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Structural evidence for perinuclear calcium microdomains in cardiac myocytes

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ABSTRACT

At each heartbeat, cardiac myocytes are activated by a cytoplasmic Ca^{2+} transient in great part due to Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptors (RyRs) clustered within calcium release units (peripheral couplings/dyads). A Ca^{2+} transient also occurs in the nucleoplasm, following the cytoplasmic transient with some delay. Under conditions where the InsP3 production is stimulated, these Ca^{2+} transients are regulated actively, presumably by an additional release of Ca^{2+} via InsP3 receptors (InsP3Rs). This raises the question whether InsP3Rs are appropriately located for this effect and whether sources of InsP3 and Ca^{2+} are available for their activation. We have defined the structural basis for InsP3R activity at the nucleus, using immunolabeling for confocal microscopy and freeze-drying/shadowing, T tubule "staining" and thin sectioning for electron microscopy. By these means we establish the presence of InsP3R at the outer nuclear envelope and show a close spatial relationship between the nuclear envelope, T tubules (a likely source of InsP3) and dyads (the known source of Ca^{2+}). The frequency, distribution and distance from the nucleus of T tubules and dyads appropriately establish local perinuclear Ca^{2+} microdomains in cardiac myocytes.

1. Introduction

Calcium ions (Ca^{2+}) are versatile second messengers that play a key role in a variety of cellular functions, including gene expression, differentiation, neurotransmitter release, muscle contraction, motility, and communication between cells [1,2]. In cardiac muscle, Ca^{2+} directly contributes to electrical and contractile activity and is the intracellular messenger in excitation–contraction (E–C) coupling. Dyads and peripheral couplings, consisting of apposed T tubules/plasmalemma and sarcoplasmic reticulum (SR) [3,4] and collectively called Calcium Release Units (CRUs), are the structural and functional units for E–C coupling. When plasmalemma and T-tubules are depolarized, Ca^{2+} current flows through the L-type Ca^{2+} channels, and this in turn activates ryanodine receptors (RyR) producing a transient increase in cytoplasmic Ca^{2+} [5]. By virtue of their high, localized content of Ca^{2+} release channels, the activity of CRUs results in a temporarily highly elevated level of Ca^{2+} within a short distance (of the order of 1 μm) from the source. This limited space constitutes a Ca^{2+} microdomain, as defined in [6].

When cardiac myocytes are activated through depolarization, in addition to the cytoplasmic Ca^{2+} transient, a second transient also occurs in the nucleoplasm [7–9], but with some delay and a slower decay [10]. Nucleoplasmic transients are thought to involve diffusion from the cytoplasm through the nuclear pore complexes and to be totally dependent on the overall Ca^{2+} changes in the cytoplasm [7,11,12]. The nuclear envelope, although continuous with the ER/SR [13], is a specialized compartment that contains functional $InsP_3R$ and produces $InsP_3$ dependent Ca^{2+} signals in different cells and thus it is expected to take an active role in the evolution of the nucleoplasmic Ca^{2+} transients [14–22]. This $InsP_3$ dependent nuclear transient, which modifies the E–C coupling derived transient, but is not independent of it, seems to be essential for transcription through a calmodulin kinase II (CaMK II) dependent phosphorylation of histone deacetylase 5 (HDAC5), leading to its translocation out of the nucleus and to the derepression of genes that underlie hypertrophic growth, such as those under the control of myocyte enhancer factor-2 (MEF2) [23,24].

In the case of adult cardiac myocytes there is functional evidence for an effect of either direct stimulation with $InsP_3$ or the stimulation of its production using endothelin-1 (ET-1) on nuclear calcium signals characterized by a large amplitude, a temporal lag behind the cytoplasmic Ca^{2+} signal and specific inhibition by $InsP_3R$ blocking agents [12,24,25]. Even more directly, Zima et al. [25] showed that $InsP_3$ addition to isolated cardiac nuclei induced Ca^{2+} signals spreading to the nucleoplasm.

