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1206-Pos Board B50

SRP-35 A Putative NAD(P)H Binding Protein Of Skeletal Muscle Sarcoplasmic Reticulum Membrane

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SRP-35 (sarcoplasmic reticulum protein of 35 kDa) is a newly identified integral membrane protein constituent of skeletal muscle sarcoplasmic reticulum. We have deduced the primary structure of the protein from cDNA clones isolated from mouse and rat skeletal muscle cDNA libraries. Primary sequence prediction analysis indicates that the NH2- terminal sequence of SRP-35 encompasses a transmembrane spanning segment or a signal sequence. In addition, SRP-35 is homologous to proteins belonging to the short-chain dehydrogenase/reductases family. Members of this protein family has two domains: the first involved in binding the nucleotide co-factor NAD(P)H, the second responsible for the catalysis of the substrate. SRP35 contains only a putative NAD(P)H binding site. Analysis of tissue distribution of SRP-35 by western blot analysis with affinity purified Ab shows that SRP-35 expression is specific for skeletal muscle since our Ab did not stain any protein in other tissues including heart, brain, liver, kidney, lung, spleen and stomach. Immunohistochemistry of primary cultured mouse myotubes transfected with SRP-35 EGFP construct indicates that SRP-35 is distributed on sarco(endo)plasmic reticulum membranes. Staining of western blot of sarcoplasmic reticulum membrane subfractions isolated from adult mouse skeletal muscle revealed that SRP-35 is associated with heavy sarcoplasmic reticulum. In addition, we found that SRP-35 is an integral membrane protein since it was extracted neither by $NaCO_3$ nor by high salt treatment of isolated sarcoplasmic reticulum membrane, but was solubilised by non-ionic detergent such as CHAPS, DDM and DHPC. We propose that , SRP-35 protein might provide the co-factor to enzymes involved in the generation of local reactive oxygen species within cellular subdomains

1207-Pos Board B51

ATP Release and P2X/P2Y Receptor Activation Account for Slow Calcium Transients Evoked by Electrical Stimulation in Skeletal Muscle Cells

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ATP released to the extracellular medium participates in cell signaling through activation of plasma membrane P2X (ion channels) or P2Y (metabotropic) receptors. Skeletal muscle cells express several P2X and P2Y receptor subtypes and ATP is profusely released during muscle activity. We have previously shown that depolarizing stimuli induce two calcium signals in skeletal myotubes: a fast signal associated with contraction and a slow signal that regulates gene expression. Here we show that extracellular nucleotides released by electrical stimulation are in part responsible for intracellular calcium signals. In rat myotubes, a tetanic stimulus (45 Hz, 400, 1 ms pulses) rapidly increased extracellular levels of ATP, ADP and AMP from 15sec to 3 min, with different half-life times. Exogenous ATP applications induced a dose-dependent increase in intracellular calcium, with an EC_{50} of 7.8 $\pm$ 3.1 μM. Exogenous ADP, UTP and UDP also promote calcium transients. By RT-PCR, we detected mRNA expression for $P2X_{1\text{-}7}$ and $P2Y_{1,2,4,6,11}$ in these cells. Both fast- and slow-calcium signals evoked by tetanic stimulation were partially inhibited by either 10-100 μM suramin (non selective P2X/P2Y blocker) or 2U/ml apyrase (nucleotidase that metabolizes ATP and ADP to AMP). In hemidiaphragm preparations, we demonstrated that apyrase reduces both twitch- and tetanus-evoked increase in tension. Our results suggest that nucleotides released during skeletal muscle activity act through P2X and P2Y receptors to modulate both calcium homeostasis and muscle physiology.

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1208-Pos Board B52

Role Of K_{ATP} Channels During Fatigue And Metabolic Inhibition In Chicken Slow Skeletal Muscle

