Three-dimensional electron microscopy reveals new details of membrane systems for Ca\textsuperscript{2+} signaling in the heart

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Summary
In the current study, the three-dimensional (3D) topologies of dyadic clefts and associated membrane organelles were mapped in mouse ventricular myocardium using electron tomography. The morphological details and the distribution of membrane systems, including transverse tubules (T-tubules), junctional sarcoplasmic reticulum (SR) and vicinal mitochondria, were determined and presumed to be crucial for controlling cardiac Ca\textsuperscript{2+} dynamics. The geometric complexity of T-tubules that varied in diameter with frequent branching was clarified. Dyadic clefts were intricately shaped and remarkably small (average 4.39 x 10\textsuperscript{5} nm\textsuperscript{3}, median 2.81 x 10\textsuperscript{5} nm\textsuperscript{3}). Although a dyadic cleft of average size could hold maximum 43 ryanodine receptor (RyR) tetramers, more than one-third of clefts were smaller than the size that is able to package as many as 15 RyR tetramers. The dyadic clefts were also adjacent to one another (average end-to-end distance to the nearest dyadic cleft, 19.9 nm) and were distributed irregularly along T-tubule branches. Electron-dense structures that linked membrane organelles were frequently observed between mitochondrial outer membranes and SR or T-tubules. We, thus, propose that the topology of dyadic clefts and the neighboring cellular micro-architecture are the major determinants of the local control of Ca\textsuperscript{2+} in the heart, including the establishment of the quantal nature of SR Ca\textsuperscript{2+} releases (e.g. Ca\textsuperscript{2+} sparks).

Introduction
Cumulative evidence suggests that highly elaborated cellular membrane systems play crucial roles in the regulation of excitation-contraction (E-C) coupling and other functions in cardiomyocytes (Sommer and Johnson, 1979; Forbes and Sperelakis, 1984; Bers, 2001; Soeller and Cannell, 2004; Franzini-Armstrong et al., 2005; Song et al., 2005). Our current model posits that the depolarization of the plasma membrane triggers a Ca\textsuperscript{2+} influx through the dihydropyridine receptor (DHPR), which in turn activates a bulk Ca\textsuperscript{2+} release from sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR) – a process known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) (Fabio and Fabiato, 1975; Bers, 2001). The ‘local control’ theory asserts that the CICR is an aggregate of discrete small functional units and consists of mathematically predictable couplings of DHPRs and RyRs in clusters, which were termed ‘coupflons’ by Stern et al. (Stern, 1992; Stern et al., 1997); the theory readily gained popularity along with the successful visualization of unitary Ca\textsuperscript{2+} release events, i.e. ‘Ca\textsuperscript{2+} sparks’ using fluorescent chemical indicators (Cheng et al., 1993; Bers, 2001; Song et al., 2005).

Immunolabeling and electrophysiology have estimated that more than 50% of cardiac DHPRs in adult mammalian ventricular cardiomyocytes are located in transverse tubules (T-tubules), the uniquely developed network structure made of polymorphic tubular invaginations of the plasma membrane in cardiac and skeletal myocytes (Leeson, 1978; Sommer and Johnson, 1979; Forbes and Sperelakis, 1984; Soeller and Cannell, 1999; Brette and Orchard, 2007). T-tubule membranes appose the junctional cisterns of SR (jSR) with ~15 nm gaps in cardiomyocytes, which Langer and Peskoff termed ‘dyadic clefts’ (Langer and Peskoff, 1996). Electron-dense processes (i.e. junctional processes) or ‘feet’ are observed between the jSR and T-tubules across the dyadic cleft, and scientists have assumed these structures represent RyRs, partly based on the electron microscopic observations of purified RyRs (Inui et al., 1987; Bers, 2001). Thus, one can conclude the dyadic cleft is the structural correlate of a couplon formed between internal sarcolemmal membrane and SR.

