troponin (TnI +TnT +TnC) and actin. After uv-irradiation, SDS-gels of the Tm146* system showed 3 new high MW bands confirmed to be Tm146*-TnI, Tm146*-TnC and Tm146*-TnT using both fluorescently labeled TnI and mass spectrometry. In contrast, for Tm174* one main crosslinked band predominated attributed to Tm174*-TnT. Interestingly, the Tm146*-TnI band was much more intense when crosslinked in the absence of Ca^{2+}, in contrast to the other crosslinked species which did not show a Ca^{2+} dependence. These data show that in the thin filament:

5. a region of TnI interacts with Tm near position 146 in the absence but not in the presence of Ca^{2+};
6. a region of TnC is near Tm 146;
7. regions of TnT are near both Tm positions 146 and 174.

These data support a model in which the TnI-Tm interaction near Tm146 is involved in the Ca^{2+}-dependent Tm movement and will also aid in the location of the Tn complex along Tm in the muscle thin filament.

Muscle Regulatory Proteins - II

673-Pos Tropomyosin Pseudo-phosphorylation Alters its Interaction Within the Thin Filament

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Board B517

The head-to-tail overlap region of the tropomyosin dimer is critical to striated muscle contractile function. Tropomyosin contains a single phosphorylation site at Ser-283 that is located directly within the head-to-tail overlap region. During cardiac muscle development tropomyosin phosphorylation is elevated while phosphorylation is decreased in the adult suggesting a significant role for tropomyosin phosphorylation in either sarcomeric assembly or in the regulation of cardiac muscle contraction. To retain physiological function the tropomyosin molecule must be acetylated at its N-terminal amino acid residue. To date, all but a single study have investigated the biochemical properties of phosphorylated tropomyosin using either tropomyosin purified from muscle, that is likely to contain other post-translational modifications, or recombinant E. coli expressed tropomyosin containing an AS- tag. To investigate the effects of native N-terminal acetylated tropomyosin phosphorylation in the absence of other potentially confounding post-translational modifications we purified wild type and recombinant alpha tropomyosin containing either phosphorylation-null (S283A) or pseudo-phosphorylation (S283D) mutations expressed in insect cells. ELISA epitope analysis demonstrates tropomyosin pseudo-phosphorylation alters the binding of the monoclonal antibody CH1 to a central epitope in the tropomyosin molecule suggesting a long-range conformational effect of Ser-283 phosphorylation on the central tropomyosin molecule. ELISA solid phase protein binding assays demonstrate a significant increase in maximal binding of the pseudo-phosphorylated tropomyosin to both troponin T and the troponin complex compared to wild type and phosphorylation-null tropomyosin in the absence of a significant effect on binding affinity. These results demonstrate for the first time that recombinant pseudo-phosphorylated tropomyosin exhibits altered interaction with the troponin complex, further suggesting a role for tropomyosin phosphorylation in cardiac muscle function.

674-Pos Effects of the Novel Tropomyosin-Kappa on Modulating the Mechanical Properties of the Heart

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Board B518

Tropomyosin-kappa (TPM1κ) is a newly discovered TM isoform that is exclusively expressed in the human heart. Preliminary studies indicate an increased expression of TPM1κ in hearts of patients with dilated cardiomyopathy (DCM). TPM1κ results from the alternative splicing of the α-TM gene where the skeletal muscle exon 2b is replaced with the smooth muscle exon 2a. In this study we sought to determine the effect of the presence of TPM1κ isoform on sarcomeric response to Ca^{2+}. We generated transgenic (TG) mice expressing TPM1κ in the cardiac compartment. About 60% of the native TM was replaced with TPM1κ. We compared force-pCa relations in detergent extracted fiber (skinned) bundles isolated from hearts of non transgenic (NTG) controls (n=13) and TG-TPM1κ (n=11) at a sarcomere length of 1.9 μm. Our data (mean ± SEM) demonstrated a significant decrease in the Ca^{2+} sensitivity of the myofilaments (NTG: $pCa_{50}= 5.89 \pm 0.01$; TG: $pCa_{50}= 5.72 \pm 0.01$; $\Delta pCa_{50}= 0.18 \pm 0.01$; $P<0.0001$) with no change in the maximum developed tension (NTG: $F_{max}= 32.12 \pm 0.66$ mN/mm^2; TG: $F_{max}= 29.92 \pm 0.43$ mN/mm^2). To determine the effect of isoform switching on TM phosphorylation, we performed two-dimensional difference gel electrophoresis (2D-DIGE) followed by Pro-Q staining. There was a decrease in the total phosphorylation of TPM1κ compared with that of α-TM. Our results fit with reports showing a linkage of DCM to point mutations that decrease Ca^{2+} sensitivity, and suggest a potential role of altered TM phosphorylation.

