing, prolonged poststimulus AF lasting 0.5-15 min was detected. At the same time, no arrhythmias were registered in the left ventricle (for details see Tribulova *et al.* 1999).

Myocardial ultrastructure alterations. Normal appearance of atrial subcellular structure was observed in basal conditions (Fig. 2B). As also seen in Figure 1A,B, cardiomyocytes exhibited uniform pattern of sarcomeres, either in contracted or relaxed forms. Both types of gap junctions were observed, i.e. intercalated disc related end-to-end form (Fig. 2A) and lateral side-to-side gap junctions (Fig. 2B). Loss of integrity of lateral connections (Fig. 2D) was sporadically present. Prolonged burst pacing that was accompanied by transient post-stimulus arrhythmias resulted in subcellular injury of the cardiomyocytes of various degrees. Some cardiomyocytes were more and some less affected. Subcellular changes consisted of mitochondria alterations, nonuniform sarcomere shortening and impairment of cardiac cell-to-cell coupling (Fig. 5A,C). These changes preceded occurrence of prolonged AF, which aggravated further injury of the cardiomyocytes and their junctions (Fig. 5D).

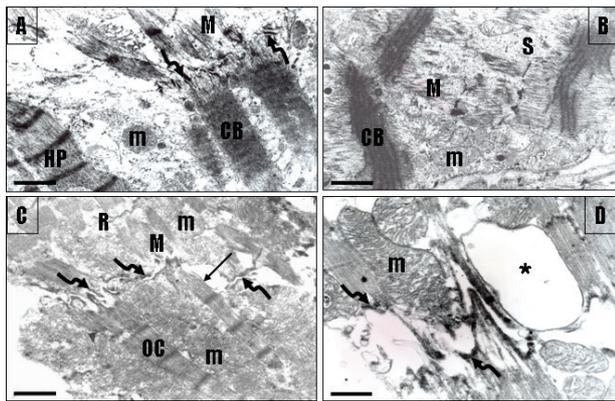


Fig. 5. Representative electron microscopic images indicating disordered contraction of myofibrils and abnormalities of intercellular junctions in the heart that underwent burst atrial stimulation to induce AF. **A,B** – Note contraction bands (CB) and disruption of myofilaments (M) as well as stretched (S) sarcomeres. **C** – Cell-to-cell electrical uncoupling at gap junctions is recognized when neighboring cardiomyocytes are connected with over-contracted (OC) ones. Moreover, dissociation of fascia adherens junctions (curved arrows) and loss of integrity of gap junctions (thin arrow) is seen. **D** – Aggravation of Ca^{2+} overload-related injury with pronounced deterioration of cell-to-cell adhesive (curved arrow) and gap junctions (asterisk) is detected during AF. Scale bar – 1 μm .

Intramyocardial noradrenaline infusion and occurrence of VT in open-chest pig heart

Focal left ventricular intramyocardial infusion of noradrenaline in the presence of calcium in open-chest pigs consistently produced VT within 60 s. Prior to the development of VT, triggered activity was recognized according to focally enhanced contractility in the area of infusion. Premature beats initiated VT that could be maintained for 30 min (Podzuweit 1980, Tribulova *et al.* 2004).

Myocardial ultrastructure alterations.

Noradrenaline infusion resulted in prominent Ca^{2+} -overload-related changes consisting of nonuniform pattern of myofibrils in individual cardiomyocytes, i.e. exhibiting dramatic shortening of sarcomere in the vicinity of relaxing sarcomeres (Fig. 6A,B). Impairment of electrical coupling was assumed when neighboring cardiomyocytes exhibited hypercontracted state (Fig. 6C). This was observed particularly during VT (Fig. 6D). Overall feature was characterized by non-uniform alterations of the cardiomyocytes and their junctions throughout myocardium in the area of infusion.

Discussion

In this study we characterized the subcellular changes in the hearts subjected to various acute

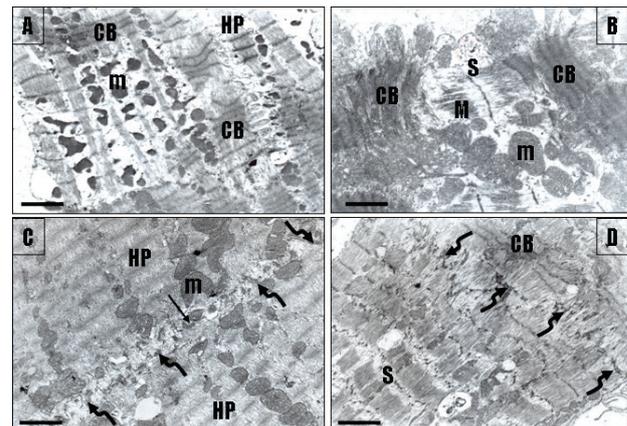


Fig. 6. Representative electron microscopic images show nonuniform sarcomere shortening indicating Ca^{2+} related disordered contraction of myofibrils in the heart submitted to local intramyocardial noradrenaline infusion. **A** – Note three distinct patterns of sarcomeres, i.e. contracted in the vicinity of hypercontracted (HP) and those exhibiting of contraction bands (CB). **B** – Disordered contraction of myofibrils with the presence of stretched sarcomeres (S). **C** – Cell-to-cell electrical uncoupling is assumed when neighboring cardiomyocytes are in hypercontracted state (HP) and integrity of intercellular adhesive (curved arrows) and gap junction (thin arrow) is impaired. **D** – Deterioration of disordered contraction of sarcomeres and integrity of cell-to-cell junctions (curved arrows) is seen during VT. Scale bar – 1 μm .