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The slow-twitch muscle fibers in contrast to the fast-twitch muscle fibers are fatigue-resistant. During fatiguing exercise, the ATP cost of contraction is reduced. This reduction in ATP can be sensed by K_{ATP} channels (ATP-sensitive potassium channels), which couple the metabolic state of the cell to its electric activity, causing its activation. Therefore, it has been proposed that K_{ATP} participate in fatigue in the fast-twitch skeletal muscle besides to ischemia in nervous system. In slow-twitch muscle fibers there are few studies related to muscle fatigue phenomena. Thus, in this study we designed an *in vitro* model for fatigue in chicken slow-twitch skeletal muscle to investigate the role of K_{ATP} channels in the fatigue process. We studied the effects of glibenclamide, which blocks K_{ATP} channels, on twitch and tetanus tension in the anterior *latissimus dorsi* slow muscle induced to fatigue. The results show that glibenclamide increases tension in the fatigued muscle. Also, the slow muscle was exposed to metabolic poisoning by cyanide, a condition in which the ATP formation is inhibited and when its intracellular concentration is diminished K_{ATP} channels are activated which in turn produces a reduction in muscle tension. The addition of glibenclamide in these conditions abolished the effect produced by cyanide. Moreover, we studied the possible role of intracellular calcium by studying the effects of glibenclamide on the contractures evoked by caffeine, which is known that releases calcium from sarcoplasmic reticulum. In these conditions glibenclamide increases tension. Thus, we are showing evidences of the role of K_{ATP} channels in the fatigue process, since glibenclamide increases twitch and tetanus tension in chicken fatigued slow muscle and during metabolic inhibition. These effects could be mediated by an increase in the calcium release from sarcoplasmic reticulum.

1209-Pos Board B53

Insulin Resistant Skeletal Muscle: Mitochondria Structure, Dynamics And Calcium Homeostasis

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Oxidative metabolism operated by mitochondria is altered in insulin resistant skeletal muscle, and these alterations are crucial for phenotype development. Mitochondria interconnected network is required for cell calcium homeostasis, whereas calcium modulates oxidative metabolism as well as mitochondria fission and fusion process. Skeletal muscle fibers, present two subpopulations of mitochondria: subsarcolemmal (SSM) and intermyofybrillar (IMF) mitochondria. SSM electron chain activity is decreased in insulin resistant and diabetic patients; moreover, mitochondria fusion protein Mfn2 is repressed in obese, hyperinsulinemic rats. We evaluated the structure-function relation between mitochondria dynamics and excitation-contraction coupling (ECC) associated calcium transients.

We developed an insulin resistance animal model based on high fat feeding of C57BL/6J mice and we studied mitochondria form skeletal muscle fibers. Proteins associated to mitochondria fission machinery, Drp-1 and Fis1 presented elevated levels in total muscle extracts from insulin resistance animals, as well as particular subsarcolemmal accumulation, evaluated using inmunohistochemistry. Furthermore, we developed an image processing method to quantify mitochondria network continuity in both SS and IMF mitochondria.

When we analyzed calcium transients associated to ECC in flexor digitorum brevis (FDB) fibers, we found a differential effect of insulin. Fibers from control animals, presented a decrease in calcium transients amplitude after cells were treated with insulin; however, fibers from insulin resistant animals, presented unaltered calcium transients.

Taken together, these evidences indicate that there is a differential distribution of mitochondria fission machinery proteins in control and insulin resistant skeletal muscle fibers, and that ECC associated calcium transients show differential susceptibility to insulin in control versus insulin resistant fibers that may be due to altered mitochondria structure adaptation.

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1210-Pos Board B54

Sarcoplasmic Reticulum Calcium Content in Normal and Dystrophic Mammalian Skeletal Muscle Fibers

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The evaluation of the maximal amount of releasable Ca^{2+} (Ca^{2+}-content) that is stored in the sarcoplasmic reticulum (SR) of skeletal muscle fibers is a topic of great importance in muscle physiology. We have developed a method to quantitatively estimate the SR Ca^{2+} content in isolated mammalian muscle fibers based on Ca^{2+} dependent fluorescence measurements performed in the presence of high concentrations of Ca^{2+} buffers, and model calculations of Ca^{2+} flux underlying the fluorescence changes. Single fibers were enzymatically isolated from murine FDB muscles and impaled with two microelectrodes. One electrode, filled with 1M KCl, was used to record the membrane potential throughout

the procedure. The other electrode was used to dialyze the fiber with a solution containing either 6-15 mM BAPTA, or up to 60 mM EGTA (pCa=7.0), and adjusted to pH=7.0 with 20 or 60 mM MOPS, respectively. Free myoplasmic Ca^{2+} concentration ($[Ca^{2+}]$) changes were measured with the low affinity indicator OGB-5N (200 μM). SR Ca^{2+} release was elicited by either 20 mM caffeine, or 1 mM 4-chloro-methy-phenol (4CmC), added to the external solution. The maximal fluorescence change of the Ca^{2+} indicator was assessed at the end of the protocol by exposing the fibers to saponin (0.1 mg/ml in isotonic $CaCl_2$, pH=7.0 with 20 mM MOPS). To prevent changes in shape of the fibers under these conditions, they were pretreated for 1 min with 1% formaldehyde (in Tyrode). The experiments were conducted at 20 °C. A single model compartment was used to estimate (from the $[Ca^{2+}]$ changes) the total Ca^{2+} released in response to caffeine/4CmC application. We obtained values of SR Ca^{2+} content in the range of 15-27 mM for normal FDB fibers. Interestingly, comparable values were obtained in fibers from *mdx* mice.