675-Pos Oxidation Of Tropomyosin By Reactive Oxygen Species In The Myocardium

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Board B519

Reactive oxygen species [ROS] generation is linked to acute and chronic cardiac pathophysiological change and injury, and is known to be increased in aging tissue. Sarcomeric components such as tropomyosin [Tm] are critical in cardiac function and may be a target of modification by ROS. The data presented test the hypothesis that ROS in ventricular tissue act through oxidation of sarcomeric

proteins. We investigated the oxidation state of Tm in ventricular homogenates, comparing wild-type [WT] mice and a novel transgenic model, DN-p67. DN-p67 mice cardiac-specifically express a dominant negative form of $p67^{phox}$, a required subunit of the key ROS-generating enzyme NADPH oxidase. Comparisons between young (<3 mo) and aged (>1 yr) animals were also performed. We employed immuno-blot studies to analyze the formation of disulfide bonds by oxidized cysteine residues. Results demonstrated several high molecular-weight bands present under non-reducing conditions that reacted with an antibody to tropomyosin. These putative oxidized Tm products were decreased in one-year-old DN-p67 mice compared to WT mice. In both DN-p67 and WT, oxidized products were observed in aged mice that were not found in young mice. Western blots employing "diagonal" gels, in which SDS-PAGE was run successively under non-reducing and reducing conditions, enabled us to more accurately describe the extent of Tm S-S dimer formation. Diagonal results were also consistent with those described above in that Tm dimers and other oxidized products detected with anti-tropomyosin antibodies in aged mice were not seen in young mice. Our findings represent evidence for the oxidation of tropomyosin in murine ventricular tissue to a greater extent with age and with higher ROS levels. This suggests a potential route through which ROS may trigger a decline in cardiac function, and may indicate a target for therapeutic intervention.

676-Pos F-actin Binding And Regulatory Properties Of An Amino-terminally Truncated Tropomyosin

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Board B520

Omp T-digested rabbit skeletal tropomyosin (residues 7 - 284) does not bind to F-actin at micromolar concentration as judged by sedimentation assay. Interaction is fully restored by rabbit skeletal troponin complex at moderate ionic strength (~70mM, pH 7). Induction is calcium-sensitive, being ~six-fold weaker at low pCa compared to high pCa. At higher concentrations of neutral salt (ionic strength, ~160 mM) and for the same range of protein concentration, saturation is observed only at high pCa. Troponin-I is able to reinstate binding of Omp T-digested tropomyosin to F-actin in the absence of the other two troponin subunits (ionic strength ~70mM). However, the amino-terminal fragment of troponin-T (residues 1-158) does not enhance the level of induction incurred by sub-saturating amounts of troponin-I, as reported for carboxy-terminally shortened tropomyosin (residues 1 - 273; Heeley et al (1987) J. Biol. Chem. *262*,9971–9978). Thin filaments (EGTA) reconstituted with either shortened tropomyosin (residues 7-284) or full-length tropomyosin exert equivalent inhibition of the steady-state actomyosin MgATPase activity (ionic strength, ~20 mM; myosin-S1, 0.5uM; regulated actin, zero to 80uM). Interestingly, the structural discontinuity produces a two-fold increase in Ca2+ activation. The difference is mainly due to a change in Vmax. These data suggest that the amino terminal region of tropomyosin is an inhibitory element of the thin filament and comprises a troponin-T binding surface.

677-Pos Cooperative Thin Filament Behavior Examined By Tropomyosin Fluorescence

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Board B521

Striated muscle contraction is controlled by highly sensitive alterations in thin filament conformation that involve tropomyosin and depend upon troponin-Ca2+ and actin-myosin binding. To study these cooperative transitions, 5-OH tryptophan (OHW) was incorporated into striated muscle alpha-tropomyosin. Single Trp tropomyosin mutants A269W and Q135W were generated and expressed in Trp auxotrophs (Farah and Reinach, *Biochemistry* 1999, 10543.) OHW incorporation was 76%. Thin filaments containing either tropomyosin retained excellent function: 12-fold Ca^{2+} regulation in myosin S1-thin filament MgATPase assays. Ca^{2+} had no effect on OHW fluorescence (315 nm ex) in thin filaments with A269W tropomyosin, but decreased the fluorescence of Q135W Tm thin filaments 14%. Titrations with nucleotide-free myosin S1 were performed in presence of troponin, F-actin, each of the mutant tropomyosins, and both EGTA and Ca^{2+}. Similar titrations using wt Trp-free tropomyosin were obtained to correct for light scattering and contaminating Trp fluorescence from actin, troponin, and myosin S1. Myosin had minimal effect on A269W filament fluorescence, and minimal effect on Q135W filaments in the presence of Ca^{2+}. In EGTA, myosin cooperatively decreased the OHW fluorescence of Q135W filaments, by 19%. This transition was 45% complete upon 20% actin-saturation with S1, and 76% complete upon 40% actin-saturation. OHW Q135W tropomyosin is a novel tool for monitoring thin filament behavior. Troponins with defective inhibitory activity are under study using this tropomyosin, to measure alterations in thin filament states. Finally, acceptor-labeled myosin S1s are being prepared for tropomyosin-myosin FRET experiments. To examine myosin binding to specific actins within the regulatory unit, and thereby address the unknown pattern of distribution of bound myosin, both Q135W and A269W tropomyosins will be used to titrate FRET as increasing myosin S1 is added to thin filaments. Probe placement is being guided by actin-myosin and actin-tropomyosin molecular models.

