steady-state and time-resolved Förster Resonance Energy Transfer (FRET) measurements and stopped-flow kinetic studies, we measured Ca^{2+}-induced changes in FRET distance from the residues 160 and 167 in the regulatory region of cTnI to the residue 89 of cTnC to monitor cTnC and cTnI interactions. The measurements were done with the reconstituted thin filament containing PAK3 pseudo-phosphorylation of cTnI(S151E). We hypothesized that the charge modification at the interface between troponin C (cTnC) and cTnI caused by the phosphorylation at the N-terminus of the regulatory region of cTnI may affect the binding of the regulatory region of cTnI to cTnC. Our results showed that the pseudo-phosphorylation of cTnI(S151E) favors the binding by shortening the distances between the regulatory region of cTnI and cTnC and increasing Ca^{2+} sensitivity of the structural change. Furthermore, the pseudo-phosphorylation showed similar kinetic effects as the strongly-bound crossbridges on the thin filament regulation by significantly slowing down the kinetics of the Ca^{2+} dissociation-induced structural transitions of the regulatory region of cTnI. This is consistent with the decreased tension cost observed in the tension measurements of cardiac muscle fiber bundle reconstituted with the pseudo-phosphorylated cTnI, which suggest a decrease in crossbridge detachment rates. Our results provide novel information on the potential molecular mechanism underlying modulation of cardiac thin filament regulation by PAK3 phosphorylation of cTnI.

768-Pos

Fast-To-Slow Fiber Type Switch Increases Fatigue Resistance as a Compensatory Adaptation In Gsα-Deficient Soleus Muscle

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Genetically modified mice with Gsα-specific deficiency in skeletal muscle showed reduced glucose tolerance, muscle atrophy and force reduction, along with a fast-to-slow fiber type switch (Chen et al., AJP 296:C930-40, 2009). We further investigated a hypothesis that the switching to more slow fibers is an adaptive response with functional significance. Corresponding to the muscle type switch evident by myosin isotyping, the thin filament regulatory proteins troponin T and troponin I both had significant changes to their slow isoforms in the atrophic soleus muscle of 3-month-old Gsα-deficient mice. This fiber type switching progressed and soleus muscles of one-year-old Gsα-deficient mice expressed only slow isoforms of troponin. Functional characterization of soleus muscle of 3-month-old Gsα-deficient mice showed slower contractile and relaxation velocity in twitch and tetanic contractions than wild type controls. Examination of fatigue tolerance showed that Gsα-deficient soleus muscle was more resistant to intermittent fatigue stimulation with faster and better recovery as compared with wild type controls. Our results suggest that fast-to-slow type switch improves fatigue resistance of skeletal muscle as a compensatory adaptation to muscle glucose intolerance and atrophy in Gsα-deficiency, suggesting a mechanism for improving muscle function in diabetic patients.

769-Pos

Strong Crossbridges are Required to Recapitulate the Ca^{2+} Affinity Changes Produced by HCM-cTnC Mutants in Skinned Fibers

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This spectroscopic study examines the steady state and kinetic parameters governing the crossbridge effect necessary to increase the Ca^{2+} affinity of hypertrophic cardiomyopathy-cardiac troponin C (HCM-cTnC) mutants to the level seen in skinned fibers. Previously, it was shown by Landstrom, *et al.* (*J. Mol. Cell Card.* 45:281-288; 2008) and Pinto, *et. al.* (*J. Biol. Chem* 284(28): 19090-19100; 2009) that the cTnC mutations A8V, C84Y, E134D and D145E do not increase the apparent Ca^{2+} affinity of isolated cTnC (D145E shows a slight increase) as monitored by 2-(4′-(2″-iodoacetamido)phenyl)aminonaphthalene-6-sulfonic acid (IAANS) fluorescence. Follow-up experiments showed that when cTnC mutants are incorporated into regulated thin filaments (RTF), only the A8V mutant increased the apparent Ca^{2+} affinity. Addition of myosin subfragment-1 (S1) to mutant RTFs (in the absence of ATP) increased the apparent Ca^{2+} affinity to similar levels seen in cTnC mutant reconstituted skinned fibers. Therefore, strong crossbridges were required to fully alter the apparent cTnC Ca^{2+} affinity and recapitulate the changes observed in the Ca^{2+} sensitivity of tension. Stopped flow fluorescence techniques were also used to measure the kinetics of Ca^{2+} binding to troponin complex (cTn) and RTF prepared with IAANS labeled cTnC mutants. At the cTn level, both A8V and D145E cTnC decreased the rate of Ca^{2+} dissociation; while in the RTF, only A8V decreased the rate of Ca^{2+} dissociation. Future experiments will determine the rate of Ca^{2+} dissociation from RTFs in the presence of S1. This study indicates that although these HCM-cTnC mutants display similar phenotypes in skinned fibers, they utilize different molecular mechanisms to alter the Ca^{2+}-sensitivity of skinned muscle. Supported by NIH HL-42325 (JDP) and AHA 0825368E (JRP) and AHA 09POST2300030 (MSP).