1211-Pos Board B55

The Absence of Utrophin Does Not Further the Impairment of Ca^{2+} Release Displayed by *mdx* Muscle

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The double mutant utr$^{-/-}$/mdx mouse has been postulated to be a better model of Duchenne Muscular Dystrophy than *mdx* mouse because it displays a progression of pathological features comparable to that in humans. We previously demonstrated that FDB fibers from *mdx* mice show limitations in action potential (AP) elicited Ca^{2+} release. Here we investigated the properties of the APs and the ability to release Ca^{2+} (30 mM [EGTA], 20ºC) in response to single and trains of APs (20 pulses, at 33Hz and 100Hz) in FDB fibers isolated from control, *mdx* and utr$^{-/-}$/mdx mice. Single APs of normal amplitude but longer duration were recorded in utr$^{-/-}$/mdx fibers, whereas the amplitude of the Ca^{2+} release was ~37% smaller than in normal fibers, but comparable with that found in *mdx* fibers. Fibers from the three strains sustained trains of Ca release at 33 Hz in which the amplitude of individual Ca^{2+} release transients decayed exponentially towards a sustained release amplitude with two time constants (τ_1=10ms, τ_2=200ms). In response to 100Hz trains, the amplitude of Ca^{2+} release in normal fibers decayed still with a double exponential (τ_1=3.5ms, τ_2=41ms) in which the amplitude of the 2nd and 20th transient along the train were ~50% and ~35% that of the first one, respectively. In contrast, fibers isolated from both *mdx* and *utr$^{-/-}$/mdx* mice could be divided in two groups according to their tetanic response to 100Hz trains: approximately 75% of *mdx* and 33% of utr$^{-/-}$/mdx fibers showed a behavior similar to that observed in normal fibers; in the remaining 25% of *mdx* and 67% of utr$^{-/-}$/mdx fibers, respectively, the amplitude of the 2nd Ca^{2+} release transient was ~20% of the first one, and this amplitude was sustained throughout the train.

1212-Pos Board B56

Deficits in Ca2+ Release and in vivo Muscle Strength in Heterozygous I4895T RyR1 Knock-In Mice

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The mutation from isoleucine to threonine of the skeletal isoform of the ryanodine receptor (RyR1) at residue 4898 results in severe Central Core Disease (CCD). Under homozygous expression (IT/IT), we reported a lack of Ca^{2+} release in response to electrical and pharmacological activation despite SR Ca^{2+} store content indistinguishable from control. Here we used heterozygous knock-in mice for the I4895T (IT/+; analogous to human I4898T) RyR1 mutation to determine the effects of the mutation on muscle strength and Ca^{2+} handling in flexor digitorum brevis (FDB) and interosseous muscle fibers.
We compared *in vivo* muscle strength of wild-type (WT) and IT/+ mice. IT/+ mice exhibited significant weakness in both upper body and grip strength assays (4-paw peak grip force: 2400 ± 70 mN, n=8 and 2040 ± 80 mN, n=14 in WT and IT/+ mice, respectively). We also determined the magnitude of action potential- and ligand-evoked Ca^{2+} release in single intact FDB fibers using Ca^{2+} fluorometry. The magnitude of both electrically- and ligand-evoked Ca^{2+} release was significantly reduced in IT/+ fibers. Moreover, the maximum rate of change in mag-fluo-4 fluorescence during the rising phase of the electrically-evoked Ca^{2+} transient was significantly reduced in IT/+ fibers (WT 0.17 ± 0.01 ΔF/F/ms vs IT/+ 0.11 ± 0.01 ΔF/F/ms, n= 53, 56, respectively). Finally, the frequency (1.9 ± 0.5 and 0.8 ± 0.3 events/scan) and Ca^{2+} spark mass (5.9 ± 0.3 and 4.6 ± 0.2 μm^3) of local Ca^{2+} release induced by osmotic shock (440 mOsm with sucrose, 750 lines/sec) were reduced in acutely dissociated IT/+ interosseous fibers compared to that of WT fibers. Together, these findings are consistent with the hypothesis that the IT mutation in the putative RyR1 selectivity filter significantly reduces Ca^{2+} flux through the channel.