proarrhythmogenic conditions. Comparative findings suggest that there is a common feature of ultrastructural alterations that precede occurrence of life-threatening cardiac arrhythmias regardless the species and cardiac atria/ventricles related differences in Ca^{2+} handling and intercellular coupling. Most importantly, findings demonstrate the impact of disturbances in $[\text{Ca}^{2+}]_i$ and cell-to-cell coupling (key factors implicated in the development of AF and VF) on ultrastructure of the cardiomyocytes and their junctions.

To strengthen this approach, we selected representative electron microscopic images from four types of experiments together in one panel for clear and comparative demonstration. To avoid interference resulting from heart disease, we used healthy animals with intact heart for investigation. Heart was exposed to acute conditions, such as hypokalemia, sudden increase of catecholamines or fast beating rates that in some way imitate clinical proarrhythmogenic events. K^+ deficient perfusion resulted in a decrease of heart rate, prolonged repolarization, lengthened action potential duration and increased $[\text{Ca}^{2+}]_i$ (Tribulova *et al.* 2003, 2009). In parallel with these changes, there was an increase in the incidence of triggered electrical activity manifested by early after-depolarization that was linked with the occurrence of

ventricular premature beats (Tribulova *et al.* 2003, 2009). Focal intramyocardial infusion of noradrenaline resulted in regional positive chrono- and inotropic effects due to an increase of cAMP and shortening of action potential duration promoted delayed after-depolarization and VT induction (Lewartowski and Zdanowski 1990). Cardiac burst pacing *ex vivo* or chronic fast pacing *in vivo* are current models to induce and investigate the mechanisms of AF (Tribulova *et al.* 2008). After prolonged episodes of rapid electrical activity, the atrial action potential was shortened because of a reduction in the I_{Ks} type calcium current (Janse 1999). Moreover, AF is initiated by triggered activity most likely due to Ca^{2+} disturbances.

The hallmarks of normal heart function are both rapid contraction/relaxation of the “working” cardiomyocytes and rapid electrical impulse propagation between them. In such conditions contractile units, sarcomeres are in uniform synchrony. Calcium is crucial for normal cardiac contraction while disturbances of excitation-contraction coupling result in both contractile dysfunction and arrhythmogenesis particularly in cardiac disease but also in acute heart failure (Hohendanner *et al.* 2015). The latter is supported by our study that demonstrates disturbances in synchronized contraction and cardiac cell-to-cell communication in the setting of K^+ deficiency, increased levels of catecholamines or abnormally fast beating rates. These conditions are accompanied by alterations in Ca^{2+} transport systems and cytosolic free calcium that affect not only synchronous heart function but even facilitate occurrence of malignant arrhythmias. Both, AF and VF aggravate further subcellular injury of the cardiomyocytes and thereby their function. Growing lines of evidence suggest that the enhancement of Ca^{2+} influx, spontaneous Ca^{2+} oscillations, Ca^{2+} sparks (i.e. Ca^{2+} leaks from sarcoplasmic reticulum) and Ca^{2+} overload jeopardize systole and diastole and can trigger arrhythmias (Laflamme and Becker 1996, Sarai *et al.* 2002, ter Keurs and Boyden 2007, Bers 2014).

Calcium changes and triggered activity have been investigated mostly in multicellular preparations (e.g. cultured cardiac cells, isolated cardiac muscle) and rarely in whole heart, either ventricle (Minamikawa *et al.* 1997) or atria (Jiang *et al.* 2014). Our approach based on ultrastructure investigation of samples from whole heart experiment allowed detecting overall feature of myocardial alterations as well as alterations on the level of individual cardiomyocytes. Pacing-induced non-uniform Ca^{2+} dynamics in rat atria have been revealed by

the rapid-scanning confocal microscope (Jiang *et al.* 2014), while our examination revealed consequences of such changes by detecting non-uniform sarcomere shortening. The irregular contraction and hypercontraction of sarcomeres strongly indicate intracellular Ca^{2+} disorders. Moreover, we suppose that spontaneous Ca^{2+} waves might initiate observed hypercontractions of sarcomeres while severe Ca^{2+} overload results in extreme sarcomere shortening and contraction bands. Consequently, there was a pronounced asynchrony of sarcomeres within individual cardiomyocytes as well as between neighboring cardiomyocytes. It should be emphasized that myocardial heterogeneity of ultrastructural alterations was apparent in all investigated acute models, i.e. there were more and less affected cardiomyocytes. This heterogeneity may be in part attributed to the degree of defects in cardiac cell-to-cell coupling.

