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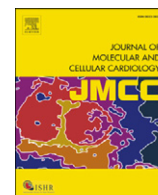

Review article

Adding a new dimension to cardiac nano-architecture using electron microscopy: Coupling membrane excitation to calcium signaling

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ABSTRACT

Advances in microscopic imaging technologies and associated computational methods now allow descriptions of cellular anatomy to go beyond 2-dimensions, revealing new micro-domain dynamics at unprecedented resolutions. In cardiomyocytes, electron microscopy (EM) first described junctional membrane complexes between the sarcolemma and sarcoplasmic reticulum over a half-century ago. Since then, 3-dimensional EM technologies such as electron tomography have become successful in determining the realistic nano-geometry of membrane junctions (dyads and peripheral junctions) and associated structures such as transverse tubules (T-tubules, aka. T-system). Concomitantly, super-resolution light microscopy has gone beyond the diffraction-limit to determine the distribution of molecules, such as ryanodine receptors, with 10^{-8} meter (10 nm) order accuracy. This review provides the current structural perspective and functional interpretation of membrane junction complexes, which are the central machinery controlling cardiac excitation-contraction coupling via calcium signaling. This article is part of a Special Issue entitled “Calcium Signaling in Heart”.

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1. Introduction

Prior to discussing the latest findings of 10^{-9}~10^{-8} meter (1–10 nm) resolution structures of the cardiac excitation-contraction (E-C) coupling system, particularly in 3-dimensions (3D), we briefly touch on the history of how electron microscopy (EM) contributed to establishing the initial concept of E-C coupling. Nonetheless, we do not get into the detail of classic cell anatomy, which was determined by the use of conventional thin-section transmission EM. This has been extensively reviewed in excellent articles (see e.g. [1–3]).

2. Membrane systems for local myofilament activation

The general concept of E-C coupling in muscles was established in the middle of the 20th century, well in advance of the identification of its molecular constituents including ion channels involved in calcium-induced calcium releases (CICRs), sarcolemmal and sarcoplasmic reticulum ion transporters, and their upstream regulators [4]. How EM then

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provided important insights into muscle physiology, first in skeletal muscle followed soon by cardiac muscle, is vividly described in articles of that era [3,5,6].

The membrane organelle, which was first linked to the control of myocyte contraction and relaxation was the sarcoplasmic reticulum (SR). Following the somewhat obscure first EM demonstration of SR in cardiac muscles [7], which resembled that of skeletal muscles presented by Bennett and Porter [8], a landmark paper of Porter and Palade depicted a uniquely formulated two-component membrane structure in cardiomyocytes, which they named a "dyad" [9]. The dyad was identified as a dilated vesicle and a thinner vesicular element enwrapping the vesicle at the I band level of myofibrils. The dilated vesicle, which we now know as a "transverse tubule (T-tubule, aka. T-system)" was thought to be part of the SR back then. Similarly, "triads" were also identified in skeletal muscles [9].

Similarities between the middle element of triads and the T-system [10], as well as the continuity of T-system to surface sarcolemma in skeletal muscles, led Huxley and Taylor to hypothesize that these cellular components conduct membrane depolarization inward and "locally activate" the contraction of myofibrils [11]. Direct connection between inner cellular membranes and sarcolemma was also hypothesized in the cardiac muscle [12]. However, it took several years for scientists to obtain compelling evidence of the direct communication of T-system lumen to the extracellular space, using EM [13–17] and fluorescent microscopy [18]. Since the diameter of the cardiac T-system is 3–5 times thicker than that of skeletal muscles, the connection of sarcolemmal invaginations to T-system was more easily discernible in cardiac muscles including human ventricular cardiomyocytes [3,19,20].

3. Cellular architectures to restrict local calcium releases

Since Ringer's signature study in frog hearts [21], calcium ions have been recognized as the key intracellular messenger for cardiac muscle contraction. In the most simplified working model, the pathway from membrane excitation to contraction is the coupling of two cellular mechanisms involving calcium fluxes. CICR from SRs through ryanodine receptors (RyRs) [22,23] is an autonomic cyclic reaction due to SR refilling by the action of the sarco-endoplasmic reticulum calcium-dependent ATPase (SERCA). Another major calcium flux, i.e. voltage-gated influx of calcium ions from the extracellular space, primarily involves L-type calcium channels (LTCCs), which are sensitive to dihydropyridine. EM played a significant role in locating these channel molecules in different membrane components and connecting CICR with the voltage-gated trans-sarcolemmal calcium influx.

