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Original article

Nanoscale analysis of ryanodine receptor clusters in dyadic couplings of rat cardiac myocytes

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ABSTRACT

The contractile properties of cardiac myocytes depend on the calcium (Ca^{2+}) released by clusters of ryanodine receptors (RyRs) throughout the myoplasm. Accurate quantification of the spatial distribution of RyRs has previously been challenging due to the comparatively low resolution in optical microscopy. We have combined single-molecule localisation microscopy (SMLM) in a super-resolution modality known as dSTORM with immunofluorescence staining of tissue sections of rat ventricles to resolve a wide, near-exponential size distribution of RyR clusters that lined on average ~57% of the perimeter of each myofibril. The average size of internal couplons is ~63 RyRs (nearly 4 times larger than that of peripheral couplons) and the largest clusters contain many hundreds of RyRs. Similar to previous observations in peripheral couplons, we observe many clusters with one or few receptors; however ≥80% of the total RyRs were detected in clusters containing ≥100 receptors. ~56% of all clusters were within an edge-to-edge distance sufficiently close to co-activate via Ca^{2+}-induced Ca^{2+} release (100 nm) and were grouped into 'superclusters'. The co-location of superclusters with the same or adjacent t-tubular connections in dual-colour super-resolution images suggested that member sub-clusters may be exposed to similar local luminal Ca^{2+} levels. Dual-colour dSTORM revealed high co-localisation between the cardiac junctional protein junctophilin-2 (JPH2) and RyR clusters that confirmed that the majority of the RyR clusters observed are dyadic. The increased sensitivity of super-resolution images revealed approximately twice as many RyR clusters (2.2 clusters/μm^3) compared to previous confocal measurements. We show that, in general, the differences of previous confocal estimates are largely attributable to the limited spatial resolution of diffraction-limited imaging. The new data can be used to inform the construction of detailed mechanistic models of cardiac Ca^{2+} signalling.

1. Introduction

The ryanodine receptor (RyR) is the primary calcium (Ca^{2+}) release channel responsible for the release from internal stores in many different cell types including cardiac muscle [1]. The arrangement of these giant tetrameric channels has been classically described as a quasi-crystalline clustered organisation at the junctional (dyadic) clefts between the sarcoplasmic reticulum (SR) and t-tubules [2]. Such clusters are thought to underlie the functional observation of Ca^{2+} sparks [3], producing at a given site (and in a given state of the cell) fairly stereotypic microscopic Ca^{2+} release events [4]; an idea that has been refined in the theory of local control [5]. RyR clusters are seen as punctate labelling densities in confocal immunofluorescence images of rat [6–8] and mouse [9] cardiac myocytes. Spread of regenerative Ca^{2+} waves is thought to correlate with elevated luminal Ca^{2+} levels in the SR [10,11] and the probability of such regenerative propagation may be facilitated by the effective shortening of the longitudinal distances between RyR clusters resulting from the helical dislocations of the z-lines [12,13]. The relatively large inter-cluster distances seen in confocal data (mean of 0.6–1 μm, [6,8]) were thought to serve to reduce the propensity of spontaneous propagation of Ca^{2+} release in myocytes [14,15] as recently reviewed [16].

The understanding of excitation-contraction (E–C) coupling and its complex interplay with nanoscale subcellular structure has evolved with more recent volume electron-microscopy (EM) and other high resolution images of RyRs within ventricular myocytes. Super-resolution microscopy based on single molecule localisation (e.g. dSTORM) has allowed us to study RyR distributions within striated muscle at nanometre-scale resolution, with the larger fields of view typical for light microscopies and the high molecular specificity of immunofluorescence imaging [17–19]. EM studies have suggested a less rigid or static arrangement of receptors within each cluster [20],

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which may reside within nanometre distances from their neighbours [9]. The wide distribution of cluster sizes within peripheral couplons, many containing only a few receptors [18], has raised several fundamental questions: (a) Is a similar distribution observed in the dyadic couplons (in the myocyte interior) that account for a large fraction of the cytosolic Ca^{2+} transient? (b) Are these clusters functionally organised in local 'superclusters'? (c) And if so, how close are clusters (or superclusters) to each other, specifically are they close enough to support the spread of regenerative Ca^{2+} waves via Ca^{2+} induced Ca^{2+} release (CICR) across the z-disk? An additional focus on RyR cluster formation and maintenance is warranted based on the recently reported effects of overexpression of the junctional protein junctophilin-2 (JPH2) on RyR arrangement and dyad area [21] highlighting a need for a more explicit investigation of the spatial relationship between JPH2 and RyR2 in the normal myocardium.

In this study we have combined super-resolution imaging with immunohistochemistry to visualise the Ca^{2+} release sites within extended regions of rat ventricular myocytes at near single receptor resolution. We found that throughout the cross-section of each myocyte RyR clusters are lining a consistent fraction of the myofibril perimeter, at much smaller edge-to-edge spacing than previously suggested by confocal data. The mean cluster size is substantially larger in dyadic couplings than in peripheral couplings. Multicolour dSTORM images also provide evidence that the supercluster grouping of RyR clusters reveals surprisingly large clusters that are strongly aligned with the local t-tubule geometry. With a near-perfect co-localisation with RyR, super-resolution images of JPH2 distribution underline a putative role in the assembly and modulation of RyR cluster function. We also present an analysis to reconcile the data from previous confocal studies with the new super-resolution data presented here. The new data provides a mechanistic basis for detailed models of EC coupling in myocytes, with a particular relevance for the transition to regenerative Ca^{2+} wave spread which is of great importance to understand the development of arrhythmogenic behaviour.