770-Pos

Changes in the Conformation of Troponin C on Activation of Skeletal Muscle

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Skeletal muscle contraction is regulated by calcium-dependent changes in the structure and thin-filament location of troponin and tropomyosin. The structural changes in the isolated calcium-binding subunit of troponin (TnC) are well characterized, but those of TnC in the native thin filament are much less clear. We measured the *in situ* orientation of the C-terminal lobe of TnC (CTnC) by polarized fluorescence from bifunctional rhodamine (BR) probes cross-linking pairs of cysteines at TnC residues 96-103, 116-123, 132-139, and 119-135. Each BR-labeled TnC was exchanged into single permeablized fibers from rabbit psoas muscle, and polarized fluorescence from the BR-TnCs was measured during relaxation and maximal calcium activation. The orientation distribution of CTnC with respect to the thin filament axis was calculated by maximum entropy analysis using the *in vitro* structure of CTnC in the troponin core complex (Vinogradova *et al.* (2005) *PNAS***102**:5038-5043). The peak angle between E helix of CTnC and the filament axis was 49° in relaxed muscle and 64° during maximal activation. Comparison with the results of our previous study of the orientation of the N-terminal lobe of TnC (Ferguson *et al.* (2003) *Mol. Cell***11**: 865-874) suggests that the central D/E helix of TnC is bent by about 30° in relaxed muscle and becomes straight during maximal activation.
Supported by MRC (UK).

771-Pos

The Perturbation of the Open-Closed Transition of Troponin C by the Mutation L48Q Leads to an Enhanced Troponin I Affinity

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Cardiac muscle contraction is regulated by Ca^{2+} binding to the N-domain of troponin C (cNTnC). Following Ca^{2+} association, the relocation of troponin I from actin to troponin C triggers contraction. In a diseased heart, there is a desensitization of the myocardium for Ca^{2+}, and one treatment strategy is to use pharmaceuticals that stabilize the open conformation of cNTnC, and thus enhance its interaction with troponin I ($cTnI_{147\text{-}163}$). Another option would be to engineer variants of troponin C that resemble the drug-induced open state of cNTnC. One possible mutant, L48Q, has been shown to increase thin filament Ca^{2+}-sensitivity. L48 is involved in forming crucial hydrophobic interactions with F20 and A23 in both the apo and Ca^{2+}-bound forms of cNTnC. The replacement of leucine with glutamine decreases the hydrophobicity in this region, and therefore may destabilize the closed state of cNTnC. We used nuclear magnetic resonance (NMR) to investigate how the L48Q mutation might increase thin filament Ca^{2+}-sensitivity. We found that the affinity of L48Q-cNTnC for $cTnI_{147\text{-}163}$ was enhanced by ~3 fold, with a $K_D \sim 50 \in \mu M$ (wt-cNTnC; $K_D \sim 150 \in \mu M$). We have developed a computational method to predict the tertiary structural changes in cNTnC by comparing the $^1H,^{15}N$ - HSQC spectra with control spectra from open and closed forms of cNTnC. The chemical shift patterns of residues in the defunct Ca^{2+}-binding site I of L48Q-cNTnC resemble the $cTnI_{147\text{-}163}$-bound form of wt-cNTnC, indicative of a more open state. We conclude that the L48Q mutation disrupts the hydrophobic packing of cNTnC such that it stabilizes a more open state of cNTnC, and it is this structural perturbation that is responsible for the enhanced affinity of L48Q-cNTnC for $cTnI_{147\text{-}163}$.

