Excitation-Contraction Coupling I

2816-Pos

Inhibition of Calsequestrin Phosphorylation Leads to its Trafficking from Rough Endoplasmic Reticulum

Timothy P. McFarland, Steven E. Cala.
Wayne State University, Detroit, MI, USA.

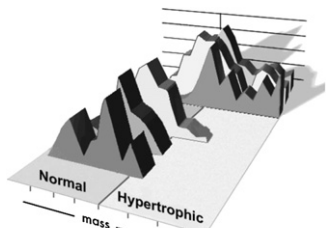
Calsequestrin 2 (CSQ) is a protein in heart cells that concentrates within the sarcoplasmic reticulum (SR), where it incorporates into Ca2+-release complexes. CSQ has a single N-linked glycan that can be used to determine the extent of its trafficking through the secretory system, and multiple C-terminal protein kinase CK2 sensitive phosphorylation sites. Recent data suggests that polymerization of CSQ defines its localization, and leads to its retention within discrete secretory compartments. In these studies, we show that phosphorylation may be responsible for localization of cardiac CSQ within the rough ER of nonmuscle cells and cardiomyocytes. Triple point mutations were made to wild-type canine CSQ (CSQ-WT) phosphorylation sites, either mimicking (CSQ-aPP, S378,382,386E) or inhibiting phosphorylation (CSQ-nPP, S378,382,386A). Overexpression into COS cells showed that, compared to CSQ-WT, mannose trimming of the CSQ glycan increased for CSQ-nPP, reflecting movement out of rough ER; whereas no change was seen for CSQ-aPP. This apparent phosphorylation-dependent change in subcellular localization in COS cells could be observed by confocal immunofluorescence microscopy. The CK2 specific inhibitor tetrabromocinnamic acid (TBCA) blocks phosphorylation of CSQ in vitro by CK2 or by endogenous CSQ kinases with an identical dose response, supporting the identity of CK2 as the CSQ kinase. TBCA (100μM) also inhibited phosphorylation in cultured cells by roughly 80%. Analysis of CSQ overexpressed in these cells showed an effect on CSQ trafficking that was highly similar to the effects of the phosphorylation-site mutant CSQ-nPP. These data support a hypothesis that CSQ phosphorylation by protein kinase CK2 acts to retain cardiac CSQ in rough ER. In cardiomyocytes, growth-dependent phosphorylation of CSQ produces rough ER retention resulting in redistribution of CSQ from junctional SR to the nuclear envelope, the site of IP3-dependent ER Ca2+ storage.

2817-Pos

Cardiac Hypertrophy Causes Newly-Synthesized Calsequestrin to Remain Around the Nucleus in Rough Endoplasmic Reticulum

Steven E. Cala[1], Sony Jacob[1], Naama Sleiman[1], Hani Sabbah[2].
[1]Wayne State University, Detroit, MI, USA, [2]Henry Ford Health Systems, Detroit, MI, USA.

Hypertrophy (LVH) leads to use of perinuclear Ca2+ distinct from junctional sarcoplasmic reticulum (jSR). Little is known about Ca2+-handling proteins associated with regulation of perinuclear Ca pools. Calsequestrin (CSQ), the Ca2+-binding protein of jSR, exists as a polymorphic collection of mass isoforms that results from actions of phosphatases and mannosidases following its biosynthesis in rough ER. To test the hypothesis that LVH triggers changes in CSQ structure by regulating its biosynthesis, we induced heart failure (LVEF <35%) in dogs by intracoronary microembolizations, resulting in increased cardiomyocyte cross-sectional area (60 ± 10%). Detergent-solubilized CSQ was purified from LV, and electrospray mass spectrometric analysis resolved individual CSQ protein structures in 3 failed and 3 normal hearts. In hypertrophic hearts, only higher mass CSQ structures were present, characteristic of perinuclear rough ER of cardiomyocytes (Figure). CSQ glycoforms and phosphoforms in LVH showed underprocessed structures characteristic of newly formed CSQ in rough ER that were more highly phosphorylated by CK2, a growth-activated kinase. These changes in CSQ processing in LVH may be part of a transformation from jSR Ca stores to perinuclear IP3-sensitive stores needed to maintain an altered phenotype.