1213-Pos Board B57

Malignant Hyperthermia Mutation Alters Excitation-coupled Ca2+ Entry In MH RyR1-R163C Knock-in Myotubes

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Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle triggered insusceptible individuals by inhalation anesthetics and depolarizing skeletal muscle relaxants. This syndrome has been linked to a missense mutation in the type 1 ryanodine receptor (RyR1) in more than 50% of cases studied to date. We have examined how the R163C MH-RyR1 mutation alters the Ca^{2+} transient during $[K^+]_e$ depolarization using Fluo4 and excitation-coupled Ca^{2+} entry (ECCE) using manganese-quench of Fura2 Wt and $RyR1_{R163C}$ knock-in myotubes. Exposure of WT and $RyR1_{R163C}$ myotubes to low Ca^{2+} solution ($8.7x10^{-6}$ M) and then to high $[K+]_e$ did not modify initial Ca^{2+} transient ($Ca^{2+}{}_{peak}$), but dramatically altered the time course of the Ca^{2+}-transient, making the duration shorter and the rate of decay faster in all genotypes. However, these changes were more evident in $RyR1_{R163C}$ than Wt myotubes. The rate of Mn^{2+} quench of Fura2 associated with K^+ depolarization (ECCE), was membrane potential dependent and always greater and faster in $RyR1_{R163C}$ myotubes than in Wt. Incubation of Wt and MH $RyR1_{R163C}$ myotubes with 15 μM ryanodine overnight, to block RyR1 Ca^{2+} release, enhanced the amplitude but did not significantly change in the rate of ECCE in either genotype. However, the increment in amplitude was greater in Wt than $RyR1_{R163C}$ knock-in myotubes. We conclude that the pre-existing conformational change caused by the $RyR1_{R163C}$ MH mutation alters the properties ECCE as consequence of significant changes in the retrograde signaling between RyR1 and DHPR making it less sensitive to the conformational change caused of ryanodine.

1214-Pos Board B58

Malignant Hyperthermia and Heat Stroke in Calsequestrin-1 Knockout Mice

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Malignant hyperthermia (MHS) and environmental heat stroke (EHS) in humans present as similar life threatening crises triggered by volatile anesthetics and strenuous exercise and/or high temperature, respectively. Many families (70-80%) diagnosed with MH susceptibility (MHS), and a few with EHS, are linked to mutations in the gene that encodes the type 1 ryanodine receptor (RYR1) located in the sarcoplasmic reticulum (SR) of skeletal muscle. However, mutations in the RYR1 gene are not found in all MH families, suggesting that alternative genes remain to be identified. Here we investigated whether a MH/EHS-like phenotype results from deficiency in skeletal muscle calsequestrin (CASQ1), a SR Ca^{2+}-binding protein that modulates RYR1 function. Exposure of CASQ1-null mice to halothane or heat stress triggers lethal episodes characterized by elevated core temperature, whole body contractures, and severe rhabdomyolysis. Both heat- and halothane-induced episodes are prevented by prior dantrolene administration, the standard antidote used to treat MH episodes in humans. *In vitro* studies indicate that CASQ1-null muscle exhibits increased contractile sensitivity to caffeine, temperature-dependent increases in resting Ca^{2+}, and an increase in the magnitude of depolarization-induced Ca^{2+} release. These findings validate CASQ1 as a candidate gene for linkage analysis in MH/EHS families where mutations in RYR1 are excluded.

1215-Pos Board B59

Expression Of Calsequestrin-1 (CS1) In CS1-null Mice: Restoration Of Ca^{2+} Release Unit Architecture And Amplitude Of Ca^{2+} Transient In Fast-twitch Muscle Fibres

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Amplitude of calcium (Ca^{2+}) transients and width of the Sarcoplasmic Reticulum (SR) lumen in Ca^{2+} release units (CRUs) are significantly reduced in Calsequestrin1 (CS1)- null mice, moreover increase in fatigue resistance is characteristic of the CS1-null-model (*Paolini et al 2007*). We extend the study of the null model at molecular level: decrease in expression of CS2, Triadin, Sarcalumenin were detected in CS1-null FDB muscles in comparison to wild type (wt) and differential FDB (null/wt) expression of 13400 mRNAs was assayed by microarray profiling. To rescue the CS1-null phenotype, exogenous mouse CS1 was expressed in adult null-FDBs by *in vivo* DNA electrotransfer. CS1 expression and correct targeting to CRUs was verified by confocal