678-Pos Effects of Mid-Region Tropomyosin Mutations on Actin Binding

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Board B522

In addition to the short, heptad residue, repeating pattern responsible for tropomyosin's coiled-coil structure, tropomyosin contains longer, actin monomer-spanning quasi-repeats believed important for

the weak but physiologically significant attachment of tropomyosin to actin. Based in part on the high resolution structure of tropomyosin's mid-region, Brown et al (PNAS 2005) proposed specific charged and surface hydrophobic residues that may facilitate tropomyosin-actin binding, and axially aligned tropomyosin on the actin filament. Here this proposal is tested by mutagenesis. In each of several mutants in the fifth quasi-repeat, 2 or 3 residues were altered: charged residues 167, 180, 181 (changed to Gln), and/or hydrophobic residues 170-171 (converted to polar residues). None of these residues is in the coiled-coil dimerization core. All are hypothesized actin-interacting residues. Binding of the tropomyosins to actin or to actin-myosin S1 was measured by competition. Increasing amounts of unlabeled bacterially expressed tropomyosins, control or mutant (all with N-terminal ala-ser as functional substitute for N-acetylation) were added to actin or actin-S1 samples that were saturated with ^{3}H-tropomyosin (Cys 190 labeled). Competitive displacement was assessed by ultracentrifugation. The results support a role for the altered residues in interacting with F-actin: in both the presence and absence of S1, the mutant tropomyosins' affinities for actin were decreased on average 3-fold relative to wt. In contrast, sixth quasi-repeat mutants designed to improve the surface chemical match for actin bound actin more strongly than did the above, fifth repeat mutants. That is, small, variable changes in tropomyosin affinity (range 46% - 156% of wt) were produced by introduction into the sixth quasi-repeat of polar or hydrophobic residues at positions 206, 208, 211-213, corresponding to residues from the exon 6b region of tight-actin binding nonmuscle tropomyosin 5a. In summary, the mutagenesis results largely support the 2005 model.

679-Pos Effect Of Tropomyosin Isoforms On Actomyosin Interaction In An In Vitro Motility Assay

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Board B523

Mutations within the head to tail overlap region of tropomyosin (Tm) have been shown to lead to cardiac disease. However, naturally occurring nonconservative Tm differences at this same region of interest exist between isoforms. Transgenic studies demonstrate that varying α- and β- Tm isoform ratios alters whole heart function. However, potential regulatory differences between isoforms have yet to be described at the molecular level. Here bovine cardiac tropomyosin was purified to achieve physiologically relevant ratios for cardiac muscle (15%–20% βTm) and additional Tm was further purified to separate α- and β- isoforms. In an in vitro motility assay, altering Tm isoform from α- to αβ- to β- in the presence or absence of cardiac troponin (cTn) did not significantly affect sliding speed (5.42 ± 0.70 μm) or fraction of moving filaments (0.75 ± 0.22) at maximal Ca2+ activation (pCa 5.0). Additionally, each Tm isoform was able to fully inhibit motion at pCa 9.0. However, a significantly higher concentration of βTm (400 nM) was needed to stop regulated filaments at pCa 9.0 than for either αTm or αβTm groups (300 nM). Finally, western blot analysis indicated that all Tm isoforms were not phosphorylated in a normal heart despite potential phosphorylation sites within αTm not found in βTm. The results suggest that βTm is a less efficient regulator of the actomyosin interaction than αTm, which may lead to the functional differences found in past transgenic studies. The current work investigated motility at only maximal calcium activation using skeletal myosin. Ongoing work investigates the role of cardiac (vs. skeletal) myosin, sub-maximal calcium levels and micro-needle force measurements. Further investigations will also look at the role of Tm phosphorylation levels on mechanics in response to post-infarction remodeling.

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680-Pos The Role of Tropomyosin Isoforms and Phosphorylation in Force Generation in Thin-filament Reconstituted Bovine Myocardium

