S-transferase-2 is stably associated with them (Clayton et al., 1998). Then an alternative explanation is that these isoforms also exist in *Ctenacroscelis*, and they are alternately arranged and only one of them binds GST-2. The scattering object remains to be identified, but the alternating arrangement of tropomyosin isoforms is the most conceivable mechanism to provide the periodicity needed to create the peculiar meridional reflection.

1187-Pos Board B31

Effect Of Tropomyosin On The Binding Force Of Unphosphorylated Myosin To Actin

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Smooth muscle (SM) is unique in its ability to maintain force for long periods of time at low energy cost. This property is called the latch-state. One of the assumptions of the latch state model of Hai and Murphy is that myosin must first be phosphorylated in order to attach to the thin filament. However, we previously demonstrated that unphosphorylated (unPHOS) myosin can attach to unregulated actin filaments. The goal of this study was to measure the binding force of unPHOS SM myosin to tropomyosin-regulated actin filaments. A microsphere captured in a laser trap was attached to an actin filament decorated with SM tropomyosin-α and β. The filament was brought in contact with a pedestal coated with unPHOS pig antrum myosin. The pedestal was then moved away from the trap at constant velocity (0.5 $\mu m.s^{-1}$). Despite pulling the pedestal away, the microsphere did not move until the force exerted by the trap on the microsphere was sufficient to overcome the binding force of myosin on the actin/tropomyosin filament. At this point, the microsphere sprang back to its unloaded position. The force of unbinding was calculated as the product of the trap stiffness and the maximal displacement of the trapped microsphere, as assessed by displacement of its center of mass. The average force of unbinding per myosin molecule (F_{unb}) was obtained by dividing the measured force of unbinding by the number of myosin molecules estimated per actin filament length. We found that F_{unb} was greater in presence (0.222 pN $\pm$ 0.018; mean $\pm$ SE) than in absence (0.142 pN $\pm$ 0.019; $p<0.001$) of tropomyosin. These results demonstrate that tropomyosin strengthens the bond between unPHOS myosin and actin. Future studies will investigate the role of other regulatory proteins of the thin filament.

1188-Pos Board B32

Tropomyosin Flexibility Evaluated by Electron Microscopy Image Analysis

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Movement of tropomyosin (Tm) on thin filaments in response to Ca^{2+}-binding to troponin and myosin binding to actin is an inherent feature of muscle regulation. As part of this process, the cable-like mechanical properties of the Tm coiled-coil are thought to underlie cooperative on and off switching of contraction. In principle, movement of Tm over the flat surface of actin may not require significant molecular flexibility. However, local perturbations caused, for example, by myosin binding on actin may necessitate some plasticity of the Tm molecule. In contrast, any large-scale Tm flexibility might dampen its cooperative movement. In the current study, we directly assessed the flexibility of Tm by examining EM images of both rotary shadowed and negatively stained molecules. Single Tm molecules in both image sets showed no obvious signs of sharp bending or kinks, and displayed contours close to those predicted from a high-resolution 3D model of Tm (Lorenz *et al.*, 1995). Short multimeric strings of end-to-end bonded Tm were commonly observed in the rotary shadowed images. These showed no pronounced bending or joints at the intermolecular junctions. The persistence length of Tm was calculated to be over twice the length of the molecule based on these EM images. The data as a whole imply that Tm molecules exhibit an intrinsic stiffness sufficient to contribute to cooperativity on thin filaments and thus are consistent with models of muscle regulation (e.g. Lehrer & Geeves, 1998; Lehman *et al.*, 2000). A tropomyosin mutant in which the coiled-coil interface was destabilized by introduction of Ala clusters in the 2nd and 3rd periodic repeats (Y60A-L64A-L106A; Singh & Hitchcock-DeGregori, unpublished) was also examined by EM. No obvious extra bending was observed, although the calculated persistence length was significantly shortened, showing the sensitivity of the technique.

1189-Pos Board B33

The Fast Skeletal Troponin Activator, CK-1909178, Increases Skeletal Muscle Force *in-vitro* and *in-situ*

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Previously, we have discovered small molecules that increase cardiac contractility by directly activating the cardiac sarcomere; this mechanism is now being investigated as a therapy for treating systolic heart failure. Using this precedent, we have focused on the identification of compounds that directly increase skeletal muscle contractility for the potential therapy of diseases that result in muscle weakness and fatigue.