2818-Pos

Effect of Azumolene on RyR1-Dependent Store Operated Calcium Entry in RyR1-Expressing, Non Excitable Cells

Balazs Lukacs, Daniela Requena, Sutanu Samanta, Jerome Parness.
Childrens's Hosp Pitt-UPMC, Pittsburgh, PA, USA.

Store-Operated Calcium Entry (SOCE) restores Ca^{2+} to depleted endoplasmic reticulum (ER) from the extracellular space via a multiprotein complex involving plasma membrane Orai1 and TRPC1, and ER membrane resident STIM1. Dantrolene and azumolene suppress the rise in intracellular Ca^{2+} seen during skeletal muscle during excitation-contraction coupling and in malignant hyperthermia, a hypermetabolic pharmacogenetic sensitivity to volatile anesthetics. Azumolene inhibits a component of SOCE coupled to activation of RyR1, the skeletal muscle sarcoplasmic reticulum Ca^{2+} release channel, but not Ca^{2+} release itself. Classical SOCE, activated by SR Ca^{2+}-ATPase inhibitors, is unaffected. Thus, azumolene distinguishes between two mechanisms of cellular signaling to SOCE in skeletal muscle, one that is coupled to and one independent from RyR1. We used CHO cells stably transfected with RyR1 (C1148) and wild type (CHO-wt) to determine whether these distinguishable mechanisms of Ca^{2+}entry are present universally, or only in excitable cells. SOCE was measured using Mn^{2+} quenching of Fura-2 fluorescence. C1148 cells expressing RyR1, but not CHO-wt, had high intrinsic Mn^{2+}-quenching that is inhibited by azumolene and by the specific SOCE inhibitor BTP2, while low intrinsic Ca^{2+} entry of CHO-wt was unaffected by these drugs. On contrast, SOCE stimulated by the SR Ca^{2+}-ATPase inhibitor, CPA (10μM), was inhibited by BTP2, but not by azumolene. Knockdown of STIM1 levels using shRNA demonstrates inhibition of RyR1-coupled SOCE. Immunocytochemistry of C1148 cells shows colocalization of RyR1 and STIM1 proteins in the presence of the RyR1 agonists, caffeine and ryanodine, and these proteins co-immunoprecipitate, suggesting they are interacting proteins. Thus, in RyR1-expressing, non-excitable cells, azumolene inhibits RyR1-dependent SOCE, but not Ca^{2+}-ATPase-dependent SOCE, and suggests that STIM1, as one of the components of the SOCE machinery, may need to interact with RyR1 in this pathway.

2819-Pos

Impact of Calsequestrin on the SR Calcium Concentration in Skeletal Muscles Fibers Monitored with a Genetically Encoded Fret Based Indicator

Carlo Reggiani[1], Marta Canato[1], Michele Scorzeto[1], Marta Giacomello[1], Feliciano Protasi[2], Ger J M Stienen[3].
[1]University of Padova, Padova, Italy, [2]University of Chieti, Chieti, Italy, [3]Free University, Amsterdam, Netherlands.

The cytosolic free Ca^{2+}-concentrations transients elicited during muscular contraction are well characterized but little is known about the free $[Ca^{2+}]$ dynamics inside the sarcoplasmic reticulum (SR). FRET-based Ca^{2+} indicators (Cameleons) allowed us to investigate SR Ca^{2+}-handling with high time resolution (9 ms or less). The impact of calsequestrin (CS) on SR $[Ca^{2+}]$ was studied in enzymatically dissociated FDB muscle fibers from wild type (WT), CS type $1^{(-/-)}$ (KO) and double CS type 1/CS type $2^{(-/-)}$ (DKO) mice 7 days after transfection with the cDNA coding for D1ER.

$[Ca^{2+}]$ measurements were performed at rest and during repetitive stimulation at 1, 5, 20 and 60 Hz, and the YFP(535nm)/CFP(480nm) ratio was deemed as a measure of the free SR $[Ca^{2+}]$.

