Three-Dimensional Reconstruction of Cardiac Sarcoplasmic Reticulum Reveals a Continuous Network Linking Transverse-Tubules

This Organization Is Perturbed in Heart Failure

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Rationale: The organization of the transverse-tubular (t-t) system and relationship to the sarcoplasmic reticulum (SR) underpins cardiac excitation–contraction coupling. The architecture of the SR, and relationship with the t-ts, is not well characterized at the whole-cell level. Furthermore, little is known regarding changes to SR ultrastructure in heart failure.

Objective: The aim of this study was to unravel interspecies differences and commonalities between the relationship of SR and t-ts networks within cardiac myocytes, as well as the modifications that occur in heart failure, using a novel high-resolution 3-dimensional (3D) imaging technique.

Methods and Results: Using serial block face imaging coupled with scanning electron microscopy and image analysis, we have generated 3D reconstructions of whole cardiomyocytes from sheep and rat left ventricle, revealing that the SR forms a continuous network linking t-ts throughout the cell in both species. In sheep, but not rat, the SR has an intimate relationship with the sarcolemma forming junctional domains. 3D reconstructions also reveal details of the sheep t-t system. Using a model of tachypacing-induced heart failure, we show that there are populations of swollen and collapsed t-ts, patches of SR tangling, and disorder with rearrangement of the mitochondria.

Conclusions: We provide the first high-resolution 3D structure of the SR network showing that it forms a cell-wide communication pipeline facilitating Ca\(^{2+}\) diffusion, buffering, and synchronicity. The distribution of the SR within the cell is related to interspecies differences in excitation–contraction coupling, and we report the first detailed analysis of SR remodeling as a result of heart failure. (Circ Res. 2013;113:1219-1230.)

Key Words: 3D imaging ■ electron microscopy ■ excitation contraction coupling ■ heart failure ■ mitochondria ■ sarcoplasmic reticulum ■ t-tubules

Transverse-tubules (t-ts) are invaginations of the plasma membrane, which in cardiac muscle facilitate transmission of the action potential from the exterior to interior of the cell. Dyads or couplons are formed along regions of the t-ts by the close apposition of the functional portion of the sarcoplasmic reticulum (jSR). It is within the compartment formed by these 2 structures, the dyadic cleft, that the contractile function in the heart is regulated by the interplay between the L-type voltage-gated Ca\(^{2+}\) channel (LTCC) localized to the t-ts and the ryanodine receptor (RyR2) anchored in the jSR. Critical to this regulation is the arrangement of the jSR and t-t membranes, which are held in a precise geometric organization separated by a gap of 12 to 15 nm. Changes to the microanatomy of the 2 membranes, and hence the spatial relationship between the LTCCs and RyR2s, are associated with Ca\(^{2+}\) handling abnormalities and impaired contractile function, although the effect on systolic Ca\(^{2+}\) remains equivocal.

Our current understanding of the 3-dimensional (3D) organization of t-ts is mainly derived from studies using confocal microscopy. Elegant experiments have revealed that the t-ts form regular arrays with a periodicity that correlates to the position of the Z-lines. The dimensions and branching patterns are varied and species-dependent. For example, rat myocytes are shown to have a more geometrically complex arrangement compared with the human t-t system, which has a radial distribution within the cell, akin to the spokes of a wheel. Recently, the development of stimulated emission depletion microscopy has pushed the resolution frontier to ≈60 nm in the focal plane, providing ultrastructural details of the t-t system in control and failing rat hearts showing...
progressive t-t remodelling with disease.\(^{10}\) Complementary to these studies has been the application of transmission electron microscopy involving the study of fixed cells,\(^{11}\) for producing details of the architecture of the couplon structure with electron tomographic methods facilitating 3D reconstructions of individual dyadic clefts in mouse ventricle.\(^{12}\) Surprisingly, the dyads were found in clusters at irregular intervals along the Z-lines and their size and number was variable. Whether this organization is species dependent is not yet clear, nor is the relevance of dyad volume heterogeneity that could lead to variable numbers of RyR2s within each dyad and thus effect \(\text{Ca}^{2+}\) spark generation.\(^{13}\) Although higher resolutions are attainable from electron tomography compared with confocal microscopy techniques (ie, 4 to 5 nm), the amount of information provided by the reconstruction is ultimately limited by the thickness of the section, typically 250 to 500 nm which is a fraction of the length of a t-t.

Compared with the t-t system, there is little 3D data describing the SR. Much of the information pertaining to SR morphology has also come from 2-dimensional (2D) TEM images, revealing that it is an extended membranous network.\(^{14,15}\) In addition to the junctional regions, the SR is composed of network SR which is found near the myofibrils and the mitochondria. Expansions of the network system form corbular regions, but these are not associated with or near the t-ts or sarclemma. Both the network and corbular SR are often collectively referred to as free SR (fSR). Although portions of fSR have been seen to cross the Z-lines,\(^{16}\) it is usual for the SR to be depicted as an isolated mesh within each sarcomere. Recently, the SR network of isolated rat cardiac myocytes has also been visualized by confocal microscopy, which has led to the suggestion that the SR forms a vast mesh.\(^{17}\) However, using this approach, only the SR structure was imaged and the relationship with the t-t organization was not defined.

