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Highlighted Article

Rough endoplasmic reticulum to junctional sarcoplasmic reticulum trafficking of calsequestrin in adult cardiomyocytes

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ABSTRACT

Cardiac calsequestrin (CSQ) is synthesized on rough endoplasmic reticulum (ER), but concentrates within the junctional sarcoplasmic reticulum (SR) lumen where it becomes part of the Ca^{2+}-release protein complex. To investigate CSQ trafficking through biosynthetic/secretory compartments of adult cardiomyocytes, CSQ-DsRed was overexpressed in cultured cells and examined using confocal fluorescence microscopy. By 48 h of adenovirus treatment, CSQ-DsRed fluorescence had specifically accumulated in perinuclear cisternae, where it co-localized with markers of rough ER. From rough ER, CSQ-DsRed appeared to traffic directly to junctional SR along a transverse (Z-line) pathway along which sec 23-positive (ER-exit) sites were enriched. In contrast to DsRed direct fluorescence that presumably reflected DsRed tetramer formation, both anti-DsRed and anti-CSQ immunofluorescence did not detect the perinuclear CSQ-DsRed protein, but labeled only junctional SR puncta. These putative CSQ-DsRed monomers, but not the fluorescent tetramers, were observed to traffic anterogradely over the course of a 48 h overexpression from rough ER towards the cell periphery. We propose a new model of CSQ and junctional SR protein traffic in the adult cardiomyocyte, wherein CSQ traffics from perinuclear cisternae, along contiguous ER/SR lumens in cardiomyocytes as a mobile monomer, but is retained in junctional SR as a polymer.

1. Introduction

Calsequestrin (CSQ) is a major protein of the junctional sarcoplasmic reticulum (SR) lumens in heart [1–3]. Its role in cardiomyocyte function is not yet clear, and may be multi-faceted [4–6]. Overexpression studies in cultured myocytes support a role as a buffer of luminal Ca^{2+} [7], whereas data from CSQ knockout mice suggest a role in regulation of release of Ca^{2+} through the ryanodine receptor (RyR) Ca^{2+} channel [8]. Interestingly, transgenic CSQ overexpressing [9] and knockout mouse models [8], also exhibit unexplained changes in the levels of other junctional SR proteins, such as triadin, junctin, and RyR. These larger changes in junctional SR protein levels highlight our poor understanding of the biosynthetic/secretory system of the adult cardiomyocyte, of which endoplasmic reticulum (ER), free SR, and junctional SR are all a part [10].

Polymerization of CSQ is now thought to play a critical role in its biology, and is thought to occur in response to high luminal ER/SR Ca^{2+} [4,5,11]. Although difficult to study in myocytes, studies of CSQ overexpression in nonmuscle cells have clearly shown how polymerization can also determine CSQ localization [12–14]. In mammalian nonmuscle cells, each CSQ isoform (cardiac or skeletal muscle) undergoes polymerization that leads to its concentration in a specific cellular compartment. Cardiac CSQ concentrates exclusively in the ER of all nonmuscle cells, whereas skeletal muscle CSQ concentrates in the ER-Golgi intermediate compartment (ERGIC). The distinct sites of localization for the two CSQ isoforms were the result of their specific polymerization properties [13]. In this same way, polymerization of cardiac CSQ in adult cardiomyocytes might occur specifically in junctional SR puncta, brought about by the ionic milieu of junctional SR lumens.

