microscopy, whereas restoration of CRUs architecture - i.e. shape and width of junctional SR (jSR) containing CS1 - was assessed by electron microscopy. Exogenous CS1 was correctly targeted to CRUs and positioned at the jSR, in close proximity of Ca^{2+} release sites. Size of the SR lumen was increased. At proteomic level CS2, Sarcalumenin, Triadin and Junctin did not change upon CS1 expression. Ca^{2+} transients induced by electrical stimulation were recorded in mock-transfected , and CS1-transfected fibres: successfully, average peak height and baseline showed significant increase upon CS1 expression resembling wt fibres. The present results provide strong evidences that expression of CS1 directly controls size of jSR terminal cisternae, influences resting cytosolic Ca^{2+} and modulates the amplitude of Ca^{2+} transient in response to electrical stimulation in fast-twitch muscles.

Paolini, C et al. 2007 J Physiol, 583: 767.

1216-Pos Board B60

Deletion Of Triadin Results In Marked Alterations In Tetanic Contraction And Global Calcium Handling

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In our recent work (Shen et al., JBC, 282(52), 2007), we examined the role of triadin in skeletal muscle EC coupling in a pan-triadin null mouse. No contractile dysfunction was evident during single twitches *in vitro*, however a reduction in Ca^{2+} transient magnitude was observed. Analysis of single myofibers revealed a decrease in SR Ca^{2+} content and an increase in sarcoplasmic $[Ca^{2+}]$, suggesting that triadin is not critical to EC coupling, yet might play a modulatory role at the RyR. In this investigation, we test our hypothesis that an increase in functional demands will unmask an EC coupling phenotype in muscle null for triadin (Tdn). We used tetanic stimulation to examine the contractile characteristics of EDL muscles *in vitro* and TA muscles *in situ*, as well as global calcium transients. The tetanic force vs. stimulation frequency (FF) relationship (250msec train; 0.5ms pulse @ 1-300Hz) between WT and Tdn EDLs was evaluated *in vitro*. Tdn EDLs were unable sustain the initial peak achieved during each 250msec train. The magnitude of the tetanic fade was progressive with increasing stimulation frequency. At peak tetanic stimulation (300 Hz) this tetanic fade resulted in a ~23% decrease in the tension-time integral; a finding that was replicated with nerve evoked tetanic stimulation of the tibialis anterior muscle *in-situ*. Similarly, our assay of myoplasmic $[Ca^{2+}]$ in FDB myofibers (MagFluo4AM) demonstrated that the plateau of the tetanic $[Ca^{2+}]$ was not sustained in triadin null FDBs, even though the peak $[Ca^{2+}]$ amplitude during a 250msec tetanic train (80Hz; field stim.) was not different between genotypes. We believe that the contractility deficits in the Tdn muscles are due to a decrease in SR Ca^{2+} release. Funded by grants from NIH-NIAMS to C.W.W, R.M.L, P.D.A, and C.P.

1217-Pos Board B61

Triadin Function In Sarcoplasmic Reticulum Structure?

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Muscle contraction is achieved when an efficient excitation signal at the plasma membrane triggers intracellular calcium release. This process called ''excitation-contraction (E-C) coupling'' relies on a multimolecular protein complex, spanning the plasma membrane and the sarcoplasmic reticulum (SR), containing the calcium channel of the SR, the ryanodine receptor (RyR). Triadin is a member of this complex, present in the SR membrane and interacting with RyR in skeletal muscle. We have shown that overexpression of triadin in cultured myotubes abolishes E-C coupling, although RyR is still functional. Moreover in knock-out mice, deletion of the triadin gene leads to disorganisation of SR membranes in skeletal muscles. In the present work, we have expressed triadin in COS-7 cells to dissect its intrinsic properties on membrane organisation. We show that triadin expression leads to important modification of the endoplasmic reticulum (ER) morphology, already observed with the expression of proteins regulating ER morphology, and known as ''rope-like structures''. These modifications of ER morphology are correlated to alteration of the microtubule network. Indeed, in cells expressing triadin, microtubules are bundled, often running parallel to the plasma membrane, and more stable than in untransfected cells. Surprisingly, suppression of the cytosolic N-terminal part of triadin did not reverse this phenotype. Using splice versions of the triadin protein and C-terminal deletion mutants, we show that ER/microtubules modifications depend on an intra-lumenal sequence. Altogether, our work lead to the hypothesis that modifications of ER morphology and microtubule dynamics observed in cells expressing triadin are mediated by an intermediate protein currently under investigation. Expression of triadin in COS-7 cells can modify endoplasmic reticulum morphology. It thus suggests that in skeletal muscle, triadin could play a role in the structure of sarcoplasmic reticulum to allow efficient E-C coupling.