The SR $[Ca^{2+}]$ at rest did not differ between WT (n=26), KO (n=25) and DKO (n=21) fibers while during electrical stimulation they were rather small in WT, reflecting powerful buffering of SR $[Ca^{2+}]$. In KO and DKO fibers, a significant reduction in the SR $[Ca^{2+}]$ occurred, which increased in parallel with the stimulation frequency. At 60 Hz the SR became virtually depleted of Ca^{2+}, both in KO and DKO fibers. Calcium reuptake during and after the trains of stimuli was governed by 3 rate constants of 50 s^{-1}, 1-5 s^{-1} and 0.3 s^{-1} (at 26°C). In conclusion, CS-KO fibers represent a unique model to resolve the kinetics of SR release and reuptake.

2820-Pos

Initial Characterization of CASQ1/CASQ2 Knockout (double CASQ-Null) Mice

Cecilia Paolini[1], Marco Quarta[2], Marco Dainese[1], Mirta Tomasi[3], Marta Canato[3], Alessandra Nori[3], Bjorn C. Knollmann[4], Paul D. Allen[5], Carlo Reggiani[3], **Feliciano Protasi**[1].
[1]University G. d'Annunzio, Chieti, Italy, [2]Stanford University, Stanford, CA, USA, [3]University of Padova, Padova, Italy, [4]Vanderbilt University, Nashville, TN, USA, [5]Harvard Medical School, Boston, MA, USA.

In muscle, Calsequestrin (CASQ), the major Ca^{2+}-binding protein of the sarcoplasmic reticulum (SR) terminal cisternae, is expressed as two different isoforms. CASQ1 is the only isoform present in adult fast-twitch fibers. On the other hand, CASQ2 expression, abundant in all fibers before birth, decreases progressively after birth and in the adult is only found in slow twitch fibers, co-expressed with CASQ1. Lack of CASQ1 results in: a) significant structural and functional alterations to the excitation-contraction coupling machinery (Paolini et al., 2007. *J Physiol* 583:767); b) higher rate of spontaneous mortality in males; and c) malignant hyperthermia (MH)-like phenotype (Dainese et al., 2009. *Faseb J* 23:1710). However, in CASQ1-null mice CASQ2 is still expressed in slow twitch fibers. We have now generated a mouse lacking both CASQ isoforms (double CASQ-null), by cross-breeding our mice with

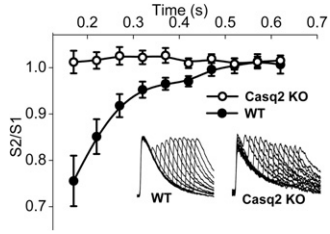
CASQ2-knockout animals (Knollmann et al., 2006. *J Clin Invest* 116:2510). Lack of both CASQ isoforms was confirmed by western blot. The double-null mice are viable and breed normally, however the rate of spontaneous mortality of male animals is higher than CASQ1-null animals. Whereas the overall phenotype of mice is similar to that of CASQ1-null mice, significant differences are found in Soleus. From the structural point of view, in Soleus muscle we find many fibers (about 30%) with severe structural damage that were not found in CASQ1-null animals. Functional studies indicate significant prolongation in twitch time parameters, increased twitch tension and impaired tension generation during prolonged tetani both in EDL and Soleus, likely related to abnormal calcium release kinetics. These findings suggest that: a) expression of CASQ2 is essential for the maintenance of a subpopulation of Soleus fibers; and b) lack of both CASQ1 and 2 exacerbates the overall phenotype of CASQ1-null mice.

2821-Pos

Refractoriness of Sarcoplasmic Reticulum Calcium Release in Cardiac Muscle Due to Calsequestrin

Oleksiy Gryshchenko, Dmytro Kryshtal, **Bjorn C. Knollmann**.
Vanderbilt University, Nashville, TN, USA.