Another biochemical property of cardiac CSQ that is likely to play a role in its biology is phosphorylation on two or more serines in the cardiac-specific tail [15–17]. These C-terminal protein kinase CK2 consensus sequences are present in all cardiac isoforms of CSQ and are exquisitely sensitive to CK2 *in vitro*. This modification is believed to occur at the rough ER of the cardiomyocyte where it may affect CSQ translocation or CSQ trafficking from rough ER to junctional SR [17]. CSQ glycans show trimming by cellular mannosidases with no other modification. Because N-acetyl glucosamine (GlcNAc) would be found on CSQ glycans were CSQ to traffic to early Golgi compartments

Abbreviations: CSQ, calsequestrin; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; RyR, ryanodine receptor; GlcNAc, *N*-acetyl-D-glucosamine; Man, mannose; Ad.CSQ-DsRed, adenoviral calsequestrin-DsRed; Ad.CSQ-HA, Adenoviral calsequestrin-hemagglutinin; MOI, multiplicity of infection; TRAP, translocon-associated protein complex; TRAM, translocating chain-associated membrane protein; HRP, horseradish peroxidase; ECL, electrochemiluminescence; DAPI, 4′-6-diamidino-2-phenylindole; IP3R, inositol trisphosphate receptor.

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[12,18], it becomes likely that CSQ remains inside classical mammalian ER compartments.

In a canine tachycardia-induced model of hypertrophy that leads to heart failure, CSQ glycosylation and phosphorylation are very significantly altered [19]. CSQ glycan structures in the hypertrophic heart show a two-fold increase in ER-localized CSQ and a roughly two-fold increase in levels of phosphorylated CSQ in the ER [19]. The cellular processes underlying these changes have remained uncertain due to the lack of understanding of CSQ biosynthesis and trafficking in the adult cardiomyocyte.

In the present study, we used confocal imaging techniques to investigate the direct fluorescence of CSQ-DsRed along with a complementary indirect immunofluorescence, to outline ER and SR regions in adult cardiomyocytes that parallel putative CSQ compartmentation based upon on its biochemistry.

2. Materials and methods

2.1. Heart cell preparation and culture

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animal research was approved by the Wayne State University Animal Investigation Committee (protocol #A 06-07-07). Excised Sprague–Dawley (*Rattus norvegicus*) rat hearts were perfused using a Langendorff apparatus for 5 min before enzymatic dissociation using a 40 ml solution consisting of Liberase Blendzyme (Roche) types 1 and 2 (3 mg/6 mg) in Hank's buffer containing 5 mM pyruvic acid, 1.2 mM $MgSO_4$, 5 mM creatine, 11 mM Glucose, 5 mM taurine, 5 mM carnitine and 0.1 mM $CaCl_2$ (20 min). Cells were titurated for 10 min at 37 °C, filtered, pelleted at $300\times g_{max}$, then washed in phosphate buffered saline (PBS) containing gradually increasing $CaCl_2$ during 3 consecutive washes, to a concentration of 500 μM $CaCl_2$, after which cells were resuspended in Medium 199 containing 2% bovine serum albumin, 2 mM carnitine, 5 mM creatine, 5 mM taurine, 2 mM L-glutamine, 2 mM Glutamax-1 (Invitrogen), ITS mixture (Sigma I3146), 100 units/ml penicillin G, 0.1 mg/ml streptomycin and plated on laminin-coated dishes at 37 °C with 5% CO_2.

2.2. CSQ plasmid and adenoviral constructs

CSQ constructs were generated from wild type canine cardiac CSQ cDNA λgt10 clone IC3A [20]. Adenoviral CSQ-DsRed (Ad.CSQ-DsRed) (from Discosoma) was created using the AdEasy XL Adenoviral Vector System (Stratagene) after canine cardiac CSQ cDNA had been cloned into pDsRed2-N1 vector as previously described [13]. Adenoviral CSQ-hemagglutinin (Ad.CSQ-HA) was previously described [12].

2.3. Adenoviral-mediated overexpression

Recombinant adenoviral treatment was carried out in cultured adult rat primary cardiomyocytes as previously described [16]. Adenoviruses were added directly to dishes 2 h post-plating at a multiplicity of infection (MOI) of 100. Virus treatments were routinely carried out for 48 h, before harvesting for biochemical analysis or fixing for microscopy as described below. Individual dishes and coverslips were incubated for shorter or longer times as indicated.

