Ultrastructural remodelling of Ca\(^{2+}\) signalling apparatus in failing heart cells

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Aims

The contraction of a heart cell is controlled by Ca\(^{2+}\)-induced Ca\(^{2+}\) release between L-type Ca\(^{2+}\) channels (LCCs) in the cell membrane/T-tubules (TTs) and ryanodine receptors (RyRs) in the junctional sarcoplasmic reticulum (SR). During heart failure, LCC–RyR signalling becomes defective. The purpose of the present study was to reveal the ultrastructural mechanism underlying the defective LCC–RyR signalling and contractility.

Methods and results

In rat models of heart failure produced by transverse aortic constriction surgery, stereological analysis of transmission electron microscopic images showed that the volume density and the surface area of junctional SRs and those of SR-coupled TTs were both decreased in failing heart cells. The TT–SR junctions were displaced or missing from the Z-line areas. Moreover, the spatial span of individual TT–SR junctions was markedly reduced in failing heart cells. Numerical simulation and junctophilin-2 knockdown experiments demonstrated that the decrease in junction size (and thereby the constitutive LCC and RyR numbers) led to a scattered delay of Ca\(^{2+}\) release activation.

Conclusions

The shrinking and eventual absence of TT–SR junctions are important mechanisms underlying the desynchronized and inhomogeneous Ca\(^{2+}\) release and the decreased contractile strength in heart failure. Maintaining the nanoscopic integrity of TT–SR junctions thus represents a therapeutic strategy against heart failure and related cardiomyopathies.

Keywords

Heart failure • Ultrastructure • Calcium channel • Excitation–contraction coupling

1. Introduction

In the heart, periodic Ca\(^{2+}\) transients determine the pace and strength of cell contraction.\(^1\) These Ca\(^{2+}\) transients are generated by the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism,\(^2\) in which the Ca\(^{2+}\) influx through voltage-dependent L-type Ca\(^{2+}\) channels (LCCs) in the cell membrane or T-tubules (TTs) activates ryanodine receptor (RyR) Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR).\(^3\) Intuitively, CICR is a positive feedback, and local Ca\(^{2+}\) signals would be expected to evoke explosive chain reactions.\(^4\) However, the Ca\(^{2+}\) release of RyRs in heart cells is modulated precisely in the form of both global Ca\(^{2+}\) transients and local Ca\(^{2+}\) release events, known as Ca\(^{2+}\) sparks.\(^5\) As the key mechanism to avoid regenerative self-excitation, RyRs and LCCs are found to cluster into discrete CICR units in TT–SR junctional structures.\(^6\) Within each junction, the Ca\(^{2+}\) influx through LCCs travels across an \(~15\) nm junctional cleft, and activates RyR Ca\(^{2+}\) release at a high signalling fidelity.\(^6\) Between adjacent junctions, however, the longer distance effectively prevents CICR crosstalk.\(^7\) These structural features of junctions enable the local control of RyR Ca\(^{2+}\) release, and are therefore among the fundamental factors determining the healthy status of CICR and contractility in heart cells.

Heart failure is the final stage of cardiac remodelling caused by hypertension, ischaemia, cardiomyopathy, and other chronic factors.\(^8\) Numerous cellular and molecular changes have been identified in failing hearts, and the key symptom that eventually leads to cardiac death is the progressive decrease in myocardial contractility.\(^9\) In failing heart cells, the degraded contractility is at least

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partly attributed to defective CICR signalling, in which the LCC Ca\(^{2+}\) influx cannot trigger sufficient Ca\(^{2+}\) release from RyRs.\(^\text{12–14}\)

It has long been hypothesized that the defective CICR in failing heart cells might be due to a prolonged mean distance between LCCs and their neighbouring RyRs.\(^\text{13}\) However, this hypothesis has not yet been tested experimentally in failing heart cells. Although previous electron microscopic studies have shown that the TTs and SRs may either be enlarged\(^\text{5,16}\) or unchanged\(^\text{7–19}\) depending on different experimental models of hypertrophy, the ultrastructural change of TT–SR junctions in heart failure has not been examined. Recently, it has been shown that the TT system in failing heart cells is destructed\(^\text{20}\) due to decreased expression of junctophilin-2 (JP2), a membrane protein anchoring SRs to TTs in cardiac cells.\(^\text{21}\) JP2 knockdown indeed leads to disrupted TTs\(^\text{22}\) and defective CICR.\(^\text{23}\) Despite these important progresses, whether and how the Ca\(^{2+}\) signalling ultrastructure is remodelled in failing heart cells and how the structural remodelling contributes to defective contraction remain unknown.