Functional evidence for $InsP_3$ dependent nuclear Ca^{2+} signals in skeletal muscle [14] and in cardiac muscle [25] raises the question of whether myocardium contains $InsP_3R$ in the nuclear envelope, particularly in mouse heart, which is used as a model for showing the gene transcription pathways where $InsP_3Rs$ seems to be involved

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[24,26]. However, so far, location of $InsP_3Rs$ in the nuclear envelope of adult tissue has been demonstrated only in rabbit [27], but not in mouse myocardium. Here we demonstrate the specific location of $InsP_3R$ in the nuclear envelope, but not in the nucleoplasm of mouse cardiac myocytes and we further establish that the close proximity of T tubules and dyads to the nuclear envelope creates a perinuclear microenvironment appropriate for $InsP_3R$ activation.

2. Material and methods

2.1. Adult mouse ventricular myocyte isolation

Adult ventricular myocytes were isolated using a modification of the method described by Mitra and Morad [28], see [29]. Mice were IP injected with 100 units of heparin and euthanized with pentobarbital 50 mg/kg before heart excision. Excised hearts were mounted on a Langendorf apparatus and perfused with Ca^{2+}-free Tyrode's solution for 6 min at 3.0–3.5 ml/min at a temperature of 36°–37 °C, followed by 12–15 min of perfusion with Ca^{2+}-free Tyrode's solution containing: 0.35 mg/ml collagenase B and 0.25 mg/ml collagenase D (Roche Chemical Co.) plus 0.05 mg/ml protease (Type XIV, Sigma Chemical Co.). When the hearts appeared pale and flaccid they were quickly removed from the Langendorf apparatus, the ventricles dissected away and kept in Ca^{2+}-free Tyrode's solution with 1 mg/ml of bovine serum albumin (Fraction IV, Sigma Chemical Co.). The ventricles were teased into small pieces with forceps and sections of ventricular tissue were gently triturated with a Pasteur pipette to dissociate individual myocytes. Successful batches of cells were used in separate functional experiments confirming their viability.

2.2. Immunolabeling and confocal microscopy

Isolated cardiac cells were fixed in 2% paraformaldehyde in 0.1 M cacodylate buffer for 15 min at room temperature, blocked in PBS/BSA1%/goat serum 10%/Triton X-100 0.05% for 1 h and incubated with the primary antibodies overnight at 4 °C. The cells were washed with PBS/BSA1%/goat serum 10% and incubated with secondary antibodies for 1 h at room temperature, washed and mounted in anti-fading mounting medium. Two primary antibodies were used, a polyclonal anti-$InsP_3R$ type 2 ($InsP_3R2$), characterized below, diluted 1:100; and C3–33 (anti-RyR2), a gift of G. Meissner [30] diluted 1:20. The secondary antibodies were alexa-488-conjugated goat anti-rabbit (Molecular Probes) and cyanine 3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA).

For the T tubule and nuclear labeling we used $DiIC_{16}$ 12.5 μg/ml (Molecular Probes, Junction City, OR), and DAPI respectively. Both markers were used on freshly isolated cells and visualized right after mounting them.

2.3. Characterization of anti-$InsP_3R2$ antibody

A polyclonal antibody directed against the N-terminal of $InsP_3R2$ (peptide sequence: N′-C-NPDYR DAQNE GKNVK DGEI) was produced by ProteinTech Group, Chicago, IL, USA. To confirm antibody specificity we tested the antibody against homogenates from Chicken DT40 B lymphocytes with all three $InsP_3R$ isoforms genetically deleted (DT40-KO) [31], in which type 1, type 2 or type 3 rat $InsP_3R$ cloned into the pIRES2-EGFP1 vector (Clontech, Mountain View, CA), were heterologously expressed [32] (Fig. 1A). Additionally, HeK293 cells expressing mostly $InsP_3R2$ (46%) [33] and mouse heart were also tested. Cellular extracts were lysed in 100 μl ice-cold Cytobuster protein extraction reagent (Novagen) supplemented with a broad-spectrum protease inhibitor cocktail (Roche). Protein concentrations of the supernatants were determined with BSA as standard. Protein extracts were suspended in Laemmeli buffer, separated in 5% SDS-polyacrylamide gels and transferred to PDVF membranes (Millipore). Blocking was at room temperature for 1 h in 5% fat-free milk, and the membranes were incubated overnight in the antibody. Immunoreactive proteins were detected using ECL reagents (Pierce Biotechnology) according to the manufacturer's instructions.