First, EM identified SR as an organelle for calcium accumulation (Ebashi's relaxing factor) [24,25]. CICR was first demonstrated in skinned skeletal muscles as a phenomena in which calcium accumulated in the SR is released by externally added calcium [18,26], followed by a study by Fabiato, which demonstrated CICR in cardiac muscles [27]. Discovery of CICR led to the identification of RyRs as the SR calcium leak channel [28]. Subsequently, the seminal paper published by Inui et al. [29] used EM to reveal that RyRs molecularly make-up SR "feet", periodic densities residing in the SR membrane, bridging the junctional membranes in dyads, triads, and at junctions between surface sarcolemma and opposed SR (i.e. peripheral couplings). Back then, the identity of the "feet" was unknown, but cell biologists were aware of peculiar "periodic densities" residing within the 10–15 nm gaps in these membrane junctions. They were described in multiple ways, such as processes, projections, dense materials, globular densities, bridges, and then "feet", in reports of bat cricothyroid muscle and toadfish swimbladder muscles by Revel [30], in fish muscles by Franzini-Armstrong and Porter [31], and ultimately in cardiac muscle cells [3,32–34].

In contrast, freeze-fracture EM was demonstrating numerous intermembrane particles in the sarcolemma of both non-fixed and fixed cardiac myocytes [35]. Franzini-Armstrong et al. recognized that the surface membrane domains where the large-sized particles (~8.5 nm) were enriched correlated with SR foot arrays both in skeletal and cardiac muscles, and thus speculated that these large-sized particles represent LTCCs [36]. Nonetheless, there was a difference in the distribution patterns of the large-sized particles between cardiac and skeletal muscles. In cardiomyocytes, the large-sized particles did not form tetrads as shown in skeletal muscles [36].

These historical EM findings led us to our current consensus about the anatomical portrait of cardiac E-C coupling machinery, i.e. junctional and dyadic "clefts" between the sarcolemma and SR form "couplons" together with associated membrane structure [37], as theorized by Stern et al. [38]. "Fuzzy space" and "subcellular restricted space" are synonyms of the clefts but these are rather functionally defined terms and likely include both clefts and diffusion-limited cleft vicinity [39,40].

4. Advancement in EM technologies toward higher dimensions

As described above, EM historically fostered our understanding of E-C coupling. Nonetheless, many researchers currently regard EM as a purely descriptive measure and tend to distance themselves from this useful tool. In this regard, there is out a limitation of conventional thin-section transmission EM. The transmission EM works with large focus depth and projects all objects within the entire thickness of sections on to a 2-dimensional (2D) plane (a film or a CCD image sensor), causing the major loss of nano-scale details of 3D structures [41]. In other words, resolution along the electron beam axis cannot be lower than the thickness of sections, while lateral (x–y) resolution is less than one nanometer [42]. So-called ultra-thin sections are typically 60-100 nm in thickness. This limitation led to the development of "electron tomography" or "EM tomography", a computational approach to extract true 3D information from transmission EM. EM tomography relies on multiple 2D image projections which are acquired while a specimen is incrementally tilted around an axis (or axes) perpendicular to the electron beam, along with the application of the weighted back projection principle, which is used in medical computed axial tomography (CAT) scans [43,44]. The product is termed a "3D density map" or a "tomogram", a 3D mass composed of arrayed volume elements (voxels), each of which has an assigned gray-scale value.

Typically, tilt angles are in the range of $\pm$60–80 degrees with 1 or 2-degree increments: e.g. a single-degree increment tile-imaging of $\pm$60 degrees with double tilt-axes prepares 240 projection images to reconstruct a single EM tomogram. The addition of the 2nd tilt axis significantly improves the quality of tomograms by diminishing the Fourier missing wedge [44]. Intermediate high-voltage EM (IVEM) systems operating typically at 200–600 keV are often used in cell biology to ensure adequate resolution in specimens prepared as sections with sufficient thickness to contain major organelles and cellular micro-domains of interest. It is known that the 3D imaging of 3–20 nm resolution range is feasible in 500-nm or thinner sections operated at 300–400 keV.

As in the case of conventional transmission EM, samples used for EM tomography must undergo harsh processing steps including fixation, staining, dehydration, embedding, and sectioning, as well as ultimate imaging. Naturally, it is highly desirable to use the most optimal sample preparation techniques to minimize potential artifacts. Typical chemical fixation involves primary aldehyde fixation of proteins, followed by post fixation of lipids with osmification. Fixed samples then need to be dehydrated for resin infiltration. Each of these multi-step processes results in potential conformational changes in cellular structures. As a result, instantaneous fast freezing methods are preferable over chemical fixation for preserving tissues in its native state [43]. Sosinsky et al. further developed a new hybrid method, which combined low concentration (~0.1%) glutaraldehyde fixation with high pressure freezing and freeze substitution, and achieved high consistency in the structural preservation of tissue sections [45]. While we also obtain favorable

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