1218-Pos Board B62

Molecular Basis Of Protein Localization To The Junctional Sarcoplasmic Reticulum Of Skeletal Muscle Cells

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The sarcoplasmic reticulum (SR) of skeletal muscle cells is a continuous network of membranes in which some specific domains (i.e. the longitudinal SR and the junctional SR) can be clearly distinguished. Although each of these domains is characterized by a specific protein composition the mechanisms leading to protein targeting to these domains are still unknown. In particular, specific targeting mechanisms to the junctional SR are likely to be present in triadic proteins, yet no specific localization signal has been defined. In order to investigate this point we expressed wild type and deletion mutant GFP-tagged triadic proteins in rat primary myotubes and followed their localization during in vitro development. In parallel the dynamic properties of these proteins were investigated by FRAP technique. Analysis of triadin, junctin, Ryanodine Receptor type 1 and junctophilin-1 allowed us to identify specific sequences that might be responsible for targeting of these proteins to the junctional SR. Furthermore, FRAP analysis showed that deletion of some, but not all, of these sequences resulted in a significant increase in the mobility of triadic proteins. This would suggest that some of these regions, in addition to mediate protein targeting to the junctional SR, could also contribute to the establishment of protein-protein interactions within the multi-molecular complex associated with the calcium release channel. Experiments are being performed to further dissect these interactions.

1219-Pos Board B63

Intra-sarcoplasmic Reticulum Ca^{2+} Depletion In Adult Skeletal Muscle Fibers Measured With The Biosensor D1ER

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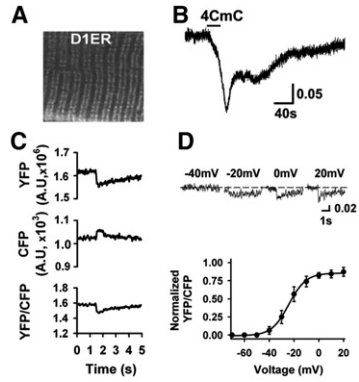
The endoplasmic/sarcoplasmic reticulum (ER/SR) plays a crucial role in cytoplasmic signaling in a variety of cells. It is particularly relevant for skeletal muscle fibers, where this organelle constitutes the main Ca^{2+} store for essential functions, such as contraction. In this work, we expressed the cameleon biosensor D1ER by in vivo electroporation in the mouse flexor digitorum brevis (FDB) muscle to directly assess intra-SR Ca^{2+} depletion in response to electrical and pharmacological stimulation. The main conclusions are: (1) D1ER is expressed in the SR of FDB fibers according to both di-8-ANEPPs staining and reductions in FRET; (2) the amplitude of intra-SR Ca^{2+} release evoked by either 4-CmC or electrical stimulation is directly proportional to resting SR Ca^{2+}, which indicates that intra-Ca^{2+} modulates RyR1-meditated SR Ca^{2+} release in the intact muscle fiber; (3) intra-SR Ca^{2+} release, as measured with D1ER, is voltage-dependent and follows a Boltzmann function; (4)



A) The triad striation pattern is evident in FDB fibers expressing D1ER; B) Calcium release evoked by 30s exposure to the RyR agonist 4CmC; C) Typical D1ER response to electrical stimulation to 20mV for 200 ms; D) Voltage-dependence of intra-SR calcium release

average SR Ca^{2+} depletion is 13% in response to 4-CmC and 4.5% in response to prolonged and maximal sarcolemmal depolarization; and (5) the time-to-peak of intra-SR Ca^{2+} release and cytoplasmic Ca^{2+} transient at maximal sarcolemmal depolarization do not differ significantly.