In cardiac excitation-contraction coupling, L-type Ca^{2+} current (I_{Ca}) triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptor (RyR) Ca^{2+} release channels. It is unclear why SR Ca^{2+} release cannot be elicited by premature stimuli, even though I_{Ca} is fully recovered. Here, we use calsequestrin null mice (Casq2 KO) and wild-type littermates (WT) to test the hypothesis that calsequestrin (Casq2) determines refractoriness of SR Ca^{2+} release. Ca^{2+} release refractoriness was measured in voltage-clamped myocytes dialyzed with Fluo-4 by applying premature extrastimuli (S2) at successively shorter S1-S2 coupling intervals following a 1 Hz train (S1 stimuli). To maintain constant trigger, Ca^{2+} release was activated with I_{Ca} tail currents that elicited maximal Ca^{2+} release during the S1 train. WT S2 Ca^{2+}release was significantly depressed with short coupling interval whereas Casq2 KO cardiomyocytes exhibit no refractoriness of Ca^{2+} release (Figure, n=11 WT, 12 KO, $p = 0.01$). At the same time, I_{Ca} current density, SR Ca^{2+} content, and steady-state Ca^{2+} transients (S1) were not significantly different from WT-myocytes. We conclude that calsequestrin is a critical determinant of SR Ca^{2+} release refractoriness in cardiac muscle (Supported by NIH-R01HL71670, R01HL88635).



2822-Pos

Effect of Triadin on Retrograde and Orthograde Signaling between RyR1 and DHPR in Cultured Myotubes

Jose M. Eltit, Hongli Li, Paul D. Allen, **Claudio F. Perez**.
Brigham and Women's Hospital, Boston, MA, USA.

Using pan triadin-null mice we previously showed that triadins ablation did not disrupt EC-coupling in muscle cells. However, calcium imaging studies in cultured myotubes did reveal that triadin-null myotubes had slightly smaller depolarization-induced Ca^{2+} transients than Wt cells. Here, using whole-cell voltage clamp, we analyze the effect of triadin ablation in skeletal EC-coupling by characterizing the retrograde and orthograde signaling between RyR1 and DHPR of triadin-null myotubes. Calcium currents elicited by 200ms depolarization steps in Wt and triadin-null cells showed slow kinetics of activation and peak current at approximately +30 mV. Although, the overall voltage dependence was preserved between Wt and triadin-null cells a leftward shift in the I/V curve was observed in triadin-null cells ($V_{1/2}$, 22.3 ± 0.8 mV in Wt vs 16.6 ± 1.1 mV in triadin-null cells, $p<0.05$). In addition, kinetic analysis of the DHPR Ca^{2+} current shows that the activation time constant of the slow component (τ_{slow}) was slightly decreased from 37 ± 2.4 ms in Wt to 26 ± 2.6 ms ($p<0.05$) in triadin-null cells.

The voltage-evoked Ca^{2+} transient, on the other hand, showed a small but significant reduction of the peak fluorescence amplitude of triadin-null cells ($\Delta F/F_{max}$, 0.72 ± 0.2 in Wt vs 0.61 ± 0.1 in triadin-null) with no differences in voltage dependence (V_m, -7.2 ± 1.1 mV in Wt vs -10.1 ± 1.9 mV in null cells). Our results suggest that the absence of triadin expression preserves the orthograde and retrograde signaling between DHPR and RyR1 nearly intact and that the effect of triadin ablation on $\Delta F/F_{max}$ would be secondary to the dysregulation of calcium homeostasis observed in triadin-null cells. These data give further support to the idea that skeletal triadins do not play a direct role in skeletal EC-coupling.

Supported by NIH Grants 5K01AR054818-02 (to CFP) and 1P01AR044750 (to PDA).

2823-Pos

Altering Skeletal Muscle EC Coupling by Ablating the Sarcoplasmic Reticulum Protein JP45 Affects Both Metabolism and Muscle Performance in Old Mice

Osvaldo Delbono[1], Zhong-Min Wang[1], Jackson Taylor[1], Maria Laura Messi[1], Susan Treves[2], M. Nishi[3], H. Takeshima[3], Francesco Zorzato[2].
[1]Wake Forest University School of Medicine, Winston-Salem, NC, USA, [2]Basel University Hospital, Basel, Switzerland, [3]Kyoto University, Kyoto, Japan.

JP45, a sarcoplasmic reticulum protein, appears to be mainly expressed in skeletal muscle. In mice, its expression is down-regulated during aging, and it interacts with the α1.1 subunit of the dihydropyridine receptor (Cav1.1) and calsequestrin, two key components of the excitation-contraction (EC) coupling machinery.