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Board B524

The thin filament extraction and reconstitution protocol was used to investigate the functional roles of tropomyosin (Tm) isoforms and phosphorylation in bovine myocardium. The thin filament was extracted by gelsolin, reconstituted with G-actin, and further reconstituted with cardiac troponin together with one of three Tm varieties: phosphorylated αTm (αTm.P), dephosphorylated αTm (αTm.deP), and dephosphorylated βTm (βTm.deP). Thus, three distinct muscle models were generated. Two-dimensional gel electrophoresis demonstrated that the Tm phosphorylation state was unaffected by the presence of 40 mM 2,3-butanedione monoxime (BDM) in the reconstitution solution. The effects of pCa, phosphate, MgATP and MgADP concentrations were examined in the reconstituted fibres at pH 7.0 and 25°C. Our data show that Ca^{2+} sensitivity (pCa_{50}: half saturation point in pCa-tension plot) was increased by 0.19±0.07 units when βTm.deP was used instead of αTm.deP ($P<0.05$), and by 0.27±0.06 units when phosphorylated αTm was used ($P<0.005$). The Hill factor decreased significantly (3.1±0.3 to 2.1±0.2) when βTm.deP was used instead of αTm.deP ($P<0.05$). The Hill factor was not altered significantly when phosphorylated αTm was used. There was no significant difference in isometric tension or stiffness between αTm.P, αTm.deP, and βTm.deP muscle models at saturating [Ca^{2+}] (pCa 4.66) or after rigor induction. Sinusoidal analysis indicated that the cross-bridge number in the strongly attached states was similar for the three models, although the kinetic constants differed up to 1.7× between αTm.deP and βTm.deP, and up to 2.7× between αTm.deP and αTm.P. Our data imply that tension and stiffness per cross-bridge are not much different among the three muscle models tested.

681-Pos H2-calponin Deficient Mice and the Effects on Smooth Muscle Tension Regulation

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Board B525

H2-calponin has been observed to play a critical role in the tension regulation of actin cytoskeleton (Hossain et al., J. Biol. Chem. *280*:42442–53, 2005; Biochemistry *45*:15670–83, 2006). To investigate the physiological function of calponin, we have developed conditional h2-calponin null mutation in mouse by gene targeting in embryonic stem cells. Western blot analysis of smooth muscle tissues from Cnn2 null mice revealed no expression of h2-calponin. The levels of tropomyosin were significantly reduced in the h2-calponin null bladder tissues compared to that in wild type controls, implying a functional correlation. There was no up-regulation of h1-calponin expression in the h2-calponin null smooth muscle cells, indicating non-redundancy and differential regulation of the calponin isoform genes. Two smooth muscles that naturally have significant levels of h2-calponin were selected to study the effect of h2-calponin knockout on smooth muscle function. The h2-calponin null neonatal bladder smooth muscle had a reduction in passive tension response to length changes as compared to the wild type control. This result suggests that the lack of h2-calponin resulted in higher cytoskeleton compliance and hence. Contractility studies using intact muscle strips showed that the norepinephrine induced contraction in adult portal vein from h2-calponin knockout mice were significantly higher compared to that of the wild type control at the same resting tension. This observation suggests that the absence of h2-calponin sensitized the effect of resting tension on the development of active tension in smooth muscle.

682-Pos Nonmuscle Myosin Regulatory Light Chain Phosphorylation Is Regulated In Smooth Muscle

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Board B526

The participation of nonmuscle myosin in force maintenance is controversial. To participate in force maintenance, nonmuscle regulatory light chain phosphorylation must be regulated in smooth muscle. To investigate this question, the regulatory light chains of smooth muscle myosin (MLC20) and nonmuscle myosin (RLC) were cloned, expressed and purified. The purified proteins could be resolved on silver stained two-dimensional gels. Intact mouse aortic smooth muscle strips were stimulated with either KCl depolarization or angiotensin II, and the 4 distinct spots were resolved on the silver stained 2-D gels. Using mass spectroscopy, these spots were identified as four distinct proteins; phosphorylated RLC, non-phosphorylated RLC, phosphorylated MLC20 and non-phosphorylated MLC20. MLC20 phosphorylation, but not RLC phosphorylation, increased during KCl depolarization. However, angiotensin II stimulation increased both MLC20 and RLC phosphorylation. These data suggest that in smooth muscle, nonmuscle myosin is not regulated by Ca^{2+}-calmodulin activated MLCK, but rather by a G-protein mediated signaling pathway, possibly phosphorylation of the RLC by Rho kinase and/or PKC.

683-Pos Phosphorylation-induced Structural Changes in Smooth Muscle Myosin Regulatory Light Chain

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Board B527

We are using site-directed spectroscopic labeling, dipolar EPR, FRET, DEER, and molecular dynamics simulations to determine the phosphorylation-induced structural transition in smooth muscle myosin regulatory light chain (RLC). Smooth muscle is activated through phosphorylation of Ser 19 on RLC, but the N-terminal 24 amino acids of RLC do not appear in any crystal structure. EPR experiments (Nelson et al., 2005) have shown that phosphorylation induces a disorder-to-order transition within the N-terminal phosphorylation domain of the RLC, in which increased helical ordering relieves inhibitory head-head interactions. To define this structural change in atomic detail, we are combining molecular dynamics simulations with spectroscopic distance constraints. Simulations on the unphosphorylated 25-residue N-terminal fragment of the RLC reveal a disordered region between residues K12-Q15, while the phosphorylated N-terminal domain maintains strong α-helicity over the same residues. The same disorder-to-order transition has been observed by both simulations extended to include the entire RLC in complex with a portion of the myosin heavy chain, as well as by dipolar EPR measurements performed on the phosphorylation domain of di-cys mutant chicken gizzard RLC. Furthermore, we have employed both FRET and DEER distance measurements on the isolated di-cys mutant RLC to provide geometric constraints for the simulations. This allows both the structure and dynamics of the regulatory domain in the absence and presence of phosphorylation to be determined.