There are established interspecies differences in excitation–contraction (E-C) coupling that have been attributed to spatial variations in, for example, LTCC localization\(^{18}\) and dependence on SR \(\text{Ca}^{2+}\) release.\(^{19,20}\) To understand fully how the structure of the SR relates to the t-t system in different species, and in disease, to unravel the mechanisms governing E-C coupling, we need detailed information describing the morphology of the SR throughout the cell volume. To this end, we have applied the novel technique of serial block face imaging coupled with scanning electron microscopy (sbfSEM) to examine the ultrastructure of control, healthy cardiomyocytes with the objective of determining the organization, and relationship, of both t-t and SR membrane network together. The sbfSEM configuration automates the process of sequential slicing through a block of tissue by positioning a micrometer within the microscope chamber so that the specimen is sectioned in situ.\(^{21}\) Scanning electron microscopy then images the surface of the exposed block after each successive thin layer has been cut away, generating high-resolution volumetric data through the tissue sample.

Here, we have reconstructed the SR, generating 3D volumes at 15-nm resolution in the \(xy\) plane, for cardiac myocytes from sheep and rat ventricular tissue. These data reveal that the organization of the SR with respect to the sarclemma differs between species, providing a new perspective of E-C coupling in the context of SR morphology. A feature found common to both sheep and rat is that the SR forms a continuous membrane system throughout the cell linking each t-t. Significantly, we have also identified that the SR undergoes ultrastructural changes in failing cells, through the study of a sheep model of tachypacing-induced heart failure.\(^{22}\) In conclusion, we provide the first high-resolution data for the SR network in the context of both health and disease and reveal that remodeling occurs not only to the t-t system but also to SR.

**Methods**

**Tissue Extraction and Specimen Preparation for Transmission Electron Microscopy**

Samples of the left ventricle from sheep (18 months; \(n=4\)) and adult male Wistar rats (12 weeks; \(n=4\)) were collected. All procedures were carried out in accordance with the United Kingdom Animals Act (Scientific Procedures) 1986 and the University of Manchester’s ethical review process. Sheep were killed with an overdose of pentobarbitone (200 mg/kg i.v.). A sheep model of tachypacing-induced heart failure was also used as described previously\(^{22}\) (\(n=3\)). Sheep hearts (control and heart failure) were removed soon after death and perfused with calcium-free Tyrode’s solution. After perfusion, 1 to 2 mm\(^3\) cubes of left ventricle were immersed in Karnowsky primary fixative with 50 mmol/L CaCl\(_2\). The addition of CaCl\(_2\) to the buffer used for sample preparation enhances the staining of jSR in myocytes.\(^{23}\) Samples were then washed 3 times in water and left in 0.5% aqueous uranyl acetate overnight, followed by dehydration in ethanol in ascending series and in propylene oxide and embedded in Taab low-viscosity resin. Rat hearts were harvested after cervical dislocation and small portions of the left ventricle treated similarly as sheep specimens. Multiple blocks were prepared from each tissue sample; 100-nm sections were cut with glass knives on a Reichert microtome and imaged using a FEI Tecnai 12 Biotwin operated at 100 kV. For each animal, a range of blocks was examined by transmission electron microscopy and sbfSEM, with each image presented in this report representative for each species.

**Specific Staining of SR Membrane System**

Sheep and rat samples were immersed in primary fixative with 50 mmol/L CaCl\(_2\). The addition of CaCl\(_2\) to the buffer used for sample preparation enhances the staining of jSR in myocytes.\(^{24}\) Samples were washed 3 times in sodium cacodylate buffer with 50 mmol/L CaCl\(_2\). From this point onward, samples were prepared as described earlier. This preparation could give images that retain the features typical of routine specimen preparation but with the advantage of having a dark staining of the SR network.

**Volumetric Data Acquisition Using sbfSEM**

To image whole cardiac myocytes, a FEI Quanta 250 FEG SEM equipped with a Gatan 3View system was used. Blocks were trimmed to \(\approx 0.5\) to 1 mm\(^3\), put on a metal pin, and gold-coated. Serial 50-nm sections were cut with the in situ diamond knife microtome. After each cut, the section was discarded and the block face imaged, generating a stack of images through the block at a calibrated magnification corresponding to 15 nm/pixel. The acquired images were in register...
through the tissue and the process was automated with no requirement for corrective focusing, because the block was in the focal plane of the microscope. Typically, between 1000 and 2000 images were collected over several days. The voxel size of the images collected here was 15, 15, 50 nm in the X, Y, Z directions, respectively. At the magnifications used serial images for between 5 to 8 cells per block face were acquired. All blocks used for sbfSEM were first screened by transmission electron microscopy for sample preservation and staining. Only regions that did not present artifacts were analyzed. Artifacts typical of routine electron microscopy specimen preparation include myofilament disruption and mitochondrial swelling characteristic of osmotic imbalance during the fixation procedure or cell death. The use of fixative with pH and molarity as close as possible to in vivo conditions, as used here, would help minimize potential deformations.