772-Pos

Effects of Cardiac TnC Variants on cTnC-cTnI Interaction;Solution and Molecular Dynamics Simulation Studies

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To better understand the complex protein interactions involved in cardiac muscle contractile activation we have developed a series of troponin C (cTnC) variants with increased or decreased Ca2+ binding affinity (in solution) that alter Ca2+ regulation of force development. We have previously reported that increasing or decreasing Ca2+ binding affinity by substitution of glutamine for leucine at residue 48 (L48Q cTnC) or isoleucine at residue 61 (I61Q) increased or decreased (respectively) Ca2+ sensitivity of steady state force in rat skinned

trabeculae. To assess cTnC-cTnI interactions that may underlie this functional effect, we have used both solution and modeling approaches. In solution, cTnC affinity for cTnI was assessed by spectrofluorimetry via labeling cTnC (C35S) with IANBD at Cys84. In both the absence and presence of Ca2+, C35S, L48Q cTnC had increased affinity for cTnI while C35S, I61Q cTnC (and other variants) had reduced affinity. To examine this in more detail we studied the molecular interactions between the (+/-Ca2+)-cTnC1-89-cTnI147-163 complex using molecular dynamics (MD) simulations. Multiple MD simulations (~ 100ns) of wild-type (WT) and the cTnC variants were performed to predict specific structural effects of each residue substitution. Results showed the 48 position of WT and the variant positions of cTnC had the most intermolecular contact pairs with cTnI(147-163). L48Q greatly increased time of contact with cTnI hydrophobic residues Ile148, Met153 and Leu157 compared with WT complex. At ~45ns simulation the B-helix of L48Q cTnC1-89 ''lifted", suggesting a move favorable ''opening up'' of the N-lobe hydrophobic patch compared with WT cTnC1-89. This could lead to stronger binding with the regulatory region of cTnI. Thus, our computational results provide novel details about specific structural alterations throughout L48Q cTnC and other cTnC variants. NIH-HL65497 (MR), AHA-09PRE2090056 (DW)

773-Pos

Structure of the Regulatory Domain of Human Cardiac Troponin C in Complex with the Switch Region of Cardiac Troponin I and the Drug W7: The Basis of W7 as an Inhibitor of Cardiac Muscle Contraction

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The solution structure of Ca^{2+}-bound regulatory domain of cardiac troponin C (cNTnC) in complex with the switch region of troponin I ($cTnI_{147-163}$) and the calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfinamide (W7), has been determined by NMR spectroscopy. The structure reveals that the W7 chloronaphthalene ring interacts with the terminal methyl groups of M47, M60, and M81 as well as aliphatic and aromatic side-chains of several other residues in the hydrophobic pocket of cNTnC, while the N-(6-aminohexyl) tail interacts with the C- and D-helices of cNTnC and with $cTnI_{147-163}$. Compared to the structure of the cNTnC•Ca^{2+}•W7 complex (Hoffman, R. M. B. and Sykes, B. D. (2009) *Biochemistry* 48, 5541-5552), the tail of W7 moves toward the surface of cNTnC, in close proximity to the N-terminus of $cTnI_{147-163}$. As a result, the N-terminus of the peptide clashes with the positively charged amino group of the W7 molecule and this repulsive interaction diminishes the helical content of $cTnI_{147-163}$ when compared to the structure of cNTnC•Ca^{2+}•$cTnI_{147-163}$ (Li, M. X., Spyracopoulos, L., and Sykes B. D. (1999) *Biochemistry* 38, 8289-8298). Thus the ternary structure cNTnC•Ca^{2+}•W7•$cTnI_{147-163}$ reported in this study provides a structural basis for the inhibitory effect of W7 in cardiac muscle contraction. The structure also offers an explanation for the ~10-fold affinity reduction of $cTnI_{147-163}$ for cNTnC•Ca^{2+} in the presence of W7. This result generates insights into the features that are useful for the design of cTnC-specific Ca^{2+}-desensitizing drugs.

774-Pos

A Second Look at the Two Phases of Ca^{2+} Binding to Fast Skinned Fibers

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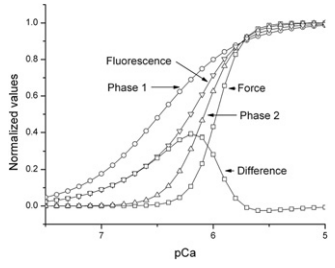
Experiments that replace native TnC with TnC_{danz} in skinned rabbit psoas muscle fibers generate pCa/Ca^{2+} binding curves that are left of and roughly parallel to the pCa/force curve (Guth & Potter, 1987). The fluorescence curve best fits two binding phases, #1 with a slope of about one and #2 with a slope of about 3 or more, a cooperative binding that is associated with major force development (Allen et al, 1992, Huang et al, 2001). Phase 1 is also accompanied with subtle increments in force. The force, fluorescence, phase 1 & 2 curves synthesized from mean fitted parameters for 22 experiments are shown. To demonstrate the difference between force and binding, we subtract the force from the fluorescence. The difference curve indicates that phase 1 Ca^{2+} binding dominates at high pCa then decreases as cooperative binding increases. We argue that the regulatory sites are shifting from normal to cooperative binding and this is why the phase 2 parameters can only approximate force. Because phase 2 binding begins on top of phase 1 the fluorescence curve is shifted left away from force.