1220-Pos Board B64

Which Low-affinity Fluorescent Calcium Indicators Accurately Track The Change In Myoplasmic Free Calcium Concentration (Δ[Ca]) In Skeletal Muscle?

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In vertebrate twitch fibers, spatially-averaged Δ[Ca] elicited by an action potential (AP) is large and brief. Consequently, Δ[Ca] is more accurately measured with low-affinity than with high-affinity Ca indicators (Hirota et al., 1989; Baylor and Hollingworth, 1998). Previous studies with low-affinity fluorescent indicators found that the time course of Δ[Ca] is quite accurately monitored with furaptra (= mag-fura-2), mag-fura-5, and mag-indo-1 (Konishi et al., 1991; Zhao et al, 1996). Because these tri-carboxylate Ca indicators have some sensitivity to free magnesium ([Mg]), we have evaluated three low-affinity tetra-carboxylate indicators, fura-5N, OGB-5N, and fluo-5N, which have negligible [Mg] sensitivity. To do so, resting fluorescence (F_R) and fluorescence changes elicited by an AP (ΔF) were measured at 16 °C in frog single fibers micro-injected with both furaptra and one of the other three indicators. Disappointingly, with the other three indicators, the full-duration at half maximum of ΔF was larger than that with furaptra, on average, by 37, 51, and 53%, respectively, increases that do not appear to arise from increased saturation of indicator with Ca. We also evaluated mag-fluo-4 (cf. Caputo et al., 2004), another tri-carboxylate indicator. Encouragingly, mag-fluo-4's ΔF time course was essentially identical to furaptra's while its signal-noise ratio with visible excitation wavelengths was an order of magnitude larger (for similar concentrations of indicator). However, because F_R of mag-fluo-4 probably arises largely from indicator molecules bound with Mg, calibration of mag-fluo-4's $\Delta F/F_R$ in terms of Δ[Ca] is likely to be more sensitive to variations in [Mg]. Also, with mag-fluo-4, unlike furaptra, fluorescence anisotropy values varied with the plane of polarization of the exciting light, thus revealing a population of mag-fluo-4 molecules that are oriented and presumably bound to structural components within the fiber.

Electron & Proton Transfer

1221-Pos Board B65

Redox Kinetics Of Cytochrome C Oxidase By Electrochemically-induced Time-resolved Surface-enhanced Infrared Absorption Spectroscopy (tr-Seiras)

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For use with surface-enhanced infrared absorption spectroscopy, SEIRAS, we designed a two-layer gold surface for use with electrochemistry which consists of a conducting underlayer onto which Au nanoparticles (AuNPs) are grown by self-catalyzed electroless deposition. This enabled us to apply time-resolved (tr)-SEIRAS to cytochrome c oxidase (CcO) from *R. sphaeroides* immobilized in a strictly oriented fashion on the two-layer gold surface. The enzyme was excited by direct electron transfer (electronic wiring) to the Cu_A redox center. Electrons then travelled through the rest of the electron transfer chain to heme a, heme a_3 and Cu_B without using any mediators. Kinetic constants were obtained by applying periodic potential pulses and recording spectral changes as a function of time. Excitation frequencies were varied in a wide range between 0.7 Hz and 2 kHz. Time resolved spectra were analyzed by phase-sensitive detection. A wide range of kinetic constants was obtained thereby separating these parameters from the contribution due to charging currents.