684-Pos The Short Skeletal-like Myosin Essential Light Chain Contributes To Lower Force In Mouse Myocardium

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To elucidate the role of the N-terminus of the essential light chain of myosin (ELC) in its direct interaction with actin, we have generated transgenic (Tg) mice expressing a 43 amino acid N-terminal truncation mutant of human ventricular ELC (Δ43) in mouse hearts. The mutant protein is similar in sequence to the short form of fast skeletal ELC (MLC3) which together with its longer counterpart

(MLC1) is expressed in fast skeletal muscle. Generated Tg-Δ43 mouse lines express from 10–40% transgene and the ELC distribution pattern of the long (endogenous) versus short (transgenic) ELC in Tg-Δ43 mouse hearts resembles that of skeletal muscle. The EDL (extensor digitorum longus) muscle of Tg-Δ43 mice looks normal and is not affected by myocardial ELC-Δ43 expression. Cardiac mRNA expression of other sarcomeric genes such as the α-myosin heavy chain, α-actin and the regulatory light chain of myosin as well as calcium handling genes, SERCA 2 and phospholamban is not affected by ELC-Δ43 expression. Skinned papillary muscle fibers from Tg-Δ43 mice demonstrated slightly but significantly decreased calcium sensitivity of force and ATPase compared to Tg-WT and NTg control mouse fibers. The largest difference was observed in force per cross sectional area which was dramatically decreased in all Tg-Δ43 lines compared to control fibers. Our results suggest that the removal of the N-terminal extension of the cardiac ELC most likely leads to decreased binding of the ELC-Δ43-containing myosin to actin. Perhaps the short ELC of Tg-Δ43 myocardium fails to make direct molecular contacts with actin resulting in a significant decrease in the myosin - actin interaction and steady state force development.

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685-Pos Contribution Of Skeletal Muscle To Cytokine Production In Heart Failure

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Board B529

Background: Patients with congestive heart failure (CHF) experience reduced skeletal muscle exercise tolerance. The mechanism underlying this is not known but studies have indicated that the extracellular matrix (ECM) may be affected. The signal from the failing heart that triggers such changes has not been defined, and it is not known to what extent the skeletal muscle itself contributes by sustaining and amplifying a local process. Hence, we have examined various components of skeletal muscle ECM and the interstitial fluid (IF) concentration of cytokines during development of CHF.

Methods: We used a post infarction model of CHF in rats. At various time points (3-112 days) after induction of CHF, blood and muscles were sampled and IF was extracted by wicks inserted into hindlimb muscles. Cytokines in plasma and IF and matrix metalloproteinase (MMP) activity and collagen content in muscle were determined.

Results: There was a transient increase in MMP activity and collagen content at 42 days after induction of CHF. Of the analyzed cytokines, vascular endothelial growth factor (VEGF) in IF was significantly lower in CHF compared to Sham at 3 and 10 days, whereas IL-18, a cardiodepressive cytokine, was significantly upregulated in plasma of the CHF animals at 112 days. Both these cytokines exhibited a large gradient from the muscle to plasma, indicating a net production in the muscle.

Conclusions: The reduced exercise tolerance in CHF seems to be associated with MMP-induced alterations in extracellular matrix. A reduced production of VEGF in muscles from the CHF animals may promote exercise intolerance. The high level of IL-18 in the IF compared to plasma, suggests that skeletal muscle may be a major contributor to the systemic level of this cytokine which in turn may further impair myocardial function.

686-Pos Impact of Oxidative Stress on Contraction, Calcium Handling and Myofilament Remodeling

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Board B530

Myofilament remodeling through post-translational modification of cardiac troponins (cTnI and cTnT) is an essential mechanism in regulating cardiac function both under physiological and pathophysiological conditions. Although mounting data implicate oxidative stress-induced myocardial injury in the development and progression of contractile dysfunction leading to heart failure, the possible intracellular targets, especially at the myofilament level, are poorly characterized. Oxidative stress can exert its effect on myofilament function directly, through protein oxidation and/or indirectly, through activation of a panel of protein kinases (ASK-1, PKC etc.) and proteolytic enzymes (MMP, caspase, calpain etc.).