We examined 12- and 18-month-old JP45 knock-out mice and compared them with age-matched, wild-type littermates. The JP45 KO mice exhibit a phenotype consistent with impaired skeletal muscle EC coupling, confirming our previous results in young JP45 KO mice. Spontaneous motor activity assessed with a running wheel revealed that the older JP45 KO group runs less and much more slowly than age-matched WT and young JP45 KO mice. *In vitro* muscle contractile property analysis showed lower twitch and tetanic absolute and specific force, evident mostly in the EDL of aged JP45 KO mice compared to age-matched WT, which correlates with type-II fiber atrophy. Cav1.1 expression and SR Ca^{2+} release in voltage-clamped flexor digitorum brevis muscle fibers of aged JP45 KO mice were reduced compared to age-matched WT. Additionally, aged JP45 KO mice exhibited decreased food intake and body weight.

Our results show that JP45 plays a role in EC coupling and regulation of body metabolism. *Supported by NIH/NIA, Japanese Science Foundation, M.U.R.S.T., A.F.M., and Swiss Muscle Foundation.*

2824-Pos

Characterization of Calumenin-RyR2 Interaction in Murine Heart

Sanjaya K. Sahoo, Taeyong Kim, Do Han Kim.
Gwangju Institute of Science and Technology, Gwangju, Korea, Republic of.

Calumenin is a multiple EF-hand Ca^{2+}-binding protein localized in the sarcoplasmic reticulum (SR). In our recent study, we showed that calumenin-knockdown (KD) of HL-1 cells led to enhanced Ca^{2+} release and Ca^{2+} uptake in the SR (*Sahoo et. al. J. Biol. Chem.*, 2009). To elucidate the underlying mechanisms responsible for the enhanced Ca^{2+} release from the SR in calumenin-KD samples, the possible interaction between calumenin and RyR2 was examined by various methods. GST pull-down assay showed a direct interaction between calumenin and RyR2. We have further found that the middle region of calumenin (aa 132-222) interacts with RyR2. GST pull-down assay also shows that RyR2 intra luminal loop-I region (aa 4519-4576) is the binding site for calumenin. Immunofluorescence study shows that RyR2 and calumenin are co-localized in the junctional region of SR in rat ventricular cardiomyocytes. The detailed amino acid residues involved in the interaction between calumenin and RyR2 are currently under investigation. (This work was supported by the Korean Ministry of Science and Technology grant, Systems Biology Research Grant, M1050301001-6N0301-0110, and the 2009 GIST Systems Biology Infrastructure Establishment Grant).

2825-Pos

Progressive Triad-Mitochondria Un-Coupling in Aging

Alessandra D'Incecco, Marco Dainese, Feliciano Protasi,
Simona Boncompagni.
University of G. D'Annunzio, Chieti, Italy.

An impairment of the mechanisms controlling the release of calcium from internal stores (excitation-contraction (EC) coupling) has been proposed to contribute to the age-related decline of muscle performance that accompanies aging (EC un-coupling theory). EC coupling in muscle fibers occurs at specialized intracellular junctions called calcium release units, or triads, which are specifically placed at sarcomere's I-A band transition. In recent publications we have shown that: a) in human muscle, the frequency of triads decreases significantly with age (Boncompagni et al., 2006; J Gerontol 61:995); and b) in mice, triads are tethered to mitochondria placed at the I band (Boncompagni et al., 2009; MBC 20:1059). Here we have studied the frequency, sarcomeric-localization, ultrastructure, and coupling of triads/mitochondria in EDL from male WT mice using transmission electron microscopy (TEM).

Preliminary results indicates that the number of triads/100μm2 of longitudinal section in aging mice (n=4, 25-35 months of age) decreases compared to the adult mice (n=5, 3-12 months of age): 92 ± 9 vs. 79 ± 8. In addition, the percentage of abnormally positioned triads (longitudinal and/or oblique) increases. On the other hand, the total volume of mitochondria does not change significantly with age. However, the number of mitochondria-profiles/100μm2 of