In order to probe the ultrastructural mechanism of the defective Ca\(^{2+}\) signalling in failing heart cells, we created rat heart failure models by transverse aortic constriction (TAC) surgery and examined the structure of TT–SR junctions by transmission electron microscopy (TEM). By applying and developing morphometric analysis, we demonstrated that the TT–SR junctions became smaller, fewer, and mis-positioned in failing heart cells, which provides novel mechanisms underlying the desynchronized and inhomogeneous Ca\(^{2+}\) release in heart failure.

2. Methods

A detailed description of the methods can be found in the Supplementary material online.

2.1 Rat heart failure model

The investigation conforms to the Guide for Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University. A model of heart failure induced by pressure-overload was created by performing TAC surgery in rats.\(^\text{14}\) About 12–15 weeks after aorta banding, a pedal pinch reflex were completely inhibited, the heart was rapidly excised from the animal and perfused on a Langendorff system. Cells were subjected to electrophysiological and biochemical measurements 48 h after infection.

2.3 Whole-cell patch clamp and confocal imaging

The cells were immersed in an extracellular solution which contains (in mM): 137 NaCl, 4.0 KCl, 1.0 CaCl\(_2\), 1.2 MgCl\(_2\), 1.2 NaH\(_2\)PO\(_4\), 10 glucose, 0.02 tetrodotoxin, and 10 HEPES, pH 7.35 adjusted with NaOH. The patch pipette solution contained (in mM): 105 CsCl, 15 TEA\(_2\)Cl, 5 Na\(_2\)ATP, 10 HEPES, 10 EGTA, 5 CaCl\(_2\), and 0.2 fluo-4 pentapotassium (Molecular Probes, Eugene, OR, USA), pH 7.2 adjusted with CsOH. Myocytes were depolarized in whole-cell patch clamp configuration by 300 ms pulses from –70 to 0 mV using an EPC7 amplifier at room temperature (25°C). Ca\(^{2+}\) spikes were recorded by confocal line-scan using a Zeiss LSM-510 inverted confocal microscope at a sampling rate of 1.54 ms/line.\(^\text{14}\) The threshold of spike detection was set at 0.2 ΔF/F0.

2.4 TEM and stereological measurement

Cardiomyocytes were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in PBS, and post-fixed in 0.1 M sodium cacodylate buffer containing 2% osmium tetroxide and 0.8% potassium ferrocyanide.\(^\text{25}\) After dehydration through an ethanol series, the specimens were embedded in Spurr resin. Thin sections were stained with uranyl acetate and lead citrate, and observed under a JEM-1010 TEM. For stereological measurement of the volume density and surface area of TTs and JSRs, we followed Mobley’s stereological method.\(^\text{26}\)

2.5 Model simulation

We used a geometry-based three-dimensional model of stochastic CICR\(^\text{27}\) to estimate the time interval between depolarization and the start point of RyR Ca\(^{2+}\) release by numerical simulation, taking into account the Ca\(^{2+}\) release by CRUs, the Ca\(^{2+}\) uptake by SERCA, and the Ca\(^{2+}\) buffer effect.

2.6 Statistics

All data are presented as mean ± SEM. Statistical analysis was performed using Student’s t-test for normal test-past data or the Mann–Whitney rank sum test, unless otherwise specified. A value of P ≤ 0.05 was considered significant.

