Experiments were carried out to investigate the possibility that store operated Ca^{2+} entry (SOCE) may be triggered by volatile anaesthetics in malignant hyperthermia susceptible (MHS) human skeletal muscle. Samples of *vastus medialis* muscle were obtained from patients undergoing assessment for malignant hyperthermia (MH) susceptibility using the standardised *in vitro* contracture test. All experiments were performed with institutional Research Ethics Committee approval and informed patient consent, according to the Declaration of Helsinki. Single fibres were mechanically skinned and confocal microscopy used to detect changes [Ca^{2+}] within the re-sealed t-tubules (with fluo-5N) or within the cytosol (with fluo-3). In normal fibres (MHN), exposure to 0.5 mM halothane failed to trigger SR Ca^{2+} release, or to induce depletion of t-tubule Ca^{2+} (n=8). However, in MHS fibres, 0.5 mM halothane induced both SR Ca^{2+} release and a rapid depletion of t-tubule Ca^{2+}, consistent with SOCE (n=8). In ~20% of MHS fibres, SR Ca^{2+} release took the form of a propagating Ca^{2+} wave and this was associated with a corresponding SOCE wave of t-tubule Ca^{2+} depletion. In MHN fibres, both SR Ca^{2+} release and SOCE could be induced by 0.5 mM halothane when the cytosolic [Mg^{2+}] was decreased to 0.2 mM (n=6). In MHS fibres, SOCE was potently inhibited by inclusion of a STIM1 blocking antibody within the re-sealed t-tubules (n=6). These data suggest (i) that in MHS fibres the degree of SR Ca^{2+} depletion induced by a clinically relevant level of volatile anaesthetic is sufficient to induce SOCE and (ii) that STIM1 located within the sarcolemma modulates SOCE.

3704-Pos

Passive Activation of Store-Operated Ca2+ Entry in Myotubes Depends on the Rate of RyR1-Dependent Ca2+ Leak

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In spite of extensive studies of store operated calcium entry (SOCE), the detailed mechanism of SOCE activation in skeletal muscle remains largely unknown. We recently reported that STIM1-Orai1 coupling is required for SOCE activation in myotubes. However, other proteins that control sarcoplasmic reticulum (SR) Ca2+ content may also contribute to SOCE activation. We hypothesized that passive SOCE activation in skeletal muscle depends on the rate of SR Ca2+ leak through the type 1 ryanodine receptor (RyR1). To test this hypothesis, we conducted a series of whole-cell patch-clamp measurements of SOCE current (ISOC) in myotubes obtained from normal and RyR1-null (dyspedic) mice. Myotubes were bathed in external solution containing (in mM): 138 TEA-methanesulfonate, 10 CaCl2, 10 HEPES, 1 MgCl2, 0.1 nifedipine, pH 7.4. The internal patch pipette solution contained (in mM): 140 mM Cs-methanesulfonate, 10 HEPES, 20 Na-EGTA, 4 MgCl2, pH7.4. SOCE was activated by passive SR Ca2+ depletion following intracellular dialysis with 20 mM EGTA. ISOC in myotubes exhibited many hallmarks of SOCE including strong inward rectification and inhibition by La3+, Gd3+, BTP-2, and 2-APB. ISOC current density at −80 mV was significantly ($p<0.01$) larger in normal myotubes (1.05 ± 0.09 pA/pF, n = 33) compared to that from dyspedic myotubes (0.74 ± 0.07 pA/pF, n = 18). Moreover, the speed of ISOC activation was slower in dyspedic myotubes. Specifically, the time to 10%, 50%, and 90% maximal activation were 4.95 ± 0.65 s, 15.3 ± 1.4 s, and 53.9 ± 7.5 s (n = 19), respectively, in normal myotubes and 67.5 ± 11.1 s, 114 ± 13 s, and 159 ± 17 s (n = 18), respectively, in dyspedic myotubes. These results indicate that RyR1 Ca2+ leak promotes passive SOCE activation.