Although the elegant ultrastructural studies by Franzini-Armstrong and co-workers (Franzini-Armstrong et al., 1999) described the anatomy of couplons using thin section electron microscopy (EM) nearly a decade ago, we are still lacking the accurate quantitative knowledge of the 3D fine structure of the individual couplon and how couplons are arranged in muscle cells. This gap in our knowledge arises primarily because the couplon is essentially a 3D object, the dimension of which is nearly equivalent to or below the diffraction limit of light microscopy. The organization of a group of couplons associated with a sarcomere spans several microns in 3D, which is beyond the general view field of conventional EM – this is a typical issue in current cell biological research, presenting a challenge to modern microscopic
technologies. In this study, we have applied an advanced EM technology (i.e. electron tomography) for the first time to cardiac membrane systems, revealed the topology of dyadic clefts and defined the anatomical basis of the cardiac E-C coupling, by achieving ~5-8 nm resolution 3D microscopic analyses (McIntosh, 2001; Frey et al., 2006) across multiple sarcomeres in mammalian cardiac muscles.

Results
3D models of cardiac membrane organelles
3D reconstructions were generated from fixed tissues obtained from the adult mouse myocardium. All tissues were stained with heavy metals to improve contrast. Volume reconstructions were obtained using either single axis tomography or dual axis tomography. Fig. 1A shows a computed slice (pixel size, 1.42×1.42 nm) generated from a typical reconstruction; the slice is ultra-thin (1.42 nm). Fig. 1B illustrates the clarity provided by the technology that enabled the identification of the outline contours of T-tubules and jSRs through manual segmentation (Fig. 1C). In this study, jSR was identified as the narrow terminal cistern of the endoplasmic membrane, which was relatively uniform in width, filled with electron-dense contents and adjoined to T-tubules across clefts with electron-dense foot connections (i.e. junctional processes) (Sommer and Johnson, 1979; Forbes and Sperelakis, 1984). The anatomy of the outer membranes of mitochondria was also determined, in light of the accumulating evidence that supports mitochondrial involvements in the local control of Ca2+ dynamics in muscle cells and in other cell types (Csordas et al., 2001; Franzini-Armstrong et al., 2005). The anatomical approximation of mitochondria to SR is already known (Forbes et al., 1985). Stacks of contours drawn on slices were meshed to create 3D models. Fig. 1D,F represents the resulting anatomical models of T-tubules (green), jSR (yellow) and mitochondria (magenta). In some cases, the segmentation was facilitated by adapting and using a selective staining protocol that accentuates T-tubules and SRs (Fig. 2A). The resulting high contrast enabled the semi-automatic segmentation of T-tubules (Fig. 2B).

Ultrasructural details of cardiac T-tubules and jSR in mice
The cardiac T-tubule system in mammals has been recognized as an elaborate network of tubular extensions of sarcolemma, with diameters ranging from 150-300 nm, locating their origins of invaginations at or near the exterior of z-discs (Leeson, 1978; Sommer and Johnson, 1979; Forbes and Sperelakis, 1984; Soeller and Cannell, 1999; Brette and Orchard, 2007). The preceding studies also show that the mouse T-tubule system is polymorphic; it can be likened to a labyrinthian latticework made of transverse and axial tubular elements with varying luminal sizes (Forbes et al., 1984). The current study took advantage of the near nanometer-scale 3D accuracy of electron tomography, and confirmed and further detailed the microanatomy of T-tubules, describing them as the pleiomorphic continuums of elongated membranous invaginations at or near the exterior of z-discs (Leeson, 1978; Sommer and Johnson, 1979) or ‘network-SRs’ (nSRs) (Forbes et al., 1985). We found that jSRs, which have commonly been described as the flat discoidal or oblong expansions of the SR, were in fact planar cisternae that were extremely polymorphous in 3D, forming multiple discrete contacts with both the transverse and the longitudinal branches of T-tubules (Fig. 1D,F; supplementary material Movies 1 and 2). From six volume reconstructions totaling 24.2 μm3, we calculated that the T-tubules occupied 1.53% of ventricular myocyte volume (μm3/μm3) with a surface area density 0.52 μm2/μm3 tissue. By contrast, jSRs occupied 0.57% of myocyte volume (μm3/μm3) with a surface area density of 0.53 μm2/μm3 tissue.