2.4. Antibodies

Monoclonal antibodies specific to cardiac CSQ and RyR were the generous gift of Dr. Larry Jones, Indiana University School of Medicine. Rabbit polyclonal antibodies raised to canine CSQ2 were purchased from Abcam (ab3516). Rabbit polyclonal anti-HA antibody was purchased from Sigma-Aldrich. Rabbit polyclonal anti-sec23 antibody was obtained from Affinity BioReagents. Rabbit polyclonal anti-DsRed antibody was purchased from Clontech. Rabbit polyclonal antibodies specific to translocon-associated protein complex (TRAP) and translocating chain-associated membrane protein (TRAM) were the generous gift of Dr. Ramanujan Hegde, National Institute of Child Health and Human Development, NIH. Alexa Fluor 488-conjugated goat anti-rabbit IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibodies were purchased from Invitrogen.

2.5. Immunoblotting

SDS-PAGE was carried out according to Laemmli [21] on 7.5% acrylamide gels, and transferred to nitrocellulose membranes (0.45 μm, Bio-Rad Laboratories) and stained with Amido black (Sigma). Immunoblotting was carried out as previously described [22] using HRP-coupled secondary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. A SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) electrochemiluminescence (ECL) kit was used according to the manufacturer's protocol to detect immune complexes, which were then visualized by autoradiography using Amersham Hyperfilm ECL film (GE Healthcare). Protein concentrations were determined according to Lowry [23].

2.6. Fluorescence microscopy

Cells were fixed on coverslips with 4% paraformaldehyde in PBS for 10 min and then permeabilized for 5 min in 0.2% Triton X-100. Coverslips were blocked in PBS with 0.2% Tween-20 (PBS/Tween) and 2% goat serum at room temperature for 1 hour. Cells were then incubated in PBS/Tween with primary antibody (1:200) for 90 min at ambient temperatures, followed by washing in PBS/Tween, and incubation with goat anti-rabbit IgG or goat anti-mouse IgG antibodies conjugated to Alexa Fluor dyes (1:500 dilution in PBS/Tween for 60 min). Cells were counterstained with 100 μM 4′-6-diamidino-2-phenylindole (DAPI) for 2 min, rinsed, and mounted to microscope slides with ProLong Antifade Mounting Kit (Molecular Probes). Confocal microscopy was performed at the Microscopy and Imaging Resources Laboratory at Wayne State University, School of Medicine. Imaging was performed using a C-Apochromat 63×/1.2 WKorr water objective on either a Zeiss LSM 510 laser scanning microscope or an Axioplan2 Imaging fluorescent microscope with Apotome imaging, or using a 63×/1.4 oil objective on a Leica TCS SP5 laser scanning microscope. Laser scanning images were acquired on a 1024×1024 pixel canvas with 8-line averaging. Apotome images were obtained with a Zeiss Axiocam MRm B/W CCD camera. Confocal images were processed and optimized offline for publication using Photoshop (Adobe Systems Inc.). Z-stack images created by the Leica TCS SP5 were compiled and optimized in ImageJ (Wayne Rasband, National Institutes of Health). The ImageJ 3D Viewer plugin was used to create three-dimensional z-stack images.

3. Results

3.1. CSQ-DsRed is localized to perinuclear regions of the cardiomyocyte

Following its overexpression in cultured adult rat cardiomyocytes, the fluorescent fusion protein CSQ-DsRed produced a unique pattern of subcellular localization in which both myonuclei were surrounded by intense red fluorescence (Fig. 1). Red fluorescence could be visualized in most cells after 32 h of virus treatment, consistent with levels of overexpression observed by immunoblotting (Fig. 2). CSQ-DsRed accumulated in cardiomyocytes to levels that were comparable to endogenous levels of CSQ after 48 h, by which time CSQ-DsRed prominently surrounded both nuclei producing a pattern that was