3705-Pos

Expression of Functional Transgenic Alpha1s-DHPR Channels in Adult Mammalian Skeletal Muscle Fibers

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Plasmids encoding for two variants of the α1sDHPR, tagged at the N-terminal with EGFP, were transfected into adult FDB muscles by *in vivo* electroporation. The wildtype variant of the channel (EGFP-α1sDHPR-*wt*) was rendered insensitive to dihydropyridines by site-directed mutagenesis (EGFP-α1sDHPR-*T935Y*). Standard and TPLSM fluorescence microscopy demonstrated that both variants were similarly expressed with high efficiency and targeted to the surface and TTS membranes of the muscle fibers. Functional evaluation of the efficiency of transgenic expression was carried out by characterizing Ca^{2+} currents and SR Ca^{2+} release in single fibers enzymatically isolated from transfected muscles. The fibers were voltage-clamped using a 2-microelectrode configuration and dialyzed internally with solutions containing 30 to 70 mM Cs-EGTA and 20 to 70 mM Cs-MOPS; they were externally bathed with isotonic TEA-Cl containing 2-12 mM Ca^{2+}. Na and Cl currents were blocked with TTX and 9-ACA, respectively. The maximal Ca^{2+} conductance (gCa_{max}), measured in 12 mM Ca^{2+}, was 0.40 ± 0.04 (mean ± SD, n=6) in control fibers isolated from non-transfected animals. This parameter was not significantly different in fibers expressing EGFP-α1sDHPR-*wt* channels (0.42 ± 0.07, n=6). In contrast, fibers expressing EGFP-α1sDHPR-*T935Y* reported a significantly smaller gCa_{max} of 0.27 ± 0.02 (n=5). Interestingly, after treatment with 1 μM of the specific DHPR blocker NP-200, the residual conductance was <5% in control and EGFP-α1sDHPR-*wt* transfected fibers, but 30-70% in fibers transfected with EGFP-α1sDHPR-*T935Y*. Our results suggest that, in adult skeletal muscle fibers, the functional expression of transgenic DHPR channels is done mostly at the expense of the expression of their endogenous counterparts. Supported by NIH grants Supported by NIH grants AR07664, and AR054816.

3706-Pos

An Important Fraction of the Mammalian Skeletal Muscle Chloride Conductance is Located in the Transverse Tubules

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The actual density of chloride (ClC1) channels in the surface and transverse tubular system (TTS) membranes of mammalian skeletal muscles is still unknown. To investigate this issue, we simultaneously recorded fluorescence signals and chloride currents (ICl) in enzymatically dissociated FDB muscle fibers, stained with the potentiometric indicator di-8-ANEPPS, and voltage-clamped using a 2-microelectrode configuration. The external solution contained (in mM) 150 TEA-Cl, 15 CsMOPS, 2 $CaCl_2$, 0.5 $CdCl_2$, and 200nM TTX. Internally, the fibers were equilibrated with a solution containing 60 CsCl, 40 CsEGTA, 40 CsMOPS, and 5 $MgCl_2$ and voltage-clamped at the chloride equilibrium potential (−20mV). gCl was maximally activated by a pulse to +60mV (150ms) and its voltage-dependence calculated from 9-ACA-sensitive tail currents (measured at the onset of a pulse to −100mV) after 200ms test pulses (−100 to +80mV in amplitude). Boltzmann distributions fitted to the data (n=8) yielded: gCl_{max}=-2.1 ± 0.4 S/F, or 8.1 ± 1.5 mS/cm^2; $V_{1/2}$=73 ± 11 mV and k=24 ± 4 mV. The amplitude (in detaF/F) of di-8-ANEPPS fluorescence transients recorded at the onset of the test pulses were plotted as a function of the pulse amplitudes. In the presence of 9-ACA, the deltaF/F vs. voltage relationship was linear over the entire range of pulse amplitudes explored (slope= −0.124 ± 0.015 /100mV), whereas in the presence of ICl the slope of the linear dependence was less steep. For hyperpolarizing pulses, associated with large instantaneous inward currents, the slope was −0.099 ± 0.03 /100mV; for depolarizing pulses (smaller positive currents) the slope was −0.11 ± 0.025 /100mV. The differential attenuation of the average TTS voltage change in the presence of ICl was predicted by a radial cable model provided that ~30% of the total gCl was in the TTS. Supported by NIH grants Supported by NIH grants AR07664, and AR054816.