CK-1909178 is a member of a class of fast skeletal troponin activators that were identified by high throughput screening of skeletal sarcomere preparations. We sought to understand how this compound altered force development in isometric skinned and live muscle fibers. Treatment of skinned rabbit psoas fibers with 0.1 μM CK-1909178 caused a dose-dependent left-shift of the force-pCa relationship without altering the Hill slope or maximum force, consistent with a calcium sensitizing effect on force production. In living rat flexor digitorum brevis (FDB) preparations, CK-1909178 (10 μM) caused significant increases in subtetanic force (150% of control at 10 Hz) without altering maximum force. Similar experiments were then performed using a rat extensor digitorum longus (EDL) preparation *in-situ*, where nervous and vascular connections were left intact and the muscle was stimulated via the peroneal nerve. Infusions of CK-1909178 up to 10 mg/kg rapidly increased sub-tetanic isometric force (190% of control at 30 Hz). In summary, we have identified a skeletal troponin activator that sensitizes the sarcomere to calcium and results in increased submaximal muscle force development *in-vitro* and *in-situ.* We believe that this mechanism may translate to improved physical power and strength in diseases where muscle function is compromised due to injury, disease or age.

1190-Pos Board B34

The Small Molecule Skeletal Sarcomere Activator, CK-1909178, is a Calcium Sensitizer that Binds Selectively to the Fast Skeletal Troponin Complex

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Striated muscle contraction is tightly coupled to the release of Ca^{2+} from the sarcoplasmic reticulum by the sarcomeric calcium sensor, troponin. This complex of three proteins (troponins T, I, and C) undergoes calcium-sensitive conformational changes that regulate the accessibility of myosin binding sites along the actin filament. We used a high throughput screen to identify compounds that activate the ATPase activity of skinned fast skeletal myofibrils; optimization of the initial hit compounds has resulted in compounds with sub-micromolar affinity. A potent representative of this chemical series, CK-1909178, shifts the calcium sensitivity of detergent skinned skeletal myofibrils by >10-fold in a concentration dependent manner. This compound specifically activates fast skeletal myofibrils, with no effect on either slow skeletal or cardiac myofibrils. A reconstituted sarcomere assay using combinations of fast skeletal, slow skeletal, and cardiac components demonstrates that the activity of CK-1909178 requires the presence of fast skeletal troponin. Isothermal titration calorimetry indicates the compound binds directly to fast skeletal troponin with a sub-micromolar dissociation constant. Consistent with its calcium sensitization effects, CK-1909178 slows the dissociation of calcium from troponin as measured by Quin-2 fluorescence. Consistent with its mechanism of action, CK-1909178 sensitizes muscle *in vitro* and *in vivo*, suggesting this mechanism may increase power or strength in diseases where muscle function is compromised due to injury, disease or age.

1191-Pos Board B35

Modulation Of Human Cardiac Troponin C-troponin I Interaction By An Analogue Of Levosimendan, (2′,4′-difluoro(1,1′-biphenyl)-4-yl) Acetic Acid (dfbp)

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The binding of Ca2+ to cardiac troponin C (cTnC) triggers contraction in heart muscle. In diseased heart, the myocardium is often desensitized to Ca2+, leading to weak cardiac contractility. Compounds that can sensitize cardiac muscle to Ca2+ have therapeutic value in treating heart failure. Of the known drugs that are proposed to accomplish this, levosimendan is the best characterized and currently in clinical use. Levosimendan interacts with the regulatory domain of cTnC and promotes association of cTnC with troponin I (cTnI). Detailed understanding of the mechanism of levosimendan has been concealed by its unstable nature; however, the use of analogues that are more stable would

provide valuable insights into its mode of action. The molecular framework of DFBP resembles that of levosimendan, thus it was chosen to mimic levosimendan to establish how the cTnC-cTnI binding equilibrium is modulated. We have utilized 2D {1H, 15N} HSQC and 2D {1H, 13C} HSQC NMR spectroscopy to examine the binding of DFBP to cNTnC•Ca2+ in the absence and presence of cTnI144-163 and of cTnI144-163 to cNTnC•Ca2+ in the absence and presence of DFBP. The results show that DFBP and cTnI144-163 bind cNTnC•Ca2+ concurrently and the affinity of DFBP for cNTnC•Ca2+ is increased ~5-10-fold by cTnI144-163. We are in the process of determining the NMR solution structure of cNTnC•Ca2+•cTnI144-163•DFBP. This structure will contribute to the understanding of the mechanism of action of levosimendan in the therapy of heart disease. It will also provide a structural basis for the design of Ca2+-sensitizing drugs in general.

