zebrafish oocytes with a splicing blocker morpholino designed to bind to RyR3 pre-mRNA. Tails from several fish at 72 hpf (hours post fertilization) were pooled for western blotting. A pan-RyR antibody (34C) reveals three bands, presumably representing RyR3 and RyR1a and b. The slowest band specifically disappears after morpholino injection. Tails from ~50 larvae at 72 hpf were collected for enzymatic dissociation of single muscle fibers. Intact 48 and 72 hpf tails and some dissociated fibers were fixed for EM. The P/N ratio in EM images of fast fibers in control larvae at 72 hpf was 0.79 ± 0.14, mean ± SD (7 fish, 70 fibers, 310 triads; 3 experiments) and decreased to 0.03 ± 0.03 (10 fish, 100 fibers, 460 triads; 4 experiments) in injected larvae. To measure Ca sparks, dissociated fibers were adhered to coverslips by matrigel, loaded with Fluo-4, and imaged confocally. 0.3 mM caffeine was used to stimulate sparks, which were readily detected in normal cells but almost absent in morpholino-treated cells. These data identify P/N as RyR3 and indicate that RyR3 activity is required for the ready-detection of sparks.

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Chemical Uncoupling the DHPR-RyR1 Complex by Substituted Halogenated Diphenylethers
Yassaman Niknam, Wei Feng, Gennady Cherednichenko, Yao Dong, Isaac Pessah.

Vet Med: Molecular Biosciences, UC Davis, Davis, CA, USA.

Ca2+ release units (CRU) of skeletal muscle have two components necessary for excitation-contraction coupling (ECC); the DHPR and RyR1 that physically interact to engage conformational coupling that involves both orthograde and retrograde regulation of Ca2+ dynamics during ECC and SR Ca2+ leak when the muscle is at rest. We previously identified that 2,2',3,5',6-pentachlorobiphenyl (PCB95) promotes long-lived stability of the full open state of RyR1 channels (Samso et al 2009). Further investigations PCB95 and its 4- and 5-hydroxy (-OH) derivatives toward rabbit skeletal muscle ryanodine receptor (RyR1) were performed using [3H]ryanodine binding and SR Ca2+ flux analyses. 5-OH metabolites have comparable activity to their respective parent in both assays; however, 4-OH derivatives are unable to trigger Ca2+ release from SR microsome in the presence of Ca2+-ATPase activity. PCB95 and derivatives are investigated using single channel voltage-clamp and clamp and mirror embryonic muscle cells. Like PCB95, 5-OH-PCB95 quickly and persistently increases channel open probability (P0>0.9) by stabilizing the full-open channel state, whereas 4-OH-PCB95 transiently enhances Po. Ca2+ imaging of myotubes show acute exposure to PCB95 (5µM) potentiates ECC and caffeine responses and partially depletes SR Ca2+ stores. Exposure to 5-OH-PCB95 (5µM) increases cytoplasmic Ca2+2, leading to ECC failure in 50% of myotubes with the remainder retaining negligible responses. 4-OH-PCB95 neither increases baseline Ca2+ nor causes ECC failure but depresses ECC and caffeine responses by 50%. With longer (3 h) exposure to 300 nM PCB95, 5-OH-PCB95, or 4-OH-PCB95 decreases the number of ECC responsive myotubes by 22%, 81%, and 51% compared with control by depleting SR Ca2+ and/or uncoupling ECC. The powerful hydroxylation of PCB95 and chemically related diphényl ethers differentially influence RyR1 channel gating kinetics, SR Ca2+ leak, and the pattern of ECC impairment, including ECC uncoupling in intact muscle cells. P01 AR52354.

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Altered Ion Channel Properties of Ryanoide Receptor from Heart Mice Lacking Calstabin2
Nathalie Saint, Albano C. Meli, Valerie Scheuermann, Alain Lacampagne.

CHU Arnaud de Villeneuve, INSERM, Montpellier, France.

The cardiac ryanodine receptor (RyR2) is the main channel for the release of intracellular calcium during excitation-contraction coupling in cardiac muscle. Calstabin2 (i.e., FKBP12.6), a member of the FK506-binding protein family, has high affinity to RyR2, stabilizing RyR2 in the closed state and reducing its activity. The goal of the present work was first to examine the ion channel properties of RyR2 from calstabin2-/- mice reconstituted into lipid bilayers in resting condition. The second aim was to evaluate the effect of adding recombinant calstabin2 on RyR2 ion channel properties. RyR2 channels from 9 months old calstabin2 deficient mice exhibited an increase of opening probability compared to RyR2 from wild-type mice in conditions of low free cytosolic Ca2+ of 150 nM to approximate the conditions in the heart during diastole. The activity of RyR2 calstabin2-/- channels was also higher with 350 mM/L and 700 mM/L cytosolic Ca2+. When channels were activated by higher cytosolic Ca2+ concentration (> 700 mM/L free Ca2+), no differences in open probability were found between RyR2 calstabin2-/- and WT channels. Addition of 1 nM ATP increased the number of long openings to the maximum conductance in both native and calstabin2-/- channels with a prevalent sub-conductance activity for the depleted calstabin2 RyR2 channels. Addition of calstabin2 did neither increase nor decrease RyR2 calstabin2-/- open probability and had no effect in preventing the sub-conductance activity. This suggests that in vitro addition of calstabin2 is not sufficient to restore the RyR2 calstabin2 complex. This conclusion should further be supported by the biochemical analyses of RyR2 macromolecular complex remodeling, performed along with age-dependency.