3. Results

3.1 Morphology properties of TT–SR junctions in failing heart cells

To characterize the ultrastructural remodelling of TT–SR junctions in failing heart cells, we created pressure-overload heart failure models by TAC surgery in rats.\(^\text{14}\) About 12–15 weeks after aorta banding, a status of heart failure at the decompensated hypertrophy stage was identified by increased left ventricle thickness and reduced fractional shortening compared with the sham-operated control group (Supplementary material online, Figure S1A and S1B). The JP2 expression in cardiomyocytes was reduced by 63% (Supplementary material online, Figure S1C), agreeing well with previous reports.\(^\text{14,22}\) Ultrathin sections were made along the longitudinal axis of isolated ventricular myocytes and imaged with TEM. We used classic stereological analysis\(^\text{26}\) to quantify the coupling structure between TTs and SRs (Figure 1A). Compared with those in the sham group, the volume density (Figure 1B) and the surface area (Figure 1C) of TTs were reduced by 19.4 and 17.0%, respectively, in failing heart cells. These reductions were attributed to the loss of TTs that coupled to SRs.
The volume density and the surface area of bald TTs (TTs apparently not coupled to SRs) were even increased by 24.3 and 30.4%, respectively. The loss of SR-coupled TTs indicated a detachment of TTs from SRs. Accordingly, the volume density and the surface area of junctional SRs were reduced by 31.6 and 29.5%, respectively (Figure 1B and C).

We noted that the TT–SR junctions, which usually appeared regularly at Z-lines in the sham group, were missing from many of the Z-line areas in failing heart cells (Figure 2A). To quantify this change, we measured the presence rate of junctions in the non-myofilament areas between two adjacent M-lines (inter-M areas, centred by Z-lines). We found that the percentage of inter-M areas that displayed junctions was decreased from 41.8 to 25.6% (Figure 2B). Due to the absence of junctions at their ‘due positions’, the apparent distance between adjacent junctions (as appearing in TEM sections) in the TAC group was prolonged, with its distribution shifted to longer distances (Figure 2C).

We went on to estimate the spatial span of individual junctions by measuring the apparent curvilinear length of parallel TT and SR membranes (yellow line in Figure 4A). The apparent junction length had a logarithmic normal distribution (Figure 4B). Notably, the peak of the distribution was shifted to smaller size in the TAC group, and the average junction length was 29.3% lower than in the sham group (Figure 4C).

### 3.2 Structure–function relationship of junctions

Within a junction of rat cardiomyocyte, LCCs are co-localized with 4–10 times more RyRs, which are self-organized to form a two-dimensional array. A 30% reduction in apparent junction length would predict a 50% decrease in junction size and thereby a proportional decrease in the numbers of RyRs and LCCs within a junction.
Figure 2  Regularity of junction appearance in failing heart cells. (A) Representative TEM images of cardiomyocytes from sham-operated (left) and TAC (right) rats. White arrows indicate TTs coupled with SRs. (B) Junction presence rate measured as the percentage of non-myofilament areas between adjacent M-lines that displayed junctions. For data analysis, 160 and 200 TEM images were taken from 6 sham and 10 TAC rats, and more than 3000 \( \mu m^2 \) of image area were analysed in each group. (C) Distribution and Gaussian fittings of inter-junction distance in sham (white bars and red curve) and TAC (black bars and blue curve) groups. The inter-junction distance was measured from more than 520 junction pairs in each group. Data were from 160 and 200 TEM images of 6 sham and 10 TAC rats, respectively. \( **P < 0.01 \) vs. sham group.

Figure 3  Positioning of TT–SR junctions in failing heart cells. (A) Representative TEM images from the TAC group showing the measurement of distance (red) between the centre of a junction cleft (yellow) and an adjacent Z-line (blue). (B) Distance between junctions and Z-lines (junction-Z distance) compared between sham (white) and TAC (black) groups. Data were measured from more than 780 junctions in each group. Data were from 160 and 200 TEM images of sham \( (n = 6) \) and TAC \( (n = 10) \) rats, respectively. \( **P < 0.01 \) vs. sham group. (C) Distribution of junction-Z distance in sham and TAC groups.
Figure 4 Measurement of individual junction size in failing heart cells. (A) Representative TEM images from the sham group illustrating the selection of a region-of-interest. The junction region (left, red box) was selected and analysed (right). The TT–SR junctional cleft was marked in yellow. The junction length was measured as the curvilinear length of the yellow line. (B) Distributions and logarithmic normal fittings of the length of junction cleft in sham (white bars and red curve) and TAC (black bars and blue curve) groups. (C) Average length of junctions. Data from more than 100 junctions from sham (n = 6) and TAC (n = 10) rats, respectively. **P < 0.01 vs. sham group.