1192-Pos Board B36

Decreased Fatigue Tolerance In Diaphragm Muscle Of Slow Troponin T Knockdown Mice

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The loss of slow skeletal muscle troponin T (TnT) results in a severe type of nemaline myopathy in the Amish (ANM). The genes encoding TnT and troponin I (TnI) are closely linked in pairs in which the 5′-enhancer region of the slow TnT gene overlaps with the cardiac TnI gene. In a mouse line with the entire cardiac TnI gene deleted, a partial destruction of the slow TnT gene promoter produces a knockdown effect. By crossing with transgenic mouse lines that over-express a core structure of cardiac TnI (cTnI-ND) under the control of cloned alpha-MHC promoter, we rescued the postnatal lethality of the cardiac TnI gene-deleted mice with no detrimental cardiac phenotypes or leaking expression in non-cardiac tissues. The double transgenic mice exhibited decreased expression of slow TnT mRNA and protein in adult diaphragm muscle. Functional analysis of isolated muscle strips showed that the slow TnT deficient (sTnT-KD) diaphragm had significantly decreased fatigue tolerance evident by the faster decrease in force and slower rate of recovery as compared with that in wild type controls. As a consequence of slow TnT deficiency, the sTnT-KD diaphragm muscle contained a higher proportion of fast TnT, decreased slow TnI with increased fast TnI, and decreased type I myosin with increased type II myosin. Consistent with the switch toward fast myofilament contents, the sTnT-KD diaphragm muscle produced higher specific tension in twitch and tetanic contractions as well as shorter time to develop peak tension in twitch contractions. The decreased fatigue tolerance of sTnT-KD diaphragm muscle explains the terminal respiratory failure seen in virtually all ANM patients and this double transgenic mouse model provides a useful experimental system to study the pathogenesis and treatment of ANM.

1193-Pos Board B37

Troponin Isoforms and Stretch-activation of Insect Flight Muscle

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Oscillatory contraction of insect indirect flight muscle (IFM) is activated by sinusoidal length changes. Work done by oscillating fibres is measured from the area of loops on a length-tension plot. At [Ca2+] above 10 μM, progressively less oscillatory work is produced because fibres contract isometrically and are unable to relax fully after each cycle of oscillation. Periodic stretches during oscillations activate fibres through the action of TnC F1, which binds one Ca2+ in the C-lobe. Activation of isometric contraction by Ca2+ acts through F2, which binds Ca2+ in both N- and C-lobes. Lethocerus IFM fibres substituted with F1gave oscillatory work, which did not decline at high [Ca2+], while fibres substituted with F2 produced more isometric tension as [Ca2+] was increased. Varying proportions of F1 and F2 gave maximal work with an F1:F2 ratio of 100:1, which is higher than the in vivo ratio of 7:1. The structure of F1, and the interaction with TnI, were determined by NMR. The N-lobe of F1 is in the closed conformation in apo and Ca2+- bound forms and does not bind TnI. Unexpectedly, the C-lobe is open in both states, and binds the N-terminal domain of TnI independently of Ca2+. The affinity of F1and F2 for a complex containing tropomyosin, TnT and TnH (Lethocerus TnI) were measured by isothermal calorimetry in the presence of Ca2+. The affinities of F1 and F2 for the complex were 5.4 μm and 65 nM respectively. This difference is likely to be due to a single TnI binding site on F1 and two sites on F2. Stretch may be sensed by an extended C-terminal domain of TnH, and transmitted to the C-lobe of F1, resulting in a change in the interaction of the TnI inhibitory domain and actin.