In the documentation of cellular membrane structures, potential artifacts induced during the sample preparation were of major concern. Thus, we tested an alternative sample preparation strategy: a hybrid of chemical fixation and high-pressure freezing/freezing substitution (HPF-FS) (Sosinsky et al., 2007). Tissues are mildly fixed in 4% paraformaldehyde (PFA) with 0.1% glutaraldehyde (GA) prior to HPF-FS. Consequently, we confirmed that the details of the 3D anatomical features of T-tubules and jSRs were comparable between the myocardial specimens prepared by the hybrid method and those prepared by the conventional chemical fixation strategy followed by dehydration and embedding (supplementary material Fig. S1).
Shape, size and 3D distribution of dyadic clefts
In cardiac EM tomograms, we carefully identified anatomical couplings composed of the opposing membranes of T-tubules and jSRs, segmented their anatomy (Fig. 1C, the criteria used for the segmentation are described in the legend) and established 3D structural models of dyadic clefts. The resulting 3D models of dyadic clefts clearly show their extensive geometric intricacy, size variations and scattered distributions (Fig. 3A-C; supplementary material Movie 2).

We measured the size of dyadic cleft spaces in six 3D volume reconstructions and revealed that their size in cardiomyocytes was highly variable, ranging from 9.3×10^3 nm^3 to 3.89×10^5 nm^3 (mean±s.e.m. 4.39×10^4±3.8×10^4 nm^3, median 2.81×10^5 nm^3, n=187) (Fig. 3D). With an estimated cleft height of 12 nm (Langer and Peskoff, 1996; Serysheva et al., 2007), we calculate the area of jSR or T-tubule membranes involved in an average dyadic junction is ~3.66×10^4 nm^2. Of importance is that more than one-third of dyadic clefts were smaller than 1.5×10^4 nm^3 (Fig. 3D). These figures are significantly smaller than the previous estimates (described previously as couplons/dyads) derived from thin-section EM observations (Franzini-Armstrong et al., 1999) [see also Langer and Peskoff (Langer and Peskoff, 1996), who estimated the junctional volume as about 1.5×10^6 nm^3].

Scientists assumed that distances between Ca^2+-releasing sites were sufficiently large in the cytosol (~1 μm) in normal cardiomyocytes and have postulated that such spacing is crucial to maintain a sufficient level of Ca^2+ diffusion between Ca^2+-releasing sites, so that neighboring Ca^2+-releasing sites are insulated in between (Izu et al., 2006; Sobie et al., 2006). As dyadic clefts constitute cell interior Ca^2+-releasing sites associated with T-tubules, we applied computations to measure 3D distances between the 187 dyadic clefts found in the six EM tomograms (Fig. 3E,F).
Two strategies were used. One was the calculation of center-to-center distance between all possible pairs of dyadic clefts, which globally represented the distribution of Ca\textsuperscript{2+}-releasing sites in the myoplasm; the other was the application of a nearest-neighbor algorithm that measured end-to-end distances between the nearest dyadic clefts. As shown in Fig. 3E, a significant fraction of dyadic clefts were closely situated within 1.0 \mu m, whereas the overall pattern of distance distribution was oscillatory, with 1.8-2.0 \mu m periodical intervals. The nearest-neighbor calculation revealed the mean end-to-end distance to the most adjacent dyadic cleft from any given dyadic cleft was 19.9±2.0 nm (Fig. 3F).

Recent studies by others have visualized RyR clusters under light microscopy and used them as the representation of the cellular distribution of Ca\textsuperscript{2+}-releasing sites in cardiomyocytes (Chen-Izu et al., 2006; Soeller et al., 2007). Accordingly, we immunostained tissue specimens that were collected for the EM tomography using antibodies against RyR and observed them in laser-scan confocal microscopy. As previously reported, the study visualized RyR clusters with patchy staining patterns (Fig. 4, insets); we then computed the nearest-neighbor distances (center-to-center) between the RyR clusters in two orthogonal oriented directions. The longitudinal spacing between RyR clusters was relatively regular and measured 2.1±0.015 \mu m (n=400) (Fig. 4A), whereas the transverse spacing was more variable with an average value of 0.76±0.012 \mu m (n=439) (Fig. 4B). The data clearly indicate light microscopic observation did not have a power to dissociate dyadic clefts that were apart with distances less than ~300 nm (note the size range under 0.3 \mu m is completely blank in the histograms shown in Fig. 4, whereas a significant fraction of measurements are within this range in EM tomograms as indicated in Fig. 3E).