Figure 5 Relationship between junction size and Ca\(^{2+}\) release performance. (A) Schematic diagrams of two junctions composed of 20 LCCs (blue dots) + 100 RyRs (orange squares) and 13 LCCs + 64 RyRs. (B) Simulated distribution of the time delay from depolarization to RyR activation (D\(_{\text{fire}}\)) in junctions composed of different numbers of LCCs and RyRs. (C) Simulated Ca\(^{2+}\) transients generated by a model system composed of junctions with different numbers of LCCs and RyRs.
To estimate the functional consequence of decreased TT–SR junction size, we used a cardiac CICR model\(^{27,29}\) to simulate the performance of junctions containing different numbers of RyRs and LCCs (Figure 5A; for details, see Supplementary material online, Methods and Tables S1 and S2). Given the stochastic nature of voltage-dependent LCC activation and Ca\(^{2+}\)-dependent RyR activation, our simulation showed that a junction with fewer LCCs and RyRs exhibited a more dispersed time delay from membrane depolarization to junction activation (Figure 5B). Assuming that a refractory period prevents the repetitive firing of a junction,\(^{30,31}\) the simulation showed that the Ca\(^{2+}\) transient generated by a set of smaller junctions was markedly lower than that generated by larger junctions, even when the Ca\(^{2+}\) spark parameters were set to be independent of the junction size (Figure 5C). These simulation results well reproduce the degraded and desynchronized Ca\(^{2+}\) release in failing heart cells\(^{14}\) (Supplementary material online, Figure S2), demonstrating the reduction in the individual junction size as a possible mechanism underlying the compromised Ca\(^{2+}\) release in failing heart cells.

To test the above idea experimentally, we sought to suppress the expression of the TT–SR linker protein, JP2, and examine the functional change of junctions. We designed a shRNA specific for JP2 mRNA, and delivered it into cultured rat cardiomyocytes via adenovirus vectors (Supplementary material online, Figure S3A). Comparing with the non-specific control group of cardiomyocytes cultured for the same length of time, the cell size was not changed (Supplementary material online, Figure S3B). JP2 expression in the JP2 shRNA group was suppressed by 42% without altering the expression levels of other CICR proteins and the SR Ca\(^{2+}\) load (Supplementary material online, Figure S4). TEM analysis showed that JP2 knockdown not only decreased the presence rate of TT–SR junctions by 37.5%, but also curtailed the average junction length by 16.7% (Figure 6A). Notably, the presence rate of TT–SR junctions in the control group (the white bar in Figure 6A) was markedly lower than that in freshly isolated cells (the white bar in Figure 2B), agreeing well with the degradation of TTs in cultured cardiomyocytes.\(^{32,33}\) To assess the functional consequence of these structural changes, we analysed the spatiotemporal patterning of local Ca\(^{2+}\) release at individual sarcomeres\(^{34}\) (Figure 6B) during a depolarization from −70 to 0 mV. Whole-cell current recording showed that JP2 knockdown did not alter LCC Ca\(^{2+}\) current (Figure 6C), yet the spike amplitude was greatly reduced in the JP2 shRNA group (Figure 6D). By calculating the percentage of sarcomeres that failed to fire Ca\(^{2+}\) spikes higher than 0.2 ΔF/F0, we derived a chance of failure of local Ca\(^{2+}\) release, which was increased by several fold after JP2 knockdown (Figure 6E). For those sarcomeres displaying Ca\(^{2+}\) spikes, the time delay from depolarization to the peak of each Ca\(^{2+}\) spike (\(D\_\text{spike}\), in Trace a in Figure 6B) was more dispersed in failing heart cells (Figure 6F), with an increased standard deviation of \(D\_\text{spike}\) (Figure 6G). The increases in the spike failure index and standard deviation of \(D\_\text{spike}\) agreed well with the absence (Figure 6A upper) and shrinkage (Figure 6A lower) of junctions, respectively, supporting the idea that the ultrastructural remodelling of individual junctions may underlie the desynchronized and inhomogeneous Ca\(^{2+}\) release in heart cells.