Image Analysis
Contrast-based segmentation techniques were used, using either Fiji or IMOD, to determine the 3D structures of both SR and t-t systems. Selective staining facilitated imaging of both t-t and SR networks for the same cell, and because the t-ts are visualized as white and SR as black, the 2 organelles can be easily differentiated. A limitation of the image analysis process is that, unless the contrast between the organelle of interest compared with the surrounding components is sufficient, automated analysis of a whole cell would not be possible and manual segmentation must be performed. Data are reported as means±SEM, and differences in organelle density and volume were tested using Student t test where appropriate with probability values <0.05 considered significant.

Results
Sheep t-t Morphology
A SEM serial image of a cross-section of sheep cardiac myocyte is presented in Figure 1A, showing a high density of mitochondria surrounding the myofilaments. Using the method described by Forbes and van Neil, there was dark staining of the SR, leading to images of sheep cardiac myocytes with a well-defined membrane system. The SR appears as thin black lines (Figure 1A inset), seen in places to follow a path around the perimeter of the mitochondria as well as traverse the myofibrils. Franzini-Armstrong and colleagues suggested that the high concentration of calsequestrin in the jSR binds the Ca and forms electron-dense structures that can be visualized by transmission electron microscopy. However, we found that this method led to staining of the entire SR network. Significantly, the t-ts did not take up any stain appearing almost white, compared with the surrounding features of the cell, which permitted effective binary segmentation of the t-t system from neighboring organelles, as shown in Figure 1B. The sheep t-ts project radially from the sarcolemma toward the center of the cell, as shown in a transverse view of the myocyte, and thus adopt a similar organization described for human as determined using confocal microscopy. We see

Figure 1. 3-Dimensional (3D) structure of the sheep transverse-tubular (t-t) system using serial block face scanning electron microscopy. A, An image of an entire sheep cardiomyocyte; mitochondria (M) are dark grey; myofibrils (m), light grey; and sarcoplasmic reticulum (SR), black. The white t-ts are highlighted by the white arrows. Inset: A magnified area (2×) illustrating the dense almost black staining of SR (dashed white arrow). B, 3D rendering of the t-t membrane system showing that the t-ts all extend from the sarcolemma toward the center of the cell decorated by nodules, with examples highlighted by dashed ellipsoids. C, A portion of the t-t network showing the t-ts, extending from the sarcolemma, revealing the periodicity of the network. Axial branches are indicated by arrows. Long axis of reconstruction is 17.3 μm. Inset: An enlarged region of the area encompassed by the dashed box showing that the branches from each t-t do not contact adjacent invaginations. D, Orthogonal view of the t-t network in (C), illustrating that each row of t-ts follows a distinct trajectory.
that the length of the individual t-ts is dictated by the distance of the sarcolemma to the apparent center of the cell because their path is interrupted by the nucleus. The sheep t-ts also vary in width along their length, with an average diameter of 244±64 nm (n=127), a feature noted from studies of rabbit t-t morphology.26 The high-resolution sbfSEM images also reveal that the t-ts are punctuated by regions that are enlarged, 380±66 nm (n=117) across, compared with the flanking tubule (P<0.05). We termed these enlarged regions nodules, which form along the length of the invaginations. Detailed reconstructions in Figure 1B show that the nodules are in register along a row of t-ts. Regions of t-t dilation have recently been identified as a feature of mice t-ts.27

Figure 1C shows an enlarged portion of the myocyte, illustrating the periodicity of t-ts as they extend out from the sarcolemma. The t-ts do not generally form contacts or fuse with an adjacent invagination, as illustrated in inset to Figure 1C. Although the tubules project at an angle almost orthogonal to the plane of the sarcolemma on rotation around the y axis by 90°, it can be seen (Figure 1D) that they twist and bend, as well as that the trajectory of t-ts forming a row is consistent and that there is no branching between the rows, implying that there are axial extensions they are in one plane only (Online Movie I). From these types of reconstructions, we calculated that the t-t system occupies 2.1±0.7% of the cell volume.

Two t-ts at 1 Z-Line

Generally, one t-t is expected at each Z-line; however, in sheep, 2 invaginations, one on either side of the Z-line, were sometimes observed. We calculated that the relative densities of a single t-t compared with 2 tubules (twins) per Z-line were 0.240±0.070 and 0.024±0.003 per μm², respectively. An example of a twin t-t imaged using conventional transmission electron microscopy is shown in Figure 2A. The presence of a twin t-t at one Z-line was also identified in the 3D reconstructions as shown in Figure 2B, illustrating that the twin t-ts originate from the same area of the sarcolemma. SEM images of serial sections are presented in Figure 2C, allowing the path of an exemplar twin t-t to be tracked as it penetrates the cell. The t-ts are positioned on either side of the Z-line and initially follow the same trajectory within the myocyte; then they start to diverge and separate from each other so that at ≈1 μm into the cell they are positioned at 500 nm apart; after this point, the trajectory of each tubule with respect to the other does not change further. These twin t-ts are distinct from an axial extension protruding from the main tubule because branches are formed on the same side of the Z-line and are not found at the sarcolemma. The significance of twin t-ts remains unknown and, to our knowledge, is a feature not reported elsewhere.