**RyR density in adult mouse myocardium**

To understand the correlation between the density distribution of RyRs and the geometry of dyadic clefts, we measured \[^{3}H\]ryanodine binding activities in mouse ventricular tissue homogenates. The saturation binding curve analyses yielded the Bmax as 681±30 fmol/mg of protein (or 56.5±2.5 pmol/g of wet tissue). The curve fitting predicted a single-class binding site within the assayed ryanodine concentration range (1-100 nM).

**Fluctuation of ‘Ca\textsuperscript{2+} spark’ origins in isolated adult mouse cardiomyocytes**

The close proximity between dyadic clefts that serve as Ca\textsuperscript{2+}-releasing sites found in the current study raised the issue of how the topological distribution of dyadic clefts is correlated with functional Ca\textsuperscript{2+}-release events. Thus, we examined the spatial distribution of Ca\textsuperscript{2+} spark origins induced by Ca\textsuperscript{2+} overload in isolated mouse ventricular cardiomyocytes. In this study, a fluorescent Ca\textsuperscript{2+} dye, fluo-4, signals were line-scanned in both the longitudinal (across z-lines) and transverse (across myofilaments) directions using high-speed confocal microscopy, similar to the previous study in rat cells (Parker et al., 1996).

We presumed that consecutive Ca\textsuperscript{2+} sparks occurred in local areas within a short time duration arose from identical hot spots and superimposed the fluo-4 signal intensity plots obtained from the uprisingle phase of successive Ca\textsuperscript{2+} sparks, as illustrated in Fig. 5. Repeated measurements in multiple mouse cardiomyocytes consistently showed that Ca\textsuperscript{2+}-spark origins migrate by several hundreds of nanometers during sequential activations within narrow restricted areas. The fluctuation of Ca\textsuperscript{2+}-spark origins in the z-disc planes (transverse direction, Fig. 5H) was nearly three times higher than that obtained along the longitudinal axis of isolated cells (Fig. 5D). Consequently, we speculate that the locally assembled Ca\textsuperscript{2+} releasing sites that we visualized as asymmetrically clustered dyadic clefts using electron tomography (Fig. 3C; supplementary material Movie 2) constitute hot spots for spontaneous Ca\textsuperscript{2+} release and for Ca\textsuperscript{2+}-spark origins migrating among neighboring Ca\textsuperscript{2+}-releasing sites.

**Fine 3D anatomy of dyads**

Dual axis electron tomography (magnification, ×20,000) provided a significantly higher level of structural details of dyads (Fig. 6; supplementary material Movie 3); the fine-tuned imaging revealed that dyadic clefts were only partially filled with RyR feet. We then determined the subregions of dyadic clefts in which RyR feet were abundantly identified (shown in light blue in Fig. 6C,E). The whole junctional structure shown in Fig. 6 is the composite of three dyadic clefts assembled around the short segment of the middle T-tubule. Fig. 6E,F demonstrate the intra-cleft anatomy of cardiac dyads after the removal of jSR cisternae that are displayed in Fig. 6D. Eight RyR rich subdomains (light blue) that vary in size are mapped on the dyadic T-tubule membrane (white); ~78% of the dyadic cleft spaces are occupied by these RyR-rich subdomains in this junctional structure.

**Membrane-associated electron-dense bridges between the mitochondrial outer membrane and neighboring T-tubules and SRs**

Previous EM tomography reports from our group (Perkins et al., 2001) and others (Csordas et al., 2006) indicated that endoplasmic reticulum and mitochondria are structurally connected in various
Cardiac membrane systems for E-C coupling

In the current study of EM tomography, we also observed electron-dense bridges between mitochondrial outer membranes and nSR (Fig. 7A,D,F), T-tubules (Fig. 7B,D,E,G) or nSR (Fig. 7C-E,H): some of them shared structural similarity to the mitochondrial ‘tethers’ reported by Csordas and co-workers in liver cells (Csordas et al., 2006).

Analyses in a larger tomographic volume revealed mitochondria-associated bridges that were formed against SR were rich at two distinct locations (Fig. 8). Groups of the bridges were laterally assembled on the outer membrane of mitochondria by forming elongated narrow attachments to nSRs (Fig. 8A,B), whereas another set of bridge structures were found adjacent to dyadic clefts (Fig. 8B,C). By contrast, bridges associated with T-tubules were much rarer and were observed at the axial end of mitochondria (Fig. 7E).