In the present study, we evaluate the impact of acute oxidative stress on contraction, Ca^{2+} handling, and myofilament remodeling in adult rat ventricular myocytes (ARVM). Exposure to H2O2 (0.5 mM) enhanced ARVM contraction, augmented systolic amplitude and maintained diastolic length. These effects occur in the absence of obvious changes in Ca^{2+} transient, indicating increased myofilament Ca^{2+} responsiveness. Our data also indicate that acute exposure of cultured ARVMs to H2O2, leads to the formation of several high-molecular weight (HMW) protein complexes that immunoreact with anti-cTnI and anti-cTnT monoclonal antibodies, but not an anti-tropomyosin antibody. Interestingly, some of the HMW protein complexes also react with anti-phospho-cTnT antibodies. Phosphorylation of cTnT was not ablated by pretreatment with PKC inhibitors (GF109203X or Gö6976) nor augmented by PMA (a PKC agonist). The presence of HMW troponin complexes, suggest the occurrence of two tightly coupled events: fragmentation and cross-linking. Cell permeable calpain inhibitors, Z-VF-CHO and Z-LLY-FMK, failed to hinder the formation of HMW complexes, signifying that calpains may not be involved in this process.

687-Pos Dynamic Skeletal Muscle Exercise And Fatigue Development

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Board B531

Skeletal muscle fatigue defined as decline in maximum force production or a decline in power output is exclusively linked to repeated isometric contractions. However, most muscles shorten during normal use and we propose that both the functional correlate of fatigue as well as the fatigue mechanism will be different during dynamic contractions as compared with static contractions. Fatigue was induced in rat soleus muscles in situ by isotonic shortening contractions. Muscles were field stimulated repeatedly for 1s at 30Hz every 2s for a total of 15 minutes. The muscles were allowed to shorten isotonically working against a load corresponding to 1/3 of maximal isometric force. Between contractions the muscles were restretched to initial length. Power output was reduced after 100s but returned to almost initial values at the end of the stimulation protocol. Maximal unloaded shortening velocity (V0), maximum force production (Fmax) and isometric relaxation rate (-dF/dt) followed the same transient pattern. Likewise ATP and CrP were reduced and lactate was increased after 100s, but recovered to initial values after 5 minutes. The rate of isometric force development, the velocity of shortening and isotonic shortening were also reduced at 100s, but in striking contrast did not recover during the remainder of the stimulation protocol. The regulatory myosin light chain (MLC2) was dephosphorylated after 100s and did not recover. While metabolic changes seem to account for the transient change of Fmax, -dF/dt and V0, dephosphorylation of MLC2 may be involved in the fatigue seen as sustained slower contraction velocities and decreased muscle shortening.

688-Pos Vicinal-thiol Groups Regulate SERCA1 Activity and Structure in Resting and Fatigued Fast Skeletal Muscle

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Board B532

During prolonged skeletal muscle contractions free radicals are produced that may lead to fatigue. Vicinal-thiol (VT) groups react preferentially with free radicals. Therefore, we examined the role of VT groups on the activity and structure of selective oxidation in skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA1). After Ca^{2+} is released from the SR to start contraction, SERCA1 pumps this cytosolic Ca^{2+} back to the SR leading to muscle relaxation. Phenylarsine oxide (PAO) reacts selectively with VT proteins forming dithioarsines, which is stable but exchanges rapidly with 2,3-dimercaptopropanol (BAL). When 0.1 mM PAO is added to isolated SR, 60% inhibition of SERCA1 hydrolytic and Ca^{2+} uptake activities is observed. This inhibition was fully reversible when 1 mM BAL was added. The properties of SERCA1 thermal inactivation determined from SR isolated from muscle at rest showed a single transition for inactivation (Ti) at 49°C±1.12°C. In the presence of 0.1 mM PAO, the Ti shifted to 34±0.9°C and an additional transition was observed at 27±1.2°C. The thermal denaturation profile of SERCA1 from muscle at rest, showed two transitions at Tm=51.5±1.3°C and 63±1.02°C related to nucleotide and Ca^{2+} binding domains respectively. Whereas SERCA1 isolated from fatigued muscle, showed three transitions at Tm=46.8±1.03°C, 60.2±0.58°C and 36.5±0.33°C. Furthermore, addition of BAL to the SERCA1 isolated from fatigued muscle resulted in a denaturation of SERCA1 with two transitions at Tm=49±1.05°C and 62.3±0.82°C with Tm=36.5±0.33, disappearing during the thermal inactivation. The present findings suggest the presence of a mechanism relating free radicals to muscle fatigue through VT groups.

689-Pos siRNA Targeting Small Ankyrin 1 Selectively Disrupts The Network Compartment Of The Sarcoplasmic Reticulum In Adult Skeletal Muscle Fibers