SR Network Connects Adjacent t-ts With Multiple Dyads Formed Along a Single Tubule

We present in Figure 3A the first high-resolution 3D reconstructions revealing the relationship between the SR and the t-t membrane systems. It was found that the SR wraps around each t-t, forming a dyad and then extends, parallel to the myofibrils, to the next t-t and so on, thereby forming a continuous link between couplons in the cell. Furthermore, as shown in Figure 3B, at each dyad the jSR appears to almost engulf the t-t, showing a dyad structure with 3 strands of fSR connected to the jSR region (Figure 3B inset). In addition to the regions of SR linking the t-ts, which run parallel to the

Figure 2. Twin transverse-tubules (t-ts), a novel feature of sheep cardiac myocytes. A, TEM image showing twin t-ts at a single Z-line. B, 3-Dimensional rendering of a section of t-t network showing that twin t-ts originate from the same region within the sarcolemma (white arrows). The dashed line indicates an example of a tubule bending and twisting so that the portion toward the center of the cell is orientated at almost 90° to the trajectory from the sarcolemma. C, Individual serial block face scanning electron microscopy images of sections revealing the trajectories of the twin t-ts as they extend through the myocyte volume; black SR is indicated by dashed arrows. Image box size=2.6×2.6 μm.
myofilaments, there are also connections that extend in a perpendicular direction, forming a complex rete permeating the entire cardiomyocyte.

TEM image in Figure 4A shows a longitudinal view of a single t-t with multiple SR patches along the tubule length. The 3D reconstruction from the sbfSEM method, as shown in Figure 4B, reveals that there are potentially multiple dyads formed along an individual t-t as it extends into the cell and that each dyad is connected by the vast SR network. Figure 4C shows a portion of the reconstruction at the sarcolemma, revealing the complex nature of the relationship between t-t, SR, and the interior leaflet of cell membrane.

SR Forms Contacts Along Longitudinal Elements of the t-t System
Figure 1C shows that there are longitudinal extensions of the t-t network. The high-resolution (15 nm) reconstructions using sbfSEM technique provide detailed images of the longitudinal elements. Figure 4Di presents examples of branched t-ts with longitudinal extensions. Figure 4Dii reveals that some of the axial extensions, which when viewed in projection appear longitudinal, are oblique. Moreover, Figure 4Diii and Figure 4Div reveals that some of these oblique and longitudinal segments are not always a result of branches extending from the transverse invagination, but are formed by the weaving trajectory of a single t-t as it penetrates the cell volume. We have calculated that the longitudinal and oblique branched components of the t-t network together account for 8.9±0.3% of the volume of the total t-t system. Furthermore, the diameters of the longitudinal/oblique branched elements (200±47 nm; n=100) are not significantly different than the transverse segments (P>0.05). Figure 4E and 4F shows that the SR engulfs the longitudinal and oblique portions of the tubules in a similar manner as the t-ts, forming what we suggest are jSR domains.

SR Has an Intimate Relationship With the Sarcolemma in Sheep Cardiac Myocytes
A portion of the outer rim of a myocyte is shown in Figure 5A, in which the SR membrane seems to be in close apposition to the inner leaflet of the sarcolemma. This close relationship between SR and sarcolemma was a feature of each sheep cardiomyocyte examined. Three-dimensional reconstructions revealed multiple extended domains, jSR, connected by network SR as shown in Figure 5B and Online Movie II. The formation of couplons at the sarcolemma, as well as populations of extradyadic RyR2s colocalized with caveolin-3 at the cell surface, has been reported, and our data present the first high-resolution images of this organization. We have calculated that the putative jSR–sarcolemma regions have an average volume of 1.57±0.63×10⁷ nm³ and surface area of 7.46±3.33×10⁵ nm² (n=36). Previous studies have determined that RyR2s form clusters averaging about 14 channels arranged in a chequered board formation. Based on these earlier calculations and using a configuration with an elongated cluster (14 RyR2s for an edge length ratio of 1.7), a 900 nm² footprint for each RyR2, and a 50 nm edge-to-edge intercluster spacing, we calculated that the jSR–sarcolemma domains can accommodate between 130 and 336 RyR2s. We also determined that the jSR domains shown in Figure 5B are juxtaposed to ≈7% of the inner surface of the sarcolemma.

Similarities and Differences Between Rat and Sheep Cardiomyocyte t-t Morphology
We next sought to determine whether using sbfSEM techniques we could resolve the structural bases underlying interspecies differences in E-C coupling in rat and sheep. Figure 6A shows a region of a rat cell illustrating how the SR, like in sheep cardiomyocytes, weaves multiple paths through the myofilaments and around the edges of the mitochondria. However, an obvious difference was that in rat more than one
The diameter of the rat t-t was 120±50 nm (n=101), which is smaller than that reported previously using confocal microscopy.9 Because of the narrowing of the rat t-ts in places, the contrast was poor; hence automated binary segmentation proved difficult for generating a 3D reconstruction of an entire cell t-t network; however, some regions of the cell were more amenable to processing (an example area shown in Figure 6B). We found that rat t-ts are also characterized by nodules that have a diameter of 278±81 nm (n=58; Figure 6C). Figure 6D illustrates how the rat t-ts form large loops and adopt a more geometrically complex system than the organization of sheep t-ts described here, although the t-t density was found to be the same in the 2 species (P>0.05; 0.27±0.01 tubule per μm² for rat compared with 0.24±0.01 per μm² for sheep). Twin t-ts were also occasionally identified in the rat with a density of 0.008±0.001 twin t-t per μm², which is at a much lower frequency than in sheep (P=0.02).