4. Discussion

In failing heart cells, the cell-wide Ca\(^{2+}\) transients exhibit a decreased amplitude, which is the major reason for the compromised myocardial contractility.\(^{13}\) At the sub-cellular level, decreased Ca\(^{2+}\) transients are associated with inhomogeneity and desynchronization of Ca\(^{2+}\) release.\(^{12,14,34}\) Studies on feline failing cardiomyocytes have shown that alterations in early repolarization of the action potential compromise LCC Ca\(^{2+}\) influx and lead to inhomogeneity and desynchronization of Ca\(^{2+}\) transients.\(^{11}\) Under whole-cell voltage clamp conditions, where the action potential difference is excluded, failing rat cardiomyocytes also exhibit increased chance-of-failure and scattered delay in activating RyRs. Given that the LCC current is unchanged in these models,\(^{13,14}\) this phenomenon suggests that additional factors underlie the inhomogeneity and desynchronization of Ca\(^{2+}\) release. The present study identified TT–SR morphology as another important factor involved in the inhomogeneous and desynchronized Ca\(^{2+}\) release in heart failure.

The junctions between TTs and SRs constitute the structural basis for highly localized LCC–RyR communication. Although early studies have characterized TTs and SRs in hypertrophic models,\(^{17,19}\) their ultrastructural change in heart failure has not been characterized. Recent fluorescent imaging studies have shown that failing heart cells have a disorganized TT system,\(^{22,23}\) leaving many RyRs detached from LCCs.\(^{20}\) This morphological remodelling of the TT system has been attributed to the decreased expression of JP2, because JP2 knockdown indeed disrupts the TT system.\(^{22,23}\) However, whether and how the TT–SR junctions are altered in heart failure were still unclear. It has been reported that the density of TTs associated with SRs was decreased in a post-infarction rat model of heart failure.\(^{36}\) In the present study, we showed further that the junctions became smaller, fewer, and displaced in failing heart cells. These morphological changes agreed well with the absence and desynchronization of Ca\(^{2+}\) release spikes. As these structure and functional remodelling were fully reproduced in JP2-knockdown cardiomyocytes, our data demonstrated that the loss of junctions due to JP2 down-regulation is a major mechanism underlying the compromised Ca\(^{2+}\) release in failing heart cells.\(^{33}\)