Discussion

First, the study confirmed extreme intricacy in the shapes of T-tubules, which have been demonstrated in previous studies at lower resolutions (Leeson, 1978; Sommer and Johnson, 1979; Forbes and Sperelakis, 1984; Soeller and Cannell, 1999; Brette and Orchard, 2007). Taking advantage of geometric accuracy of EM tomography in 3D, we determined the volume and surface density of T-tubules as 1.53% (μm³/μm³) and 0.52 μm²/μm³ of the myocyte, respectively, resolving discrepancy found among previous reports (estimates of the mouse T-tubule volume ranged from 0.81 to 3.24% and surface density calculations from 0.17 to 0.50 μm²/μm³) (Bossen et al., 1978; Page and Surdyk-Droske, 1979; Forbes et al., 1984).

A major focus of the current study was on the near-nanometer scale anatomical understanding of Ca²⁺-releasing sites in cardiomyocytes. In particular, our interest was in dyadic clefts, which serve as cell interior Ca²⁺-releasing sites associated with T-tubules and have been recognized as the major player of E-C coupling in ventricular cardiomyocytes (Langer and Peskoff, 1996; Bers, 2001; Franzini-Armstrong et al., 2005). We notice that the most reliable and frequently referred data source for the anatomy of cardiac Ca²⁺-releasing sites has been the publication by Franzini-Armstrong and co-workers, which gauged random chords of couplons in 2D EM images and postulated that a single mouse internal couplon (i.e. a dyad) held an average of 128 feet (Franzini-Armstrong et al., 1999). The study also estimated the end-to-end distances of internal couplons as 313±21 nm (n=73) in mice. As an alternative way to characterize Ca²⁺-releasing sites, two recent studies measured the size and distribution of RyR clusters in rat ventricular cardiomyocytes in light microscopy (Chen-Izu et al., 2006; Soeller et al., 2007). Taken together, one generally speculates that 100 or more RyRs are accumulated in one Ca²⁺-releasing site.
The cytoplasmic domain of an RyR tetramer was determined as follows. Based on our new topological measurements, we estimate the number of RyRs in dyadic clefts that we observed was large (Fig. 6; Movies 2 and 3); their size is generally small and varies in a wide range. We speculate that methodological differences are the major cause for the discrepancy, although species difference (mouse and 6; Movies 2 and 3), which only one RyR foot was observed. Notably, more than one-third of dyadic clefts are equal or smaller than the size that can accommodate three dyadic clefts. From the complete mesh model (D), jSR membranes are removed to expose eight RyR foot-rich subdomains (the surface meshes of RyR-rich sub-domains are shown in light blue in E) that partially occupy dyadic cleft spaces (the whole dyadic cleft spaces are indicated as the junctional regions of T-tubule membranes, in white). Both jSR meshes and meshes that identify the RyR-rich sub-domains are removed in F. Scale bars: 200 nm.

The quantitative analyses presented in the current study in electron tomography provide a different picture compared with the earlier studies. Dyadic clefts are extremely polymorphic (Figs 3 and 6; Movies 2 and 3); their size is generally small and varies in a wide range. We speculate that methodological differences are the major cause for the discrepancy, although species difference (mouse versus other mammalian species) remains as a potential reason. Thin-section EM might have overestimated the size of dyadic clefts by approximating their shape to oval discoidal geometry. By contrast, as formentioned, the resolution of conventional light microscopy is tied down by the diffraction limit and unable to discriminate closely located dyadic clefts. Based on our new topological measurements, we estimate the number of RyRs in dyadic clefts as follows.