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Sarcomeres of striated muscle are encircled by a compartment of the sarcoplasmic reticulum (SR) that takes up and stores Ca2+ – the "network SR". Small ankyrin 1 (sAnk1) is an integral protein of the network SR that binds with nM affinity to obscurin, an ~800 kDa protein that surrounds the sarcomere at M-bands and Z-disks, where the network SR is concentrated in mammalian skeletal myofibers. We hypothesize that this binding is both necessary and sufficient for the correct localization of the network SR. We tested this hypothesis by using siRNA to reduce the expression of sAnk1 in primary cultures of adult skeletal myofibers and examining the effects on the SR. sAnk1 levels were reduced in fibers treated with siRNA; remaining sAnk1 was disorganized. These effects were specific, as siRNA caused no change in the expression or organization of α-actinin, obscurin, or other proteins associated with sarcomeres, and scrambled sequences had no effect. By contrast, the organization and expression of SERCA, which also concentrates in the network SR, were significantly altered in myofibers treated with sAnk1 siRNA. Markers of the terminal cisternae of the SR, including calreticulin, calsequestrin, and ryanodine receptors, as well as Ca2+ channels (DHPR) in the transverse tubules, were not significantly affected. Physiological experiments demonstrated that the amplitude of electrically evoked global Ca2+ release, was reduced by ~ 33% ($p<0.05$) in siRNA-treated fibers, compared to controls. No alteration in basal myoplasmic [Ca2+] was seen. These results show that down-regulation of sAnk1 selectively alters the organization of the network SR and are consistent with our hypothesis that sAnk1 is required for the formation of the network SR and its alignment with nearby sarcomeres.

690-Pos Characterization of Proteins Associated with Cardiac Myofibrils using a Proteomic Approach

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Cardiac myofibrils are commonly used for in vitro studies to determine the role of different thin filament proteins on muscle mechanics. A recent report from our group showed the presence of protein kinase A in skinned porcine cardiac fibers (Gomes A.V. et al. J. Mol. Cell. Cardiol. 2005, *39*:754–765). The presence of other potential kinases and enzymes in Triton-X 100 treated myofibrils is of significant interest as these proteins could potentially be modulators of muscle contraction. A proteomics approach utilizing gel electrophoresis, tandem mass spectrometry (LC-MS/MS), and immunoblotting was employed to analyze the components of myofibrils purified from mouse hearts. Mass spectrometry and immunoblotting results revealed the presence of many proteins which do not directly participate in the contractile process such as αB crystallin and ADP-ribosylhydrolase. Several enzymes including protein kinase A and proteasome subunits were found to be associated with the myofibrils. In addition to obtaining the identity of the myofibrillar associated components, specific post-translational modifications of the sarcomeric proteins including novel modifications on myosin heavy chain were also detected. To ascertain the importance of several Triton-X 100 washes on the myofibrillar composition LC-MS/MS was also carried out on fibers which were only partially skinned (single wash with Triton-X 100). Numerous other contaminating proteins (in excess of 100) were detected in partially skinned myofibrils compared to completely skinned and purified myofibrils. Overall, these results suggest that the thick and thin filaments are more complex than previously envisioned with substoichiometric amounts of several proteins present which are likely to be important for regulating the physiological function of the cardiac muscle. These results also suggest the importance of several Triton-X 100 washes to remove contaminating proteins.

691-Pos Experiments And Simulations Show That The Force-pCa Relationship In Skeletal Muscle Requires Multiple Mechanisms Of Cooperative Activation

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The sigmoidal relationship between steady-state force and [Ca^{2+}] in myocytes indicates cooperative interactions between the proteins actin, myosin, troponin (Tn), and tropomyosin (Tm). In skeletal muscle, allosteric interactions drive cooperative thin filament activation and force production. Cooperativity may follow from activation of a regulatory unit (RU: 1Tn, 1 Tm, 7 actins) that augments the activation probability of neighboring RUs (RU-RU cooperativity), or from cross-bridge binding that augments Tm displacement (XB-RU cooperativity). Using single psoas muscle fibers, we reduced RU-RU cooperativity by extracting endogenous TnC and reconstituting thin filaments with a mixture of 20% TnC to 80% D28A, D64A mutant TnC that does not bind Ca^{2+} at the N-terminus. The reconstituted condition decreased maximal force by 70%, Ca^{2+} sensitivity by ~0.3 pCa units, and slope by ~3 units, compared to endogenous conditions. In contrast to the weakened force-Ca^{2+} response, this reconstituted condition significantly increased Pi sensitivity of force and the rate of force redevelopment. Using Tn densities similar to these experimental values, we simulated multiple forms of cooperativity using our recent spatially-explicit model of muscle contraction (*PLoS Comput. Biol.* 2007. *3*:e115). Simulations of RU-RU and XB-RU cooperativity confirmed our experimental results, showing that interactions between regulatory proteins along the thin filaments dominate cooperative activation of force in skeletal muscle. Moreover, simulating physiological levels of cooperativity required us to increase the spatial influence of RU activation from 37 to 56 nm, as suggested by our prior experiments. Taken together, our experimental and theoretical approaches indicate that multiple forms of cooperativity combine to yield physiological force-pCa relationships.