3707-Pos

Skeletal Muscle Fibers of Cold-Acclimated Mice Display Increases in Basal Calcium, Mitochondrial Content and Fatigue Resistance

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Mammals initially generate heat by repetitive muscle activity (shivering) when exposed to a cold environment. Shivering can later be replaced by heat generated in brown adipose tissue by activation of uncoupling protein-1 (UCP1). Interestingly, adaptations in skeletal muscles of cold exposed animals are similar to those obtained with endurance training. We studied the function of non-shivering flexor digitorum brevis (FDB) muscles of wild-type (WT) and UCP1-KO mice kept at room temperature (24°C) or cold-acclimated (4°C) for 4-5 weeks. Myoplasmic free [Ca^{2+}] ([Ca^{2+}]$_i$; measured with indo-1) and force were measured under resting conditions and during fatigue induced by repeated tetanic stimulation in intact single fibers. We observed no differences between fibers from WT and UCP1-KO mice. On the other hand, muscle fibers from cold-acclimated mice showed increases in basal [Ca^{2+}]$_i$ (~50%), tetanic [Ca^{2+}]$_i$ (~40%), and SR Ca^{2+} leak (~four-fold) as compared to fibers from room-temperature mice. Muscles of cold-acclimated mice also showed increases in expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), citrate synthase activity (reflecting increased mitochondrial content), and fatigue resistance. In conclusion, cold exposure induces changes in FDB muscles similar to those observed with endurance training and we propose that increased basal [Ca^{2+}]$_i$ has a key role in these adaptations.

3708-Pos

Allele Specific Gene Silencing in Autosomal-Dominant Skeletal Myopathies

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Central Core Disease (CCD) and Malignant Hyperthermia (MH) are linked to single amino acid substitutions in the skeletal muscle Ca^{2+} release channel, the type 1 ryanodine receptor (RyR1). We focus on two autosomal dominant (AD)

RyR1 mutations, Y522S (YS) and I4898T (IT), which cause MH and CCD, respectively. The AD mode of inheritance and data indicating knock-out of one RyR1 allele is well-tolerated in mice led us to hypothesize that allele-specific gene silencing (ASGS) of the mutant allele would rescue RyR1 functional defects in skeletal muscle cells from YS and IT knock-in mice.
We evaluated the functional consequences of allele-specific silencing in YS and IT muscle cells using short interfering RNAs (siRNAs). To screen potential siRNAs for relative knockdown efficacy and allele specificity, we generated cDNAs encoding fusion proteins derived from wild type (WT) (Venus-Exons-3XFLAG) and either YS or IT mutation-containing (Cherry-Exons-3XHA) exons. Simultaneous transfection of these cDNAs and siRNAs into HEK293 cells and subsequent evaluation of mRNA (semi-quantitative RT-PCR) and protein levels (fluorescence microscopy and western blotting) was used to determine knockdown efficacy and allele-specificity prior to functional rescue experiments. Myotubes derived from heterozygous YS mice (YS/+) exhibit ~4-fold increase in caffeine sensitivity (EC_{50} values were 0.5mM and 2.3mM for YS/+ and WT, respectively). Treatment with a YS-selective siRNA, normalized caffeine sensitivity (EC_{50} = 2.5mM) without decreasing peak caffeine-induced release. Similarly, YS-selective siRNA treatment rescued the increased voltage sensitivity of Ca^{2+} release in YS/+ myotubes determined in perforated-patch clamp experiments ($V_{F1/2}$: WT = −18mV, YS/+ scrambled = −35mV, YS/+ YS-selective = −18mV). These results indicate that ASGS represents a promising approach for normalization of RyR1 function in MH and CCD. Similar functional rescue experiments in adult skeletal muscle fibers are currently underway.