As one of the first 3D structures solved in cryo-microscopy, the cytoplasmic domain of an RyR tetramer was determined as 29×29×12 nm (Serysheva et al., 2007). Accordingly, we estimate the maximum possible number of RyR tetramers in an average size dyadic cleft (4.39×10^5 nm^3) is ~43. The size variation of dyadic clefts that we observed was large (Fig. 3D); the smallest in the six EM tomograms was 9.3×10^5 nm^3, in which only one RyR foot was observed. Notably, more than one-third of dyadic clefts are equal or smaller than the size that can hold ~15 RyR tetramers (15×1.01×10^5 nm^3=1.52×10^5 nm^3). We also estimated RyR numbers in dyadic clefts based on the density of high-affinity ryanodine-binding sites that was measured in the current study (56.5 pmol/g of wet tissue). Assuming that (1) 60% protein is associated with cardiomyocytes in ventricles (Bers and Stiffel, 1993), (2) the density of the myocardium is 1.05 (Vinnakota and Bassingthwaighte, 2004), and (3) there is only one high-affinity ryanodine-binding site per RyR tetramer (Lai et al., 1989) and (4) dyadic clefts accumulate ryanodine-binding sites per μm^3/m^2 (our measurement in the 6 EM tomograms), we calculate that the density of RyR tetramers in dyadic clefts is approximately 17.5 molecules per 10^6 nm^3. Multiplied by the volume, the average size mouse dyadic cleft (4.39×10^5 nm^3) is estimated to contain ~7.7 RyR tetramers. The discrepancy between the two estimates, i.e. the geometry-based calculation of RyR numbers in dyadic clefts and that deduced from the ryanodine-binding measurements, is not solved in the current study; the issue is also raised in a previous study in the rabbit ventricle (150-500 high affinity ryanodine binding sites per μm^2 in jSRs that can maximally pack 1300 RyR/μm^2) (Bers and Stiffel, 1993). Although the discrepancy may arise from the various uncertainties of variables in the arithmetic estimations, it...
is that a significant number of dyadic clefts that we identified in mouse ventricular myocytes corresponded to the size of a RyR cluster that may generate a single spark or the quanta of spark substructure. Nonetheless, our study also detected more than half of dyadic clefts were larger than the size of 20 RyRs, which need other mechanisms, such as coupled gating (Marx et al., 2001; Sobie et al., 2002), to limit the propagation of RyR activations in the cleft. Furthermore, single RyRs or isolated clusters of a few RyRs in small dyadic clefts may act similarly to the ‘rogue RyRs’ that were postulated by Sobie et al. as a mechanism for sparkless Ca\textsuperscript{2+} release from SRs (Sobie et al., 2006).

Significantly, the 3D distribution of dyadic clefts revealed in the current study (Fig. 3) predicts that the dyadic clefts that serve as cell interior Ca\textsuperscript{2+}-releasing sites are highly prone to interplay. In this regard, computational models have predicted that minor changes in distances between Ca\textsuperscript{2+}-releasing sites largely affects their functional coupling (Izu et al., 2006); the 3D alignments between Ca\textsuperscript{2+}-releasing sites probably also modulate their interplay (Koh et al., 2006). The current study estimates that the nearest end-to-end distance of cell interior Ca\textsuperscript{2+}-releasing sites associated with T-tubules (i.e. dyadic clefts) is 19.9 nm on average (Fig. 3F), which is more than one order of magnitude narrower than the previous estimates (Franzini-Armstrong et al., 1999; Izu et al., 2006; Soeller et al., 2007). Apparently, the geometric spacing is not sufficient to maintain the refractoriness of Ca\textsuperscript{2+}-releasing sites and we speculate that an additional mechanism is required for the prevention of catastrophic runaway Ca\textsuperscript{2+} releases in normal hearts and for the establishment of Ca\textsuperscript{2+} sparks. In this connection, the proximity of mitochondria to the dyadic clefts that mitochondrial membrane-associated bridges may stabilize (see Fig. 8), could enable the drainage of Ca\textsuperscript{2+} and could attenuate the Ca\textsuperscript{2+}-dependent link between of Ca\textsuperscript{2+}-releasing sites, as proposed previously (Seguchi et al., 2005).

Although these mitochondrial outer membrane-associated electron-dense structures have been witnessed (Sharma et al., 2000; Csordas et al., 2001; Lukyanenko et al., 2007), the current study is the first report to determine their 3D details and cellular distributions in cardiac cells. The density of these structures was unexpectedly high (Fig. 8); we attribute the failure of previous EM studies that rarely documented these structures to the general difficulties in understanding fine 3D structures from multiple 2D images; microstructures are also obscured within the thickness (50-100 nm) of typical thin sections produced for conventional EM observations. The molecular identity of these structural bridges associated with mitochondria remains unclear (Franzini-Armstrong, 2007).

