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Original article

A new twist in cardiac muscle: dislocated and helicoid arrangements of myofibrillar z-disks in mammalian ventricular myocytes

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ABSTRACT

Using deconvolved confocal microscopy of fluorescently labeled markers for z-disks, t-tubules and ryanodine receptors, we have examined sarcomere organization in cardiac myocytes from rat, rabbit and human. We show that sarcomeres exhibit dislocations in registration and occasionally more complex helicoidal topology. This organization was present at both slack (~1.8 μm) and long sarcomere lengths (~2.2 μm). Misregistrations in z-disks persisted over 15–20 sarcomere lengths and appeared to arise primarily from variations in fiber direction; particularly as myofibrils pass around nuclei. In addition, myofibrils twist along the cell length. T-tubules generally follow the sarcomere z-disks although additional elements bridging adjacent myofibrils and along the length of the myofibril are present to varying degrees in all cells. Ryanodine receptors (the sarcoplasmic reticulum Ca^{2+} release channel) are generally located within 250 nm of the local plane containing t-tubules and z-disks, but a small fraction (~2%) is found on longitudinal elements of the t-system between z-disks. The results are discussed with respect to the possible role(s) of such complex z-disk organization and z-disk dislocations in the maintenance of cell structure and sarcomere assembly. In addition, the non-planar organization of z-disks may be important in the propagation of local Ca^{2+} waves which may have a useful role in helping maintain the uniformity of sarcomere activation in the presence of t-tubule remodeling.

1. Introduction

The cardiac sarcomere is the fundamental unit of contraction and contains both contractile proteins as well as proteins involved in its activation and regulation (for review see [1]). The once static view of the sarcomere structure is now being replaced by a view, based on analysis of protein turnover and alterations of protein expression, to a structure that is highly regulated by a concert of regulatory proteins and chaperones (for review see [2]).

Despite these recent increases in our understanding of the regulation of protein expression within the contractile machinery of the sarcomere, knowledge of the macromolecular architecture of the cardiac myofibril (formed by sarcomeres in series) has been derived mostly from electron microscopy (EM) and thin sectioning techniques. Such studies have shown that myofibrils run from one end of the cell to another in coherent bundles with generally good sarcomeric registration (e.g., see [3]). However, in other striated muscles greater complexity in sarcomere organization is known to occur. Tiegs [4] reported a "zigzag" arrangements of striations in several species that appeared to form helices, which he (incorrectly) suggested might provide a way for the excitation impulse to propagate from the end plate. Even greater topological complexity in sarcomere organization was discovered by high voltage EM which showed that t-tubules (and by inference z-disks) could form large helicoids within the cell [5]. Laser diffraction studies of skeletal muscle also suggest complexity in sarcomere organization as fine structure in the diffraction pattern could be explained by the sarcomeres forming a Bragg crystal with tilt to the long axis of the myofibril or by sarcomere misregistration between myofibrils [6]. Both the cause and physiological significance of these observations have remained unclear and largely unstudied, although it seems likely that the fusion of small myoblasts with subsequent hypertrophy into adult skeletal muscle fibers may play a role. In cardiac muscle, immature myocytes have disorganized myofibrils which gradually align as concerted activation develops and these myofibrils become aligned with the long axis of the cell to give the familiar cross-striated appearance[7].

The contractile environment in cardiac cells is quite different from that of skeletal muscle with shear stresses developing across the ventricular wall [8,9] leading to a gradual change in fiber orientation. In addition, cardiac myocytes have centrally located nuclei whose location is quite different to the peripheral nuclei of skeletal muscle. How cardiac myofibrils adapt to these obstructions and the additional shear stresses they engender is unknown, although it has been suggested that myofibril disorganization may be important in heart failure [10]. Abnormal intra-sarcomeric mechanical forces leading to z-line bending may be transduced by the integrin system which may

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modulate ion channels [11] and the integrin-linked kinase is important for the maintenance of cardiac structure [12]. Therefore a greater understanding of how sarcomeres are arranged inside myocytes may be a key to understanding how such transduction systems normally operate. In this study, we present the first detailed description of sarcomeres at the macromolecular scale in normal mammalian cardiac myocytes, documenting the occurrence of complex, helicoidal sarcomere organization with dislocations in registration between z-disks.

2. Materials and methods

2.1. Ethical information and tissue preparation for antibody labeling

Ventricular myocytes from Wistar rats weighing 250 g were enzymatically isolated as described in [13] according to a protocol approved by the University of Auckland Animal Ethics Committee and following NIH guidelines. Following a lethal injection of pentobarbital (100 mg/kg; I.P.) hearts were quickly excised and mounted on a Langendorff perfusion system for enzymatic (collagenase + protease) cell isolation. Isolated cells were immediately transferred to phosphate buffered saline (PBS) containing 2% paraformaldehyde (PFA; w/v) fixative (pH 7.4) and incubated for 10 min at room temperature (RT). Cell yields were typically 70%–80% rod-shaped cells with optically sharp edges and striations. Fixed cells were washed in fresh PBS for 10 min prior to antibody labeling.

For animal tissue labeling, hearts from rats and 6-month-old New Zealand White rabbits were perfused with Ca^{2+}-free Tyrode's solution at 37 °C for 5 min followed by PBS containing 2% PFA for 10 min at RT. Ventricles were then cut free, diced into small blocks and incubated in PBS containing 1% PFA for 1 h at 4 °C. In order to increase sarcomere spacing beyond slack length, in some experiments hearts were inflated by a latex balloon placed in the left ventricle after the method of [14].