It has long been hypothesized that the defective E–C coupling in failing heart cells has to do with possible mis-arrangement between LCCs and RyRs.\(^{15}\) As the concentration of Ca\(^{2+}\) trigger generated by LCCs decays by orders of magnitude with the distance, the CICR is highly dependent on the distance between LCCs and RyRs.\(^{37–39}\) Three hypothetic models have been proposed to explain decreased EC coupling gain: mismatch in LCC and RyR locations, increased gap between SR and TT membranes, and orphaned RyRs due to TT reorganization.\(^{40}\) Recent morphological measurements\(^{20}\) and the present study consistently support the orphaned RyRs model. Here, our data suggested two new models: shrink in size and shift in positioning (Supplementary material online, Figure S5). We demonstrated quantitatively that the TT–SR junction size is decreased in failing cardiomyocytes. This decrease is attributable to JP2 down-regulation, because suppressing JP2 expression not only reduced the number but also shrink the size of junctions. This finding suggested that, the JP2 down-regulation-induced detachment between SRs and TTs may begin preferentially from the periphery of a junction. With the progressive partial detachment, more and more RyRs become detached from LCCs, leading to decreased efficiency of CICR. The gradual detachment would also eventually lead to the loss of junctions. In accordance, local Ca\(^{2+}\) release, visualized as Ca\(^{2+}\) spikes, were absent at increased number of sarcomeres, leading to inhomogeneous and desynchronized Ca\(^{2+}\) release in failing and JP2-knockdown cardiomyocytes.
Figure 6 Effect of JP2 knockdown on the structure and function of TT–SR junctions. (A) Presence rate (upper) and length (lower) of junctions in the inter-M area in control and JP2 shRNA groups. Data for the upper panel were collected from 134 and 171 TEM images from three individual experiments in either the control or JP2 shRNA group, and more than 2500 μm² of image area were analysed in each group, and more than 300 individual junction TEM images were analysed in each group for the lower panel. (B) Typical images (upper) and time courses (lower) of Ca²⁺ spikes evoked by depolarization from −70 to 0 mV in control (left) and JP2 shRNA (right) groups. The black/white strip beside each colour image is a positioning reference of Z-lines derived from the contrast-enhanced fluo-4 fluorescence prior to depolarization, as described by Song et al. The letters a–f denote the sampling positions of the time courses of Ca²⁺ spikes. (C) The I–V curve of $I_{Ca}$ in control and JP2 shRNA group. (D) The spike amplitude of the two groups. (E) Chance of failure of Ca²⁺ spikes measured as the percentage of sarcomeres without Ca²⁺ spikes at their Z-line areas. (F) Delay of Ca²⁺ spikes ($D_{\text{spike}}$) measured as the time from depolarization to the peak of Ca²⁺ spikes as illustrated in (B). The red and blue lines represent the three-parameter-log normal fittings of the $D_{\text{spike}}$ distribution in control and JP2 RNA groups, respectively. The normalized fit curves are compared in the right insert. (G) The standard deviations of $D_{\text{spike}}$ are compared between the two groups. Data from ≥15 cells in ≥4 animals in each group. *$P < 0.05$ and **$P < 0.01$ vs. control group.
An interesting question in the E–C coupling research field has been why LCCs and RyRs are organized into discrete junctions in which multiple LCCs control 4–10 times more RyRs. It is estimated that the mean open probability of LCCs at peak current is only around ≏ 0.03 in normal physiological conditions. This estimate implies that at least 22 LCCs are needed to secure a 50% chance of junction activation (at least one LCC active in a junction). Given the packing array, 22 LCCs controlling ~150 RyRs would occupy a ~400 nm diameter area. This calculation agrees well with the initial estimate of the LCC and RyR numbers in a junction based on electron microscopy, and is supported by the present measurement of junction size. If the RyR number in a junction is much less than ~100, as recently suggested by super-resolution microscopy, the actual chance of junction activation at peak LCC current may be markedly less than 50%. No matter how many LCCs and RyRs in a junction, a reduction in junction size by 17–30%, as occurred in failing and JP2 knockdown heart cells, predicts a 30–50% decrease in the number of LCCs and RyRs within a junction. According to our simulation, this reduction in LCC and RyR numbers would cause a profound delay and dispersion of stochastic junction activation during membrane excitation, leading to decreased global Ca²⁺ transients. This simulation result was fully supported by the Ca²⁺ spike experiments in failing and JP2-knockdown cells, demonstrating junction size as one of the major factors, in addition to those previously known, governing the efficiency of the E–C coupling.

In summary, we have quantitatively investigated the ultrastructural remodellings of the CICR apparatus, known as junctions, in failing heart cells. We demonstrated decreased volume density and displaced position of junctions during heart failure. The absence of junctions from their due positions provides a structural explanation for the inhomogeneity of Ca²⁺ release. As an important finding of the present study, we demonstrated that the reduction in junction size is a novel mechanism underlying the decreased and desynchronized Ca²⁺ release in failing heart cells. These findings emphasize the concept that the structural homeostasis of individual Ca²⁺ release units determines the global performance of Ca²⁺ signalling. Maintaining the nanoscopic integrity of TT–SR junctions thus provides a potential strategy for developing therapeutic treatment against heart failure and other related cardiomyopathies.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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