In conclusion, the current study used an advanced 3D visualization technology, electron tomography, to determine the meso-scale (from \( \sim 5 \times 10^{-9} \) to \( 10^{-8} \) m) ultrastructure of cardiac membrane systems. We uncovered novel topological complexity of membrane systems that control cardiac Ca\textsuperscript{2+} dynamics; the size of cell interior Ca\textsuperscript{2+}-releasing units is highly variable but could be anatomically defined as the quantum unit of a Ca\textsuperscript{2+} spark. We also demonstrated frequent physical links between mitochondria and other membrane organelles. In the cardiomyocyte, mitochondrial Ca\textsuperscript{2+} levels oscillate beat-by-beat (Robert et al., 2001); mitochondrial Ca\textsuperscript{2+} levels in the matrix also regulate mitochondrial oxidative metabolism (Csordas et al., 2001); reactive oxygen species generated by mitochondria may regulate Ca\textsuperscript{2+} sparks (Yan et al., 2008). Thus, further multi-scale structure-based biological studies should produce new local control models of cardiac Ca\textsuperscript{2+} dynamics that are crucial for regulating E-C coupling and other cardiac cellular functions.

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**Fig. 8.** 3D distribution of mitochondria-associated bride structures in the mouse myocardium. (A,B) 3D mesh models of mitochondrial outer membranes (magenta) and their sub-surface areas (blue) where mitochondria-associate bridge structures are populated. (C) Mesh models in a larger 3D volume (the same volume as shown in Fig. 1D and Fig. 3A-C). The mesh models of dyadic clefts (white) are displayed, together with the mitochondrial-associated bridge-rich regions (blue). Narrow strips marked by blue arrows in A,B exemplify the mitochondrial associations with nSR; orange asterisks in A-C indicate regions where bridges between mitochondria and JSR are found closely to dyadic clefts. Scale bars: 1 μm.
Materials and Methods

Standard sample preparation for EM

Adult male C57BL/6J (n=12) mice (6-8 months) were euthanized and perfusion fixed through the left ventricle with 2% (wt/v) PFA and 0.1% GA in 0.15 M sodium cacodylate buffer (pH 7.4) at a hydraulic pressure of 90 cm for 5 minutes. The left ventricles were isolated and several 1 mm³ tissue blocks were obtained from the middle free wall. The blocks were then post-fixed in the same fixative for 2 hours at 4°C. The fixed tissues were further incubated in 0.8% potassium ferrocyanide and 2% osmium tetroxide (OsO₄) in 0.15 M sodium cacodylate buffer (pH 7.4) overnight at 4°C, subjected to en bloc staining with 1% uranyl acetate (UA) for 1 hour at 4°C, dehydrated in a graded series of ethanol solutions and embedded in Durcupan ACM (Electron Microscopy Sciences, PA) in a vacuum oven, as described previously (Perkins et al., 1997). The animal procedures used in the current study were pre-approved by the University of California San Diego Institutional Animal Care and Use Committee.

Selecting staining of T-tubules and SR for EM

Mouse cardiac T-tubules and SR were selectively stained using a method modified from those described in previous reports (Forbes and van Neel, 1988; Franzini-Armstrong, 1991). Briefly, mouse hearts (129 strain, male, 6-8 months old) were perfused fixed through the apex of the left ventricle with a 0.15 M sodium cacodylate buffer (pH 7.4) containing 3% dextran, 3% dextrose and 50 mM CaCl₂ (solution A) in the presence of 3% GA. The fixed tissues were then washed in the solution A overnight, treated with 0.8% potassium ferrocyanide and 2% OsO₄ in 0.15 M sodium cacodylate buffer (pH 7.0) for 2 hours, further incubated with 1% UA, dehydrated in a graded series of ethanol solutions and embedded in Durcupan ACM, as described above.

High-pressure freezing/freezing substitution (HPF-FS)