Activated cardiac muscle exhibits a typical sarcomere-length dependence of force development. Results of mechanical measurements on single cardiac myofibrils implied that high stretching is accompanied by irreversible structural alterations within cardiac sarcomeres, most likely thick filaments disarray and disruption binding sites between myosin and titin due to changes in tertiary structure. Loss of a regular thick-filament organization may impair active force generation (Weiwad *et al.* 2000). We have demonstrated that thick myosin filaments can be disrupted in conditions of acute calcium disorders, thereby contributing to the contractile dysfunction. Furthermore, we have shown that pronounced Ca^{2+} disturbances resulting in marked sarcomere length shortening in individual cardiomyocytes are often in the vicinity of intercellular junctions.

The cycles of Ca^{2+} fluxes during normal heart beat which underlie the coupling between excitation and contraction, permit a highly synchronized action of cardiac sarcomeres. However, rapid force changes in nonuniform cardiac muscle (due to the chronic or acute pathophysiological events) may cause arrhythmogenic Ca^{2+} waves to propagate by the activation of neighboring sarcoplasmic reticulum *via* diffusing Ca^{2+} ions and contribute to inhomogeneous sarcomere length (ter Keurs and Boyden 2007). Such Ca^{2+} wave's propagation in individual cardiomyocyte might induce sarcomere alterations as demonstrated in conditions of intramyocardial noradrenaline infusion. Arrhythmogenesis in the failing heart has been associated with excessive, spontaneous SR Ca^{2+} release in the form

of waves of Ca^{2+} -induced Ca^{2+} release causing oscillations in myocyte membrane potential known as EAD and DAD (Pogwizd and Bers 2004, ter Keurs and Boyden 2007, Xie and Weiss 2009). The extent of SR Ca^{2+} leak is important because it can lead to i) reduction of SR Ca^{2+} available for release, causing systolic dysfunction; ii) elevation of diastolic $[\text{Ca}^{2+}]_i$, contributing to diastolic dysfunction; iii) triggered arrhythmias (Bers 2014). It is likely that Ca^{2+} waves might also propagate to the neighboring cardiomyocytes through functional gap junction channels and aggravate contraction disturbances. On the other hand, uncoupling at gap junction channels may inhibit both spreading of “injury” current and spreading of arrhythmogenic Ca^{2+} waves.

Importantly, ultrastructural examination revealed that conditions accompanied by Ca^{2+} overload facilitated occurrence of annular gap junctions. Gap junction plaques, consisting of cluster of connexin channels, exhibit dynamic characteristics resulting in formation and removal from cardiac cell membranes. Annular gap junctions (double-membrane intracellular structures) are in fact internalized gap junctions, and this unique process provides a mechanism for down-regulation of several hundreds of connexin channels at one time. Once internalized, connexins are degraded by lysosomes and proteasomes (Jordan *et al.* 2001). Our findings of enhanced occurrence of annular gap junctions profile in acute proarrhythmogenic conditions suggest that increase in $[\text{Ca}^{2+}]_i$ may facilitate internalization of gap junctions, i.e. to participate in down-regulation of intercellular electrical and metabolic communication between adjacent cardiomyocytes.

Finally, it has been reported that mitochondria can also be implicated in cardiomyocyte Ca^{2+} cycling (Dedkova and Blatter 2013). Mitochondrial Ca^{2+} signaling contributes to the regulation of cellular energy metabolism and participates in cardiac excitation-contraction coupling through their ability to store Ca^{2+} ,

shape the cytosolic Ca^{2+} signals and generate ATP required for contraction. However, controversy remains whether the fast cytosolic Ca^{2+} transients underlying cardiac excitation-contraction coupling in the beating heart are transmitted rapidly into the matrix compartment or slowly integrated by the mitochondrial Ca^{2+} transport machinery. Many questions arise how mitochondria can affect contraction/relaxation in diseased or acutely injured hearts. In this context it is worthwhile to emphasize that in condition of Ca^{2+} disturbances and Ca^{2+} overload that preceded malignant arrhythmia occurrence, we observed various degree of mitochondria injury, i.e. mild, moderate (potentially reversible) and severe (irreversible) alterations.

In conclusion, there is some limitation in this study since despite the demonstration of close association between abnormal Ca^{2+} handling and ultrastructural alterations we have not proved a direct causality between these variables. Nevertheless, our findings suggest that myocardial heterogeneity of high $[\text{Ca}^{2+}]_i$ -related subcellular injury of cardiomyocytes is a common feature that increases propensity of the heart to malignant arrhythmias. Non-uniform sarcomere shortening most likely reflects on cytosolic Ca^{2+} oscillations, disturbances in Ca^{2+} wave propagation and Ca^{2+} overload. These disturbances are associated with impairment of cardiac cell-to-cell coupling that facilitates occurrence of VF or AF and with a disruption of myofilaments that impairs contractile function. These results provide a novel paradigm linking arrhythmogenesis and contractile disorders that may contribute to the acute heart failure.

Conflict of Interest

There is no conflict of interest.

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