The rat SR network also forms a complex mesh connecting one end of the cell to the other, although it was noted that there were less transverse elements compared with sheep. We calculated that the rat SR occupies 4.0±0.4% of the cell volume, in close agreement with previous figures.30 This value is marginally less than that measured for sheep SR (5.7±0.7%) but not significantly different (P=0.059). We also found that individual rat t-ts are linked by SR as shown in Figure 7A and 7B, and the 3D reconstructions further reveal that the SR forms multiple junctions along a single invagination (Figure 7C). Figure 7D and 7E shows how the path of the SR membrane network closely follows the perimeter of the mitochondria. However, a key difference in SR organization between the 2 mammals is at the sarcolemma, with little SR found near the inner surface of the plasma membrane in the rat cardiomyocyte, as shown in Figure 7F. Moreover, 3D reconstructions of those regions of SR found at the sarcolemma show that it is formed mainly by network, tSR (Figure 7G).
Ultrastructural Changes to Both t-t and SR Networks Are a Feature of Heart Failure

We next sought to examine not only whether the organization of the t-t network is perturbed in heart failure, but also whether there are modifications to the organization of the SR network. We found as a feature of failing cells that the mitochondria are in clusters forming thick bands, composed of up to 6 rows of mitochondria (Figure 8A), rather than single rows sandwiched between the myofilaments. Significantly, we determined that these bands of mitochondria were not near the cell nucleus, because the sbfSEM technique permitted us to examine the entire cell volume. The volume of the cell occupied by the mitochondria increased during heart failure, being 41.1±1.7% compared with 33.1±1.9% in control myocytes (P = 0.01).

The arrangement of the SR in failing cells was largely indistinguishable from that of control myocytes. However, where the mitochondria had formed clusters, the SR was disordered, adopting a chicken wire–like structure (Figure 8A inset). Moreover, we also identified pockets of SR where the transverse elements are reinforced and predominate over the longitudinal sections, a reversal of the pattern found in control myocytes, with an exemplar area shown in Figure 8B. Further analysis found that although the SR in heart failure cells provides connections between t-ts, traversing the Z-lines, the SR is more irregular with some loss or disruption of the longitudinal elements (Figure 8C). Patches where the SR appeared to form a tangled web were also identified in these regions of disorder, as highlighted in Figure 8C. Examination of the sarcolemma found that the SR of heart failure myocytes did not present a quantifiable, clear pattern of remodeling around the perimeter of the cell. Compared to control myocytes there is a loss of SR in the failing cells occupying 4.0±0.3% of the cell volume (P < 0.05).

The t-t network was not well contrasted in any of the myocytes from heart failure tissue; as a result, automated cell-wide segmentation was not possible. However, reconstructions of portions of myocytes helped to identify regions where the morphology of t-ts was altered as shown in Figure 8D. The t-ts were swollen, forming globular-like domains that are larger, 611±106 nm (n = 118), in diameter compared with nodules identified in control membranes (P < 0.05). Moreover, the portions of t-ts before and after swollen regions were narrowed (eg, <200 nm). Figure 8D also illustrates that globular domains formed on each t-t are in register along a row. Free-standing globular densities not connected to sarcolemma were also found; these may represent collapsed t-ts. However, there could be portions of narrowed, poorly contrasted, tubule on either side of the swollen domains which were not detected. Interestingly, examination of the images of swollen t-ts (Figure 8D) reveals that there are no longitudinal or oblique segments between individual t-ts, unlike in control membranes (eg, Figure 1C). As explained later, t-ts shown here were from tissue samples fixed with low Ca2+ (2.5 mmol/L), and thus SR was not well contrasted; therefore, t-t and SR networks could not be readily imaged together. We also found that heart failure cells often contained vacuoles; however, these structures were distinct, being ≈1.5 μm in diameter, and so were easily distinguishable from swollen t-ts. We noted that the size and positions of vacuoles are compatible with the dimensions and localization of mitochondria.

Discussion

In this study, we generated the first high-resolution 3D structure of the SR network, advancing our understanding of the relationship of this organelle with both the t-t and sarcolemma and also providing a structural basis for interspecies differences in E-C coupling. A highly novel finding from this study, because of the ability to image both SR and t-t membrane networks, is that the SR connects every t-t and dyad within a cardiac myocyte, which is a feature common to both rat and sheep; a relationship that will influence how the calcium wave propagates inside the cell. Finally, our studies with an osmive model of tachypacing-induced heart failure revealed regions of remodeling of the t-t network and showed, for the first time to our knowledge, that the SR network undergoes structural rearrangement.

Organization and Remodeling of t-ts in Heart Failure

Axial branching of the sheep t-t network was identified here, but accounts for only 9% of the t-ts system. We think that the presence of twin t-ts in sheep cardiac myocytes may, therefore, represent an adaptation in response to the arrangement of the t-t system with respect to other organelles within the myocyte and also function to provide a more effective penetration of the action potential within the cell volume. We
also report here that there are regions of t-t enlargement that form nodules along the length of sheep invaginations and showed that they are a feature common to both small and large mammals.