2. Methods

2.1. Sample preparation and immuno-staining

Adult Wistar rats weighing 250–300 g were killed using an intraperitoneal injection of 100 mg/kg sodium pentobarbitone according to a protocol approved by the University of Auckland Animal Ethics Committee. The heart was quickly dissected, cannulated at the aorta, and retrogradely perfused for 10 min with 2% (w/v) paraformaldehyde (Sigma-Aldrich; St. Louis, MO) dissolved in phosphate buffered saline (PBS) at room temperature using a Langendorf perfusion system. The ventricles of the fixed heart were excised and immersed in fresh PBS to wash off excess fixative. Ventricular tissue was diced and cryoprotected in PBS containing 30% sucrose (w/v; Sigma-Aldrich) prior to freezing in methylbutane (Sigma-Aldrich) cooled in liquid nitrogen. 10 μm-thick cryo-sections were cut using a CM 1900 cryostat (Leica, Germany) at −25 °C. Sections were mounted onto No. 1.5 glass coverslips (Menzel-Gläser, Germany) coated with 0.05% poly-L-lysine (Sigma-Aldrich). Cryo-sections were hydrated in PBS and further permeabilised with 1% Triton X-100 (v/v; Sigma-Aldrich) in PBS for 15 min at room temperature and then washed in fresh PBS for further 15 min. Sections were then blocked in Image-iT FX Signal Enhancer (Life Technologies) at room temperature. Primary and secondary antibodies were dissolved in an incubation buffer (see below) and applied to the sample overnight at 4 °C and for 2 h at room temperature respectively. Following each of these steps, sections were washed three times in PBS at room temperature. Samples were then mounted within the dSTORM imaging buffer (described below) and the coverslip was sealed onto a glass microscope slide.

2.2. dSTORM imaging

Slides containing the samples were clamped onto the stage of a modified Nikon TE2000 inverted fluorescence microscope. A custom objective holder and stage bracket were used to minimize drift, with focusing controlled by a piezo focuser (P-725, Physik Instrumente, Germany). The laser beam from a solid-state 671 nm laser (Viasho, China) was focused onto the sample via a 60× 1.49NA oil-immersion TIRF objective (Nikon) in a highly inclined light sheet [22] to achieve an ~10^9 W/m^2 non-TIRF illumination within a 20 μm wide area up to several microns deep within the sample. Emission light was passed through a Q680LP dichroic mirror (Chroma Technology) and an XF3104-690ALP emission filter (Omega optical) prior to being split into two spectral channels using a custom-built splitter device built as described previously [23]. Transverse 2D dSTORM optical sections through ventricular myocytes (tissue sectioned parallel to z-disks) were acquired from regions where the local z-line plane was in focus. Shallow z-stacks of such 2D dSTORM images were constructed by stepping the focus repeatedly through a 1000 nm z-range at 150 nm steps. The emission light in the two channels was recorded onto the two halves of the cooled EM-CCD chip of an IXon DV887DCS-BV camera (Andor Technology, Belfast) in sequences of 20,000–40,000 frames at 20 frames/s.

2.3. dSTORM image analysis

Single molecule events were localised at a typical mean localisation precision of ~13 nm (Alexa 680) and ~17 nm (Alexa 750) (see Supplementary Fig. S1) and spectrally separated into fluorochrome identities using custom-written algorithms implemented in Python and described previously [23]. Residual drift during acquisition was corrected by using a correlation method where several images from events in subsets of the total series of frames were generated and spatially correlated. This allowed drift correction to better than ~5 nm with drift of several hundred nanometres [24]. To minimize the potential for drift induced errors we excluded series that exhibited a total drift exceeding 150 nm. The computed drift time course was used to correct the position of events and generate essentially drift-free coordinates for further processing.

As described above shallow image stacks were acquired by stepping the focus slightly around the estimated location of the z-disk (determined by wide-field focusing of the image prior to dSTORM acquisition). This was done to ensure capturing the z-lines at optimal focus. The data was rendered as a sequence of 2D dSTORM images in which the nominal focus changed by 200 nm between adjacent dSTORM images. We then selected the 2D dSTORM image which captured the z-disk distribution of RyRs with the highest number of in-focus events (out-of-focus events where rejected based on their Gaussian best fit diameter). Point data were rendered into a 2D greyscale TIF image with a 5 nm × 5 nm pixel size for further analysis using a protocol based on a jittered triangulation as described previously [25]. The axial resolution in the resulting 2D dSTORM image was ~600 nm (diffraction-limited).

For analysis of RyR cluster sizes and co-localisation with other proteins, binary masks of the regions of positive labelling were calculated by thresholding the image so that the masked area contained 80% of the total labelling intensity. This choice is consistent with the event densities observed in our experiments and in test simulations correctly extracted cluster extents (Fig. S2). The number of RyR channels within each region of labelling was calculated assuming an isotropic 30 nm centre-to-centre receptor packing density [26]. To convert this into a concentration value, area densities of RyRs at the z-line determined from our data were divided by the normal sarcomere length of 1.8 μm. The binary masks were also used for a colocalisation analysis according to a protocol described by Jayasinghe and others [19]. This analysis generates histograms of the percentage of labelling of protein A as a