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Modulation of DHPR Inactivation by RyR1 Activity in Mouse Skeletal Muscle Fibers
Zoita Andronache, Werner Melzer.

Ulm University, Ulm, Germany.

Malignant hyperthermia is a potentially fatal hypermetabolic state originating from excessive release of calcium stored in the sarcoplasmic reticulum (SR) of skeletal muscle. In most cases, MH susceptibility results from mutations in the type 1 ryanodine receptor (RyR1). In previous work (Andronache et al., PNAS 2009) we reported that heterozygous murine carriers of MH mutation Y524S (human Y522S) exhibit changes in steady state inactivation of the dihydropyridine receptor (DHPR), the sensor of the transverse tubular (TT) membrane potential. Availability curves were left shifted along the voltage axis suggesting that a feedback signal from RyR1 modulates DHPR inactivation. In the present study we investigated the hypothesis that junctional fluctuations of free Ca2+ concentration are involved in the feedback mechanism. We performed two-electrode voltage clamp experiments on enzymatically isolated toe muscle fibers of both WT and mutant mice (Y524S+/−) and measured L-type Ca2+ current and optical signals from fluorescent Ca2+ indicators. To test the hypothesis we applied conditions that would modify junctional Ca2+ levels. Millimolar concentrations of caffeine led to a left shift in the availability curve for L-type current indicating that drug-induced RyR1 hyperactivity can mimic the effect of the mutation. On the other hand, internal dialysis with an artificial solution containing 10 mM of BAPTA to effectively reduce the local Ca2+ transients near open RyR1 channels had little effect on the difference in steady state inactivation between WT and mutant fibers. We conclude that the altered inactivation depends on RyR1 hyperactivity but does not require the continuous presence of local Ca2+ fluctuations within the junctional gap separating TT and SR.

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Ca2+ Influx Mediated by Reverse Mode of Na+/Ca2+ Exchanger is Enhanced in Malignant Hyperthermia Skeletal Muscle
Francisco Altamirano, Jose M. Eltit, Isaac Pessah, Paul D. Allen, Jose R. Lopez.

1Department of Molecular Biosciences, University of California at Davis, Davis, CA, USA, 2Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA, USA.

Malignant hyperthermia (MH), is potentially fatal pharmacogenetic disorder of skeletal muscle, which has been associated with intracellular Ca2+ dysregulation. The Na+/Ca2+ exchange (NCX) is a bidirectional transporter that normally exudes Ca2+ from the cell (forward mode), but also brings Ca2+ into the cell (reverse mode) under special conditions such as intracellular Na+ accumulation or membrane depolarization. Resting intracellular Ca2+ concentration ([Ca2+]r) and intracellular Na+ concentration ([Na+]r) were elevated in MH susceptible (MHs) swine and mice muscles compared to Wt (MHN) muscle fibers. Lowering extracellular Na+ induced an elevation of [Ca2+]r in both MHN and MHS swine and rodent muscle fibers that could be prevented by removal of extracellular Ca2+. Local application of KB-R7943, a nonselective NCX blocker, reduced [Ca2+]r in both MHN and MHS muscle fibers and the magnitude of the elevation of [Ca2+]r observed during a MH episode. YM-2444769 an NCX blocker that preferentially inhibits NCX3 reverse mode did not reduce resting [Ca2+]r in MHN, but it does in MHS muscle fibers, and decreased the amplitude of the elevation of [Ca2+]r induced by halothane in these muscles. These results demonstrate the existence of a functional NCX in skeletal muscle, which appears to be enhanced in MHS muscle fibers. In addition, they represent the first evidence that NCX -reverse mode- contributes to the dysfunction of [Ca2+]r and [Na+]r and intracellular Ca2+ elevation induced by halothane in MHS muscle.

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Myofilament Ca2+ Desensitization is Associated with Reduced L-Type Ca2+ Channel Activity Mediated by Neuronal Nitric Oxide Synthase in Left Ventricular Myocytes from Murine Hearts
Yue Wang, Chun ZJ Jin, Sang Joon Kim, Yin Hua Zhang.

Seoul National University, College of Medicine, Seoul, Republic of Korea.