Often, patchy regions devoid of t-ts have been reported as a consequence of heart failure. The heart failure model studied here was characterized by regions of t-ts that were unchanged structurally compared with control cardiac myocytes, but had pockets of acutely swollen t-ts that did not have longitudinal branches. Therefore, the data here suggest that, rather than a transition occurring between longitudinal and transverse elements of the t-t network as identified in studies on small animals after myocardial infarction, there is primarily enlargement of the transverse invaginations and possibly collapse of the longitudinal tubules. Our data are consistent with previous reports of t-t dilation under pathological conditions as well as with studies of the t-t network from human failing hearts finding little change to the overall t-t density. We showed that the longitudinal/oblique branches were decorated by jSR domains (dyads), and although they account only for a small proportion of the tubular network, structural modifications will have the potential to contribute toward the development of abnormal Ca\textsuperscript{2+} transients. Another feature of the heart failure myocytes was the presence of vacuoles, often found arranged in rows. We suggest that the vacuoles may have formed because of mitochondria rupture or loss due to remodeling. A limitation of electron microscopy studies is the use of chemical fixatives, which, as explained in Methods section, could lead to cell damage, although none of the cells analyzed in this study showed overt features of tissue disruption. Pertinently, vacuoles were not observed in control cells (rat or sheep); however, we cannot completely rule out that the effect of fixation process on a diseased cell may be different than that on a healthy myocyte. We considered that the conditions used to visualize the SR membrane using high concentrations of Ca\textsuperscript{2+} may unintentionally have resulted in the equivalent of the calcium paradox, a phenomenon, first identified more than 40 years ago, describing how sudden Ca\textsuperscript{2+} depletion followed by its surge in heart cells could lead to severe ultrastructural modifications, including damage to the mitochondria. To eliminate this potential effect, we repeated all our sample preparations with low [Ca\textsuperscript{2+}] and found that the engorged t-ts and vacuoles remained a feature of heart failure myocytes.

**Disorganization of Localized Areas of the SR Continuum Is a Feature of Heart Failure**

The cell-wide SR organization identified here has implications for our understanding of synchronized Ca\textsuperscript{2+} release between dyad structures and across a myocyte as a whole. The SR may be thought of as a Ca\textsuperscript{2+} communication corridor, and we propose that by linking adjacent t-ts and dyads, this system would form a single circuit within a cell, which provides a structural basis for understanding SR function in terms of Ca\textsuperscript{2+} diffusion within a myocyte. An understanding of intra-SR Ca\textsuperscript{2+} diffusion and [Ca\textsuperscript{2+}] gradients between various domains of SR, the relationship to local Ca\textsuperscript{2+} release, and E-C coupling is complex,
The present work indicates that the current view of jSR morphology having a pancake-like structure connected to a single narrow fSR segment is perhaps simplistic as we reveal that there are often several fSR strands connected to a single jSR domain (Figure 3B), an organization that would have a bearing on the rate of jSR filling after each contraction. Our data also show that the SR membrane within a cardiomyocyte is a continuous network, which is consistent with electrophysiological experiments from Wu and Bers, which led to the proposal that SR functions as a single entity to regulate Ca\(^{2+}\) communication within the cell. Additionally, intra-SR Ca\(^{2+}\) diffusion along the longitudinal axis has been determined to be faster compared with the transverse direction, and we show here (Figures 2 and 3) that the network parallel to the long axis of the myofibrils, linking the t-ts, is better delineated and dominates the SR architecture. Therefore, the SR remodeling we identified here may suggest that Ca\(^{2+}\) diffusion rates through the cell may be perturbed in heart failure.

**Relationship Between SR and Sarcolemmal Membranes Underlies Interspecies Differences**

In sheep cardiac myocytes, the SR was found to be closely, and extensively, associated with sarcolemma with multiple jSR domains formed in contrast to the organization of rat cardiac myocytes. Early studies of dyad formation have described interspecies differences for couplon distribution at the plasma membrane, which were attributed to variations in the activation of contraction. Detubulation experiments have estimated that the LTCCs producing the inward Ca\(^{2+}\) current are most concentrated within the t-ts of small mammals, suggesting that the majority of Ca\(^{2+}\) transient originates along the t-ts. The arrangement of the SR in sheep would indicate that a component of the Ca\(^{2+}\) transient is also triggered at sarcolemma in a larger mammal. It has been established that in larger mammals the external Ca\(^{2+}\) has a more influential role in E-C coupling, and numerous reports showed that a greater proportion of LTCCs are associated with sarcolemma in, for example, rabbit cardiomyocytes and sheep. Therefore, the data here provide new structural insights as to how the SR adopts a species-specific organization that influences E-C coupling.