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692-Pos Stochastic Model of Contraction and Thin Filament Regulation in Exact 3D Sarcomere Geometry

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The dynamics behavior of muscle in physiologically relevant situations is extremely complex and is due to interactions among cross-bridge kinetics, thin filament regulation by Ca^{2+} and complex loading conditions. To quantitatively assess this complex behavior we have developed comprehensive stochastic model of muscle contraction and its regulation in the exact 3D sarcomere geometry. The model includes a simple three state and comprehensive nine state actomyosin cycle, extensibility of thick and thin filaments and three models of thin filament regulation. For comparison we explore Hill's two state model, McKillop-Geeves three state model and the flexible (tropomyosin) chain model (FCM). Loading conditions include isometric force development for prescribed Ca^{2+} concentrations and Ca^{2+} transients which include single and multiple twitches. All regulatory models were able to predict the force-pCa relationship, but only FCM was able to predict muscle relaxation after instantaneous reduction in Ca^{2+} concentration. The reason for this behavior is multiple myosin binding within a single troponin-tropmyosin (TnTm) unit except at very low Ca^{2+} concentrations. When multiple cross-bridges are bound within single TnTm they keep the unit open and allow reattachment of detached cross-

bridges. Therefore the TnTm unit remains open even at moderate Ca^{2+} concentrations. In contrast, flexible Tm chain prevents reattachment of the cross-bridges by partially covering actin sites within a TnTm unit. For the same reason only the flexible chain model predicted the twitch dynamics. In a multiple sarcomere model we tested the hypothesis that the observed heterogeneity of shortening of individual sarcomeres is the principal mechanism causing rapid decrease in overall force upon sudden decrease of Ca^{2+} concentrations. This model somewhat improved the relaxation predicted by rigid TnTm unit models, but only within intermediate range Ca^{2+} concentrations.

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693-Pos Spanning Length And Time Scales Of Muscle Activation With FRET, MD, And Modeling

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We approach the dynamics of allosteric change experimentally to resolve which allosteric mechanism—induced-fit and population-shift-stabilization—is employed in the regulatory switch of cardiac muscle. The cardiac Ca-regulatory switch (CRS), the Ca-sensitive binary complex of troponin C (TnC) and troponin I (TnI), is a prototype allosteric signaling network (ASN)—a semi-stable protein assembly that communicates information through coupled intra- and inter-protein domain movements. Most cell biological information processing is performed by ASN. Familiar examples of unbranched ASN include the family of G-protein coupled receptors, ligand gated ion channels, and the ribosome. The relative simplicity of unbranched ASN, compared to branched networks that integrate multiple inputs, make them useful for discovering *emergent properties* in modular hierarchical structure. We show how ASN are useful for discovering *emergent design* issues in modular hierarchical structure.

An engineered intra-molecular FRET pair is used to follow the central structural rearrangement in the CRS. Time resolved FRET measurements indicate that activation of TnC is insensitive to the ordering of Ca / TnI addition. This commutivity is exploited in stopped flow FRET experiments, which are interpreted using a quantitative phenomenology of allostric signaling to reveal that CRS activation proceeds through an induced-fit mechanism. When saturated with Ca, the CRS (15 C) exists as a rapidly inter-converting 3:2 mixture of open (active) and closed (inactive) conformers. Time resolved FRET, transient FRET, and Ca-titration FRET measurements are globally analyzed to resolve the system configurational phase state free-energy landscape (SPEL)—a systems-level partition function—of the regulatory switch. In molecular dynamics (MD) simulations with probes incorporated into structural models derived from NMR and x-ray crystallographic data, we demonstrate consistency between the measured FRET "optical distance" and molecular structure.

Ion Motive ATPases

694-Pos Expression Pattern of Fluorescently Tagged Na pump in *C. elegans.*

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The Na,K-ATPase is a transmembrane protein that is responsible for actively transporting $3Na^+$ (out) and $2K^+$ (in) of most eukaryotic cells. The subsequent Na^+ gradient plays a vital role in cell excitability, contractility, and osmotic balance. Na pump physiology is most evident within polarized epithelium where it drives the directional uptake of nutrients from lumen to blood. In order to maintain this unidirectional absorption, Na,K-ATPase expression is restricted to the basal-lateral membrane in epithelial cells. In order to investigate the membrane delivery of Na,K-ATPase further we have constructed fluorescently tagged alpha and beta subunits for expression in *C. elegans.* Two separate plasmids were constructed for injection into *C. elegans;* the α-subunit was constructed with YFP fused to the C-terminus and the β-subunit was constructed with CFP fused to the N-terminus. Both genes are under control of the heat shock promoter, hsp16–41 promoter. The α-YFP plasmid was injected with the cotransformation marker rol-6, whereas the CFP-β plasmid was injected with the lin-15 gene into the syncytial gonad of adults. Transgenic animals were isolated if they displayed the respective contrasformation marker phenotype. In order to view β-subunit expression, CFP-β transgenic worms were heat shocked at 33°C for 2 hours on non pre-warmed plates, then set at 20°C for 4 hours. CFP-β was expressed throughout the intestine of the worm. α-YFP fusion protein has also been viewed within *C. elegans.* α-YFP fluorescence was observed in pharynx, vulva, body-wall muscle cells, nerve cells and throughout the intestine. We are currently characterizing the heat shock time-course and tissue expression intensities of both constructs.

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695-Pos Interaction between Nitric-oxide Synthase Pathways and NAD(P)H Oxidase Pathways Leading to Modulation of the Na^+-K^+ Pump Activity

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