3709-Pos

Increased Fatigue in Sarcoglycan Knock Out Mouse Skeletal Muscle Fibers

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Background: The δ-sarcoglycan (δ-SG) knockout (KO) mice develop skeletal muscle histopathological alterations similar to those seen in humans with limb muscular dystrophy. Membrane fragility and increased Ca^{2+} permeability have been linked to muscle degeneration. However, little is known about the mechanisms by which the genetic defects lead to disease.
Methods: Isolated skeletal muscle fibers of wild type and δ-SG KO mice were used to investigate whether the absence of δ-SG alters the increase in intracellular Ca^{2+} during single twitches and tetani or during repeated stimulation. Immunolabeling, electrical field stimulation and Ca^{2+} transients recording techniques with fluorescent indicators were used.
Results: Ca^{2+} transients during single twitches and tetani, generated by muscle fibers of δ-SG KO mice, are similar to those of wild type mice, but their amplitude is greatly decreased during protracted stimulation in KO compared to wild type fibers. This impairment is independent of extracellular Ca^{2+} and is mimicked in wild type fibers by blocking SOC channels with 2-Aminoethoxydiphenyl borate (2-APB). Also, immunolabeling indicates the localization of a δ-SG isoform in the sarcoplasmic reticulum of the isolated skeletal muscle fibers of wild type animals, which could be related to the functional differences between wild type and KO muscles.
Conclusions: δ-SG has a role on calcium homeostasis in skeletal muscle fibers. The alterations caused by the absence of δ-SG may be related to the pathogenesis of muscular dystrophy.
Grants: CONACYT: 60880 (J.S) and 82667 (M.C.G).

3710-Pos

Nuclear Translocation and Possible Transcriptional Regulation in Skeletal Muscle by $Ca_V\beta_{1a}$

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Abstract: The classically described function of $Ca_V\beta$ subunits is to modulate the biophysical properties and enhance trafficking of voltage gated calcium channels (Ca_Vs), making $Ca_V\beta$ subunits important in a number of physiological processes. Most notably, the skeletal muscle-specific isoform $Ca_V\beta1a$ is critical for proper EC coupling. Recently, several other protein binding partners of various neuronal and cardiac $Ca_V\beta$ isoforms ($Ca_V\beta_{1b}$, $Ca_V\beta_{2a}$, $Ca_V\beta_3$, and $Ca_V\beta_{4c}$) have been described, which has revealed novel functions for these subunits beyond the aforementioned augmentation of Ca_Vs. One especially interesting finding is that these Ca_V subunits enter the nucleus of neuronal cells and may participate in the regulation of gene transcription. However, this novel role for $Ca_V\beta$ subunits has only been minimally explored in neurons, and to our knowledge has not been examined in skeletal muscle. We therefore investigated whether the $Ca_V\beta_{1a}$ subunit may also act as a transcription factor. Using an array of biochemical and molecular techniques, we examined the following: $Ca_V\beta_{1a}$'s subcellular localization during various stages of myogenic development; binding partners of $Ca_V\beta_{1a}$, which may facilitate its transcriptional regulation; and also the functional role of $Ca_V\beta_{1a}$ nuclear localization. Our results show strong evidence for $Ca_V\beta_{1a}$ nuclear localization in skeletal muscle and provide insight into the mechanism and function of this phenomenon.
Acknowledgments: this work was supported by grants from the NIA, and MDA.