Human tissue was obtained from a clinically normal non-failing heart (which could not be matched for transplantation) with the informed consent from the family of the donor as approved by the New Zealand Health and Disability Ethics Committee (NTY/05/08/050). Immediately after removal, the heart was rinsed in cold cardioplegic solution and samples cut from the midline region of the left ventricle. These samples were transferred to 2% PFA in PBS for overnight fixation at 4 °C.

2.2. Antibody and fluorescent labeling

A suspension of fixed myocytes in PBS was mixed with 3% (w/v) SeaPlaque® agarose (Cambrex, ME) in PBS at 37 °C and then cooled to room temperature to immobilize myocytes in random orientations [15]. Immunocytochemistry was performed on 3- to 4-mm blocks of agar containing fixed cells as described previously [16]. Tissue samples were cryoprotected in 30% sucrose prior to freezing in liquid nitrogen 30 μm thick cryosections were cut on a Leica CM 1900 cryostat, stuck to poly-L-lysine coated number 1.5 glass coverslips and rehydrated with PBS.

For immunocytochemistry, cells were permeabilized with 0.1% Triton X100 in PBS for 10 min, blocked in PBS containing 10% normal goat serum (NGS; Vector Laboratories, CA) and incubated with the primary antibodies overnight at 4 °C. The cells were washed 3 times in PBS for 2 h at RT and the secondary antibody was applied overnight at 4 °C. Excess secondary antibody was removed in 3 further 2-h washes prior to mounting. Rat, rabbit and human tissue sections were blocked in Image-iT® FX signal enhancer (Invitrogen, NZ) for 1 h and the primary antibodies were applied overnight at 4 °C. The coverslips were rinsed 3 times in PBS for 20 min and then incubated with secondary antibodies for 2 h at RT. The tissue was mounted in preparation for imaging following 3 further PBS washes.

Mouse monoclonal IgG primary antibody against α-actinin (EA-53; Sigma) and a goat anti-mouse IgG Alexa 488-conjugated secondary antibody (Molecular Probes, Invitrogen) were used for labeling the z-disks. T-tubules in rabbit and human tissue sections were labeled with an Alexa 647-conjugate of wheat germ agglutinin (WGA), incubated for 2 h at RT. The t-system in rat ventricular muscle was also labeled with a rabbit polyclonal anti-caveolin-3 IgG (AB2912-100; Abcam, Cambridge) and a goat anti-rabbit IgG Alexa 594-conjugate (Molecular Probes, Invitrogen) secondary antibody. Clusters of ryanodine receptors (RyR2) were labeled with mouse monoclonal IgG1 (clone C3-33, MA3916; Affinity Bioreagents, CO) and a goat anti-mouse IgG Alexa 594-conjugate (Molecular Probes, Invitrogen). Dual labeling of RyR2 and α-actinin was performed by labeling the anti-α-actinin primary antibody with Alexa 488 as described elsewhere [16].

2.3. Imaging and data analysis

Fluorescent images were recorded with either an LSM 710 laser scanning confocal microscope (Zeiss, Jena, Germany) using a Zeiss 63x 1.4 NA oil-immersion objective or a Zeiss Axiovert LSM410 laser scanning microscope and a Zeiss 63x 1.25 NA oil-immersion objective. High-resolution confocal sections of myocytes oriented vertically (parallel to the optical axis of the microscope) or in longitudinal section were recorded in 8-bit or 16-bit voxel grids at <0.08 μm in x-y and <0.25 in z.

3-D datasets were deconvolved with a maximal-likelihood Richardson–Lucy algorithm as described earlier [17] and custom analysis routines were written in IDL (ITT, Boulder, CO) and used for quantitative data analysis. Centroids of punctate RyR labeling were determined using a cluster detection algorithm [16]. The transverse planes containing α-actinin were reconstructed from the confocal image stacks and used to define the location of the z-disk [18]. Voxx2 v2.09d [19] was used for volume rendering reconstructed z-disks and t-tubular skeletons. Opendx (www.opendx.org) was used for surface rendering the reconstructed z-disks, color coding the isosurface data and creating movies provided in the supplementary data.

3. Results

In longitudinal confocal sections of isolated rat myocytes, labeling of the costameric protein α-actinin appeared as narrow bands at sarcomeric periodicity, similar to previous descriptions [20] (Fig. 1A). No labeling was observed in the nuclear and perinuclear regions. The myofibrils curved around the (typically two) nuclei and could be followed for 40%–60% of the long axis of the cell before merging with other myofibrils. Misregistrations of the z-line bands of α-actinin were present, particularly in the regions curving around nuclei. Similar misregistrations were also seen in regions where myofibrils bifurcated or where the cell tapered (data not shown). Inspection of such regions in 3D suggested a "zigzag" arrangements of α-actinin was present in neighbouring myofibrils. Such misregistrations were also seen in cells from hearts dilated by balloon, where the mean sarcomere length was increased to 2.22 ± 0.06 μm from 1.82 ± 0.03 μm (mean ± s.d. $n = 10$) (Fig. 1B). Misregistration generally persisted for ~15–20 sarcomeres before registration was re-established with an adjacent myofibril.

Transverse confocal sections of cells oriented along the optical axis yielded a detailed view of the α-actinin expression across the myofibrils. This is demonstrated by a maximum intensity projection of a z-stack of a rat ventricular myocyte across a 1.8 μm depth (Fig. 1C). Spaces occupied by individual myofibrils or tightly packed groups of myofibrils were seen as areas of irregular shape and intense staining that were arranged tightly around the nuclear or perinuclear regions (indicated as "N"). Smaller gaps with no α-actinin staining around