**Mitochondrial–SR Relationship Is Perturbed in Heart Failure**

We show here the intimate relationship between SR and mitochondria. Mitochondrial Ca\(^{2+}\) uptake is thought to be highly dependent on microdomains formed with the SR to provide...
localized regions of elevated cytosolic calcium. It is seen here how Ca^{2+} uptake from the SR by the mitochondria and diffusion of molecules between the 2 organelles readily occur. Our data also show that in the failing heart not only is there reorganization of mitochondria to form clusters, but also that the relationship with SR is modified in these regions, which may result in impaired Ca^{2+} uptake and contribute toward mitochondrial dysfunction. We also suggest that the formation of swollen t-ts may be linked to mitochondrial remodeling, providing the physical space for t-t enlargement. The acute swelling of t-ts will alter the surface curvature of the membrane in this region and disturb the precise geometric relationship required between LTCCs and RyR2s. Our previous electrophysiological characterization of this model of heart failure identified a smaller systolic Ca^{2+} transient amplitude and reduced peak L-type voltage-gated Ca^{2+} current.

Thus, our structural data provide, in part, an understanding of pathological modifications to functions identified in this heart failure model because distortions to the t-ts morphology will alter the size and shape of the specialized microenvironment of the couplon volume; which may also impact the localization of protein residents of the dyadic cleft involved directly or indirectly in orchestrating cardiac contraction and relaxation. Changes to the t-t volume will also alter the rate of diffusion of ions in and out of the intavgnation and exchange with the external cell environment.

A major finding from this work is that the SR links adjacent t-ts, forming a complex network within the cardiac myocytes for both a large and a small mammal. The data here help explain the apparent paradox of variable dyad sizes and distribution in the context of co-ordinated cell-wide contraction, since we now show that the SR connects all the
dyads within a cell which may serve to regulate synchronicity between couplons. In conclusion, we provide the first high-resolution reconstruction of the cardiac SR network, identifying morphological changes in heart failure cells, which has the potential to modify the dynamics of systolic Ca2+ release and hence contractile function. There has been much focus on adaptations to the t-t network in disease, but the new data presented here highlight the need to also put the spotlight on the 3D microarchitecture of the SR network for a fuller understanding of structural changes in the context of cardiac dysfunction.

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Disclosures
None.

References
What Is Known?

- Excitation–contraction (E-C) coupling takes place in a specialized cellular microdomain, termed a dyad, where the transverse-tubule (t-t) and sarcoplasmic reticulum (SR) membranes come into close contact.
- Remodeling of the t-t network has been identified as a hallmark of heart failure; however, the details of changes to the ultrastructure of the SR are unknown.
- Transmission electron microscopy and tomographic methods have contributed significantly to our current understanding of the 3-dimensional (3D) organization of individual dyads, but the size of the section that can be examined (200–500 nm thick) represents only a small fraction of a cell.

What New Information Does This Article Contribute?

- Using the novel technique of serial block face scanning electron microscopy (sbfSEM), we have elucidated the first high-resolution (15 nm) 3D structure of the SR for an entire cardiomyocyte, revealing the SR to be a continuous cell-wide network that connects every dyad.
- Differences in the SR–sarcolemma organization have been identified in myocytes from small and large mammals, providing a new understanding of interspecies differences in E-C coupling in the context of SR morphology.

Novelty and Significance

- The SR, in addition to t-ts, undergoes remodeling in an ovine tachypacing-induced heart failure model characterized by regions of tangle and disorder.

The SR is a major source of Ca^{2+} for contraction. There is a paucity of volumetric data describing the 3D organization of the SR within cardiomyocytes. Using sbfSEM, we sought to investigate the structure of the SR and its relationship with the t-t system and sarcolemma. We found that the SR provides a unifying connection between all dyads in the cell, with multiple dyads formed along a single t-t. These data indicate that the SR forms a continuous membrane system for housing and releasing Ca^{2+}, a feature that will influence synchronous myocyte contraction which is essential for normal cardiac function. We further identified differences between the organization of SR and sarcolemma in sheep and rat, providing a novel structural perspective to explain, in part, previously described interspecies differences in Ca^{2+} regulation. Significantly, our study showed that in the failing heart t-ts were swollen and the SR underwent remodeling, both of which will influence the characteristics of the Ca^{2+} transient and contractile properties. Therefore therapeutic strategies should be aimed at stabilizing both membrane systems to preserve the integrity of dyad structures for maintaining the contractile properties of the heart.
Three-Dimensional Reconstruction of Cardiac Sarcoplasmic Reticulum Reveals a Continuous Network Linking Transverse-Tubules: This Organization Is Perturbed in Heart Failure

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Detailed Methods

Animals

In this study adult female Welsh ewes aged 18 months (n=4) and adult 12 week old male Wistar rats (n=4) were employed. All procedures were carried out in accordance to the United Kingdom Animals Act (Scientific Procedures) of 1986 and the University of Manchester’s ethical review process. Sheep were killed with an overdose of pentobarbitone (200 mg/kg i.v.). Hearts were removed soon after death and perfused with calcium free Tyrode’s solution. After perfusion, 1-2 mm³ cubes of left ventricle were immersed in Karnowsky's primary fixative (2.5% w/v glutaraldehyde, 2% w/v paraformaldehyde (EM grade) in 100mM sodium cacodylate buffer, pH 7.2) supplemented with 50 mM CaCl₂. Samples were also prepared in the absence of CaCl₂. Rats were killed by cervical dislocation, hearts removed and tissue taken immediately from the left ventricle and immersed in Karnowsky's primary fixative as described for the ovine specimens.