Mouse hearts (129 strain, male, 6-8 months old) were fixed through the left ventricle with 4% PFA and 0.1% GA in 0.15 M sodium cacodylate buffer (pH 7.4). Sections (80 μm) were obtained from the middle free wall of left ventricles using a vibrating microtome (VT 1000S, Leica, Germany). The section was punched in 125 mm of the diameter, transferred on a small copper hat, filled with 1-hexadecene and sandwiched with the other hat. This assembly was quickly loaded into a holder and frozen instantaneously using a high-pressure freezing system (HPM010, BAL-TEC, Liechtenstein). The frozen samples were transferred to the liquid nitrogen, immersed in 0.1% tannic acid in acetone and incubated using an AFS cryo-substitution unit (EM AFS, Leica, Germany) at –90°C for 24 hours. After washing with acetone three times, the samples were submerged in a cocktail of 2% OsO₄ and 0.1% UA in acetone and incubated using an AFS cryo-substitution unit (EM AFS, Leica, Germany) at –90°C for 24 hours. The specimens were then washed with acetone, infiltrated with the graded mixtures of acetone and Durcupan ACM (16 bits, 2000 dpi) using a scanner (Supercool scan 9000, Nikon, Japan) or by a modified Tyrode’s solution (140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 5 mM glucose and 1 mM probenecid in 10 mM HEPES/NaOH, pH 7.4) containing 1.8 mM CaCl₂ to remove the excess dye. Ca²⁺ sparks were imaged in a Fluoview 1000 confocal laser scan microscopy (Olympus) equipped with a ×60 PLAPON objective (oil, NA=1.42) using a line-scan mode (2 mseconds/line, 69 nm/pixel, ex 488 nm, em 500-600 nm). All cells were electrophysiologically stimulated by field pacing (0.5Hz) for 10 minutes in a constant flow of the modified Tyrode’s solution with 3.0 mM CaCl₂.

Fluorescence signals were recorded for up to 30 seconds after the termination of the field-stimulation. Transmitted light images were also collected by a photomultiplier to obtain the differential interference contrast (DIC) images of cells simultaneously. The scan line was oriented on z-lines or in parallel with the long-axis of cells, avoiding the nuclei, using a live DIC image as a guide. The fluo-4 fluorescent images were analyzed in SparkMaster (Pich et al., 2007), which was kindly provided by Drs Pich and Bers (Loyola University of Chicago), after Gaussian filtering and plots of signal profiles were generated in ImageJ. Ca²⁺ sparks collected both in longitudinal scans (n=117) and in transverse scans (n=132) exhibited known Ca²⁺ spark characteristics in rodent cells (Cheng et al., 1996a; Cheng et al., 1996b): normalized fluorescence peak increase (∆F/F₀) refers to baseline fluorescence (0.8±0.03) and transverse (0.8±0.03) scans; half decay time of longitudinal (37±2.7 mseconds) and transverse (36±2.9 mseconds) scans; and full-width at half-maximum amplitude of longitudinal (1.36±0.08 μm) and transverse (1.4±0.06 μm) scans.

Statistics and data dissemination

All values are expressed as a mean±S.E.M., unless specified otherwise. Tomographic reconstructions and anatomical models will become available through an online data repository Cell Centered Database (CCDB: http://ecdb.ucsd.edu/index.html).

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Vibratome (80 μm) sections of mouse left ventricles (129 strain, male, 6-8 months old) were prepared after the perfusion-fixation of the hearts with 4% PFA in phosphate-buffered saline (PBS). The sections were pretreated with a blocking solution (solution B) consisting of 3% normal goat serum, 0.01% saponin, 1% BSA (Sigma), 1% fish gelatin (Sigma) in 20 mM glycine-PBS buffer (pH 7.4) at 4°C for 1 hour and incubated with a mouse anti-RyR antibody (1:100, Clone 34C, a gift from Dr Sutko or a purchase from Affinity BioReagents) (Sutko et al., 1991) in the solution B for 2 days at 4°C. The sections were then washed with a washing solution (a 10 times dilute of solution B) and incubated with a goat anti-mouse or anti-rabbit IgG antibody (1:200, Chemicon/Millipore) for 2 hours, washed again with the washing solution, incubated with a Cy5-conjugated anti-mouse IgG antibody (Invitrogen, Molecular Probes) overnight at 4°C, washed with the washing solution and then PBS, and staining was finally observed on a Radiance 2000 confocal laser-scanning microscope (Bio-Rad Laboratories, ×60 objective, oil, NA=1.40, ex 637 nm, em 660-700 nm). The staining images (62 mm/pixel) were first smoothed by Gaussian filtering and the intensity of each image was scaled to [0, 255]. A number of rectangular regions containing RyRs were selected manually and the average intensities within each region were calculated along the shorter edge of the rectangle, yielding a smoothed one-dimensional curve (for each selected region). Peaks on each curve were automatically detected and the distances between adjacent peaks were measured.
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