1194-Pos Board B38

Tracking of Qdot Conjugated Titin Antibodies in Single Myofibril Stretch Experiments Reveals Ig-domain Unfolding at Physiological Sarcomere Lengths

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The mechanical characteristics of titin in muscle sarcomeres were previously studied by us in single myofibril stretch experiments, where the extensibility of I-band titin segments was usually measured under static conditions. Here we investigated the behavior of I-band titin during and after stretch of single rabbit psoas myofibrils in real-time. The focus was on titin's proximal Ig-domain region, whose stretch dynamics were analyzed by labeling the myofibrils specifically in the N2A-titin domain using antibody-conjugated quantum dots, which stained the periphery of the myofibril but did not enter the myofilament lattice. Qdot labels were tracked to obtain the stretch-dependent change in epitope distance (across Z-disc) and sarcomere length (SL) over time. In contrast to what was expected from the current titin extensibility model, at sarcomere lengths of 2.5 and 3.8 μm, titin's proximal Ig-domain region elongated continuously, in proportion to the half I-band length. Already at ~2.6 μm SL the proximal Ig-segment length exceeded the value expected if all Ig-domains remain folded. Our results suggest that Ig-domains unfold in parallel with PEVK-titin extension at physiological sarcomere lengths and under relatively low forces. By reducing the antibody-Qdot concentration, we succeeded in observing titin Ig-domain dynamics in myofibrils at the single-molecule level.

1195-Pos Board B39

Constitutive Phosphorylation of Cardiac Myosin Binding Protein-C Increases the Probability of Myosin Cross-bridge Interaction with Actin

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Protein kinase A-mediated (PKA) phosphorylation of cardiac myosin binding protein-C (cMyBP-C) accelerates the kinetics of cross-bridge cycling and appears to relieve the tether-like constraint of myosin heads imposed by cMyBP-C (Colson *et al.*, 2008, *Circ Res.*, 103:244-251). We favor a mechanism in which phosphorylation of the 3 PKA sites in cMyBP-C modulates cross-bridge kinetics by regulating the proximity and interaction of myosin with actin. To test this idea, we used synchrotron low-angle x-ray diffraction and mechanical measurements in skinned myocardium isolated from a mouse model with phosphomimetic substitutions in cMyBP-C, i.e., the CTSD mouse. The substitutions were introduced by transgenic expression of cMyBP-C with Ser-to-Asp mutations on a cMyBP-C null background. Western blots showed that expression of CTSD cMyBP-C was 85% of wild-type (WT), and the heart weight to body weight ratio was similar (5.2 ± 0.2 mg/g) in CTSD and WT mice. Expression of WT cMyBP-C on the knockout background served as control (i.e., the CTWT mouse). Skinned myocardium from CTSD and CTWT mice exhibited similar maximum active forces (mN/mm^2: 17.7 ± 3.7 *vs* 13.2 ± 2.9), Ca^{2+}-sensitivities of force (pCa_{50}: 5.55 ± 0.03 *vs* 5.58 ± 0.04), and maximum rates of force development (k_{tr}, sec^{-1}: 20.2 ± 1.7 *vs* 22.5 ± 1.9; k_{df}, sec^{-1}: 37.6 ± 3.7 *vs* 43.2 ± 2.3). I_{11}/I_{10} intensity ratios and d_{10} lattice spacings determined from equatorial reflections from CTSD and CTWT myocardium were used to determine the effect of constitutive cMyBP-C phosphorylation on the distribution of cross-bridge mass between the thick and thin filaments and on interfilament lattice spacing. The results suggest that interactions between cMyBP-C and the S2 domain of myosin heavy chain are dynamically regulated by phosphorylations in the cMyBP-C motif. *(AHA-predoctoral fellowship (BAC); NIH-HL-R01-82900)*

1196-Pos Board B40

Obscurin Interacts with a Novel Isoform of Myosin Binding Protein C-Slow to Regulate the Assembly of Thick Filaments

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Obscurin is a multidomain protein composed of adhesion and signaling domains that plays key roles in the organization of contractile and membrane structures in striated muscles. We used adenoviral-mediated gene transfer to overexpress its extreme NH2-terminus in developing myofibers, followed by immunofluorescence and ultrastructural methods to study its effects in sarcomerogenesis. We found that overexpression of obscurin's second immunoglobulin domain (Ig2) inhibits the assembly of A- and M-bands, but not Z-disks and