2.4. Electron microscopy

Intact hearts were fixed by retrograde perfusion through the left ventricle with 3.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2; kept at room temperature for 2 h; and stored at 4 °C. Isolated cells were fixed by immersion in the same fixative immediately after isolation. The tissue and cells were postfixed in 2% OsO_4 in 0.1 M sodium cacodylate for 1 h at room temperature, stained in bloc with saturated uranyl acetate in H_2O, and embedded in Epon. For T tubule staining, isolated cardiac cells were postfixed in a mixture of 2% OsO_4 and 0.8% $K_3Fe(CN)_6$ for 1–2 h followed by a rinse with 0.1 M sodium cacodylate buffer with 75 mM $CaCl_2$ [34,35]. Samples were then dehydrated, infiltrated, and embedded as above. Ultrathin sections (50–90 nm) and semithin sections (1–200 nm) were cut in Leica Ultracut R (Leica Microsystems, Wien, Austria) using a Diatome diamond knife (Diatome, Biel, Switzerland) and stained in 4% uranyl acetate and lead citrate (only for thin sections). Sections were observed using a Philips 410 microscope (FEI Co.). Images were recorded using a Hamamatsu C4742-95 digital imaging system (Advanced Microscopy Techniques).

2.5. Quantitative analysis from thin sections

Random digital EM images were collected for the following measurements: i) nuclear length (N_L) was measured as the distance between the two farthest poles of each nucleus profile seen in thin longitudinal sections of *in situ* myocytes. In order to insure that the entire length of the nucleus was included in the measurements, we selected images from very well oriented longitudinal sections showing profiles of nuclei that were not cut tangentially as indicated by the sharp outline of the nuclear envelop profiles at the two poles. ii) Nuclear perimeter (N_P), measured from cross sections of *in situ* myocytes, using NIH image; iii) sarcomere length for *in situ* muscle and isolated cells; iv) the frequency of "perinuclear dyads" (F_D) that is the number of dyads contained within a 1 μm wide strip around each nuclear profile, as seen in transverse sections of *in situ* myocytes; v) the length of the dyads (D_L) along the longitudinal axis, measured sections of *in situ* myocytes, again assuming that the whole length is included in the section. Hearts from at least 3 different mice were used for each measurement.

From these data we calculated: i) surface areas of nuclei (S_N) calculated from the measured length and perimeter based on a simplified cylindrical shape. $S_N = N_P \cdot N$. ii) The average number of perinuclear dyads surrounding each nucleus (D/N) is estimated from $D/N = F_D \cdot N_L/D_L$. The term N_L/D_L is used to correct for the fact that nuclei and dyads appear in repeated thin sections in a manner that depend on their length in the direction perpendicular to the section plane. In this case, this means the longitudinal direction in the cell. iii) The number of dyads per unit area of nuclear surface, D_{NS}, is calculated from $D_{NS} = D/N: S_N$.

Isolated cells were examined in a Zeiss Axiovert 510 LSM Pascal confocal microscope. For the Z projections we used optical sections of 0.8 μm thickness with a spacing of 0.3 μm in Z axis, encompassing a total thickness of approximate 4 μm. Images were processed using Perkin Elmer Ultraview Software.

2.6. Nuclear isolation

Nuclei were extracted from cells by a combined hypotonic shock and mechanical disruption in a Dounce homogenizer, as previously described [14] with modifications. Briefly, isolated cardiac cells were pelleted, washed in phosphate-buffered saline and then re-suspended in hypotonic buffer (10 mM K-HEPES, pH 7.9, 1.5 mM $MgCl_2$, 50 mM