We also studied the morphological changes that occurred to the myocyte ultrastructure in heart failure using an ovine tachypacing induced heart failure model. Heart failure was induced in adult sheep by right ventricular tachypacing as described previously²⁻⁴ (n=3). Anesthesia was induced and subsequently maintained by isoflurane inhalation (1 – 4 %), animals were intubated and ventilated at 15 breaths per minute and analgesia (meloxicam 0.5 mg.kg⁻¹) and antibiosis (enrofloxacin 5 mg.kg⁻¹) provided. A single IS-1 bipolar pacing lead was fixed transvenously at the right ventricular apex and connected to a pacemaker (Kappa, Medtronic) buried in a subcutaneous pocket. Animals were allowed to recover from surgery for at least 1 week before tachypacing (3.5 Hz) was commenced. On presentation of clinical symptoms of heart failure (lethargy, dyspnoea, cachexia) animals were killed (pentobarbitone 200 mg.kg⁻¹ intravenously). Upon death hearts were removed and perfused with calcium free Tyrode’s solution and a cube of tissue taken from the left ventricle and immediately immersed in Karnowsky's primary fixative.

Sample preparation for electron microscopy

Tissues (sheep and rat) were transferred from the Karnowsky’s solution, washed 3x in fresh cacodylate buffer (15 mins each wash) and next postfixed by incubation in 1% w/v OsO₄ and 1.5% w/v K₄Fe(CN)₆ in 100mM sodium cacodylate buffer, including 50mM CaCl₂ for 1h at room temperature. This step was followed by three washes in double-distilled water (ddH₂O). The samples were then left in 0.5% w/v aqueous uranyl acetate at 4°C overnight and subsequently washed with ddH₂O (3x). The tissue samples were next sequentially dehydrated in graded ethanol in ascending series (30, 50, 70, 90% and 100% v/v in ddH₂O). The tissue was transferred into propylene oxide (PO) for 15mins (2x). The specimen was infiltrated in a series of Taab low viscosity resin concentrations (2-3 hours in each of the following...
resin:PO (v/v) mixes - 1:2, 1:1 and then overnight in a 2:3 solution). A repeat treatment for 1h in pure resin 100% was employed (3x) after which the samples were embedded and left to cure at 60°C for 48h. The same blocks were employed for both TEM and serial block face scanning electron microscopy. For TEM, sections (100 nm) were cut with glass knives on a Reichert microtome, and were deposited on 200-mesh copper grids (Agar Ltd.). An FEI Tecnai 12 Biotwin TEM operated at 100kV was used to screen sample blocks for the quality of specimen preparation and SR staining.

Serial block face scanning electron microscopy (sbfSEM)
Blocks were glued to the cryo pin using cyanoacrylate glue. When the glue had set the blocks were then trimmed to about 0.5 mm³ size using a diamond knife. The block was gold coated to provide a conductive surface and positioned within the FEI Quanta 250 FEG SEM equipped with a Gatan 3View system. The microscope was operated at 3.8kV, using spot size 3.5 with a vacuum setting of 0.5torr. We employed a magnification of 2500. Data were captured using a 4K x 4K Gatan CCD leading to a final resolution of 15 nm/pixel. The cut thickness was set to 50 nm so the voxel size was 15; 15; 50 nm in the X; Y; Z directions respectively.

Image analysis - Volumetric data were collected over 48-72h in .dm3 or .dm4 format which were then converted into a single .mrc file (16 bit) that could be immediately viewed in either Fiji⁵ or IMOD⁶. Contrast segmentation was undertaken in Fiji exploiting the difference in grey scale levels between the organelles. For example, t-tubules appeared white whereas the SR was black. Segmentation of both the SR and t-t systems was achieved by adjusting the threshold of the images (Image-Adjust-Threshold). This operation generated a binary image that was viewed in 3D using the Fiji plugin 3D Viewer. Organelles, objects, were also manually segmented using 3dmod (part of the IMOD software suite) for the 3-D reconstruction of the cardiac myocyte organelle organization using closed contours to trace features of the cell. Diameter analysis of the t-ts was determined in Fiji using the histogram function to plot the density across the tubule cross-section and the distance measured between the two peaks corresponding to the edge densities. The 3-D reconstructions of the SR in apposition to the sarcolemma carried out in Fiji were imported into IMOD. The individual jSR patches (e.g. Figure 5) were manually segmented and the surface area and volume information calculated for each object. Volume percentages of each organelle (object) were obtained by calculating the proportion between the pixels in a stack belonging to an object or a family of objects and the total number of pixels of the stack. Stacks were representative of portions of cells. Differences in measurements between samples were tested for statistical significance using Student’s t-test (GraphPad prism). P values less than 0.05 were considered significant.

Legends for video files
Movie 1: 3-D reconstruction of the t-tubule network imaged using sbfSEM of a sheep cardiac myocyte. The t-ts extend from the sarcolemma towards the center of the cell. Longitudinal branches decorating some, but not all, of the t-ts extend in one plane only. The t-ts bend and curve as they project into the cell.

Movie 2: The sarcoplasmic reticulum (SR) is in close apposition to the sarcolemma in sheep cardiac myocytes. The 3-D reconstruction of the SR (red) shows junctional regions, connected by network SR, adjacent to the inner surface of the cell membrane (grey). The viewpoint is looking from the inside of the cell towards the sarcolemma.

References