Tr-SEIRA spectra of the cytochrome c oxidase at different excitation frequencies
10 Hz 250 Hz

Ch. Nowak, Ch. Luening, D. Schach, D. Baurecht, W. Knoll, R. L. C. Naumann, electron transfer kinetics of cytochrome c in the sub-ms time regime using time-resolved SEIRAS, JPC(C) under review.

1222-Pos Board B66

Isolation and Characterization of Site-directed Mutants in the Highly Conserved Dicyclohexylcarbodiimide Binding Site in Subunit III of *Rhodobacter sphaeroides* Cytochrome c Oxidase

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Cytochrome *c* oxidase (COX) is the final electron acceptor in mitochondrial respiratory chain and in many bacterial species including *Rhodobacter sphaeroides.* Electron transfer is coupled with the pumping of protons across the membrane. Previous work has shown that reaction of beef COX with dicyclohexylcarbodiimide (DCCD) resulted in an inhibition of proton translocation by covalently binding to the conserved amino acid residue E90 located in a nonpolar region of subunit III (SIII). E90 is involved in a bonding pair with another conserved residue H212, possibly connected by a salt bridge or a hydrogen bond pair in the three dimensional structure of SIII. Our goal was to test whether the retention of the E90-H212 linkage and the spatial arrangements of these amino acid residues were critical for electron transfer and proton pumping activities of the enzyme. In the current work, we investigated the functional role of these amino acids through the creation of three mutants in SIII_H212E, E90H, and E90H/H212E. Each of the visible absorbance spectra of the three mutant proteins in the bacterial membranes exhibited similar properties as wild type COX. Conversely, the spectrum of isolated and purified COX mutant SIII E90H displayed a blue shift of 3 nm. SDS-PAGE verified that subunit III was present. Electron transfer activity assays of E90H showed an approximate 40% decrease in activity when compared to wild type enzyme and that the mutant did not undergo suicide inactivation during steady-state turnover. Proton pumping activity of the mutants reconstituted into liposomes will be discussed.

1223-Pos Board B67

Functionality of Single-Cysteine Mutants in Subunit III of *Rhodobacter sphaeroides* Cytochrome *c* Oxidase

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Cytochrome *c* oxidase (COX) catalyzes the reduction of oxygen to water using ferrocytochrome *c* and conserves the energy of this reaction by translocating protons across the bacterial or inner-mitochrondrial membrane. COX from *Rhodobacter sphaeroides* is a four subunit transmembrane protein that serves as a model for the mitochondrial enzyme. Subunit I and II contain the redox centers and proton pathways necessary for redox chemistry and proton translocation. The indispensable role of subunit III is an area still being investigated. This work examines the functionality of three mutant forms of COX - one in which all cysteines have been removed from the enzyme (CA1CS3), and two in which single cysteines are reintroduced into CA1CS3 at specific locals in subunit III (A4C, S187C). The single cysteine mutants provide a means to specifically target thiol-reactive probes to areas of interest in COX subunit III. The A4C mutant allows for a probe to be placed at the mouth of the D-channel - an important proton-conducting pathway necessary for the pumping and redox activities of COX. Bioconjugation of S187C would place a probe on an exterior loop which is thought to undergo redox-linked transient conformational changes. All three mutants were expressed and purified, and their absorbance spectra are identical to wildtype, indicating that the heme active centers are unperturbed. SDS-PAGE gels show that all three mutants retain wildtype subunit composition. The oxygen reduction activity of the mutants are also comparable to wildtype, with values between 1200-1600 $e^-/s*mol$ at pH 7.4. In conclusion, these results indicate that the cysteine-free mutant and two mutants in which single cysteines are reintroduced at non-conserved locations retain wildtype functionality, indicating that cytochrome *c* oxidase subunit III is a candidate for cysteine scanning-mutagenesis studies utilizing thiol-reactive probes.

1224-Pos Board B68

ENDOR Spectroscopy Shows that Q_A Remains in the Same Orientation Upon Reduction in Reaction Centers from *Rhodobacter Sphaeroides*

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