3711-Pos

Effects of Domain Peptide DP4 on Depolarization-Induced Calcium Transients and Calcium Currents in Voltage-Clamped Fibers from Mouse FDB Skeletal Muscle

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Skeletal muscle excitation-contraction coupling involves sequential activation of dihydropyridine receptors (DHPRs) and type-1 ryanodine receptors (RyR_1) to produce depolarization-dependent sarcoplasmic reticulum Ca^{2+} release via ''orthograde'' signaling. Another form of DHPRs-RyR_1 communication is ''retrograde'' signaling, in which RyRs modulate the gating of DHPR. Domain peptide 4 (DP4), a peptide corresponding to residues Leu^{2442}-Pro^{2477} of RyR_1, interferes with inter-domain interactions within RyR_1 that normally stabilize the closed state of the RyR_1 channel. DP4 has been shown to potentiate force in response to submaximal depolarization by ionic substitution and caffeine-induced Ca^{2+} release in peeled muscle fibers. In sarcoplasmic reticulum vesicles, DP4 directly potentiates RyR_1 opening as shown by increased ryanodine binding and sensitization of Ca^{2+} release. Here we explore possible effects of DP4 on excitation-contraction coupling in whole-cell voltage clamped adult FDB skeletal muscle fibers. Depolarization-induced fluo-4 fluorescence transients (F/F0) became detectable at smaller depolarizations, and were increased at larger depolarizations in fibers dialyzed with DP4 compared to those without DP4 in the patch pipette, as were the Ca^{2+} transients and Ca^{2+} release calculated from F/F0. The amplitude of peak Ca^{2+} release (R) depended on voltage according to a two-state Boltzmann function. DP4 increased the amplitude of maximum release rate (R_{max}) by *ca* 54% when compared to control fibers. DP4 also induced a negative 5 mV shift in the potential at which R = 0.5 of R_{max} and an augmentation of macroscopic DHPR Ca^{2+} current density at all voltages tested. Thus, DP4 potentiates both depolarization-dependent Ca^{2+} release via RyR_1 and Ca^{2+} influx via DHPR, the later possibly mediated by retrograde signaling between the RyR_1 and DHPR Ca^{2+} channels in adult mammalian muscle fibers. Supported by NIH-NIAMS Grants R01-AR055099 and T32-AR007592.

3712-Pos

Small Ankyrin 1 Organizes the Ca2+ Stores of the Sarcoplasmic Reticulum in Skeletal Muscle

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Excitation-contraction (EC) coupling in striated muscle requires the coordinated functions of the transverse (t-) tubules, sarcoplasmic reticulum (SR) and the sarcomeric contractile proteins. The transient rise in [Ca2+]i that activates contraction depends on Ca2+ release from the SR. Maintaining the structural and functional integrity of the SR is crucial to maintaining the efficiency of EC coupling in skeletal muscle. Small ankyrin 1 (sAnk1, Ank1.5), a small alternatively spliced product of the ANK1 gene, is an integral membrane protein of the network compartment of the SR (nSR). sAnk1 binds obscurin, a giant cytoskeletal protein that surrounds the sarcomere at the level of the M-band and Z-disc. Our earlier results show that reducing the expression of sAnk1 with siRNA decreases the protein level, but not the mRNA, of the SR Ca2+ pump, SERCA, and may lead to a decrease in SR [Ca2+] load. We used Fluo-5N, a low affinity Ca2+ indicator, which we specifically loaded into the SR lumen, as a reporter of SR Ca2+ stores in control and siRNA treated myofibers isolated from rat flexor digitorum brevis muscle. The intensity of Fluo-5N fluorescence and its distribution in the SR lumen were altered when sAnk1 expression was reduced, consistent with a loss of nSR. Further, fluorescence recovery after photobleaching (FRAP) experiments showed that even small changes in sAnk1 expression altered the interconnectivity of the SR. Functional measurements of SR Ca2+ dynamics also suggested an important role for sAnk1 in maintaining EC-coupling. Our results are consistent with the hypothesis that sAnk1 is essential for the integrity of the nSR compartment and its Ca2+ stores in skeletal myofibers.