775-Pos

Effect of D145E Mutation on Calcium Binding and Exchange with the C-Domain of Troponin C

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Recent discoveries of a number of hypertrophic cardiomyopathy related mutations in the C-terminal domain of cardiac troponin C suggest that sites III and IV might play a more important role than just anchoring troponin C into the troponin complex. We investigated the effects of hypertrophic cardiomyopathy related mutation D145E in human cardiac troponin C on calcium binding and exchange with the C-terminal domain sites III and IV. The calcium titration data indicated that the D145E substitution in the +z position of the calcium binding site IV dramatically decreased calcium binding affinity of that site (~1, 856-fold), and virtually eliminated magnesium binding to that site. Furthermore, the D145E substitution significantly decreased the calcium affinity of site III (~1.4-fold), correlating with ~1.6-fold faster rate of calcium dissociation from site III. Stopped-flow studies utilizing fluorescent calcium chelator Quin-2 demonstrated that the D145E mutation reduced the stoichiometry of moles of calcium per mole of the C-terminal domain by ~2-fold, both in the absence and presence of cardiac troponin I peptide (residues 34-71). Thus, binding of troponin I peptide to the C-terminal domain of D145E troponin C was not able to restore normal calcium binding to site IV. These results indicate the conservative D145E substitution has detrimental effects on calcium and magnesium binding to site IV of troponin C.

776-Pos

Effect Of Down-Regulation of a Stretch-Activated TnC Isoform on Flight of Drosophila

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Both *Drosophila* and *Lethocerus* have indirect flight muscle (IFM) that is activated by sinusoidal length changes at constant [Ca^{2+}]. IFM has two TnC isoforms. F1 binds a single Ca^{2+} in the C-lobe and is needed for the periodic stretch-activation of fibres to produce oscillatory work. F2 binds Ca^{2+} in both N- and C-lobes and is needed for producing Ca^{2+}-dependent isometric tension. We have obtained flies (from VDRC, Vienna) in which F1 is down-regulated by RNAi. Male flies of the F1 RNAi line were crossed with virgin female flies having the Dmef2 driver, which is expressed in all muscles, or a UH3 driver, which is expressed only in IFM. Crosses were maintained at 25°C and 29°C to get different levels of RNAi expression. The proportion of flies unable to fly was: wt 0%; Dmef2 87% at 25°C, 90% at 29°C; UH3 70% at 25°C, 100% at 29°C. There was no difference in time of development or viability of the different lines. Confocal microscopy of Dmef2 and UH3 flies showed myofibrils of both lines were narrower than wt; sarcomere length was normal, but Z-disc and M-line were not straight. Electron microscopy showed that sarcomere structure was disrupted more than expected. Troponin was regularly spaced at 38 nm along thin filaments, but thick and thin filaments were misaligned and Z-and M-lines shifted. Blots of IFM with anti-F1 and F2 showed F1was absent in Dmef2 flies, and greatly reduced in UH3 flies; F2 content of IFM was the same as wt. Therefore, F1 is essential for maintaining normal sarcomere structure of IFM, as well as for stretch-activation. Evidence for cross-linking between troponin components and thick filaments of *Lethocerus* IFM will be presented. Lack of F1 may affect these links.

777-Pos

The Effect of Glutathione on Skeletal Muscle Calcium Sensitivity and Myofilament Sulfhydryl Groups

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Glutathione, a critical reducing agent present in relatively high levels(~5mM) in skeletal muscle, can also attach to protein thiols via a disulfide bond. This process is referred to as glutathionylation, and is thought to be a protective mechanism to prevent irreversible protein oxidation. Prior studies have shown that when skinned fibers are exposed to reduced glutathione there is an increase in calcium sensitivity with no significant change in maximal force. These calcium sensitivity changes were largely reversible by the reducing agent DTT, indicating modification of protein thiols. We measured the force-pCa relationship of permeabilized rabbit psoas fibers treated with DTDP a thiol-specific oxidizing agent and glutathione (5mM). 2D gel electrophoresis using either IEF 4-6.5 or NEPHGE 3-10 in the first dimension was used to identify myofilament proteins whose sulfhydryl groups were modified with the oxidant-glutathione treatment. Additionally, phosphorylation of the regulatory myosin light chain was analyzed using 2d gels (IEF 4-6.5) and the phosphorylation specific stain Diamond Pro-Q. The pCa 50 of skinned psoas fibers was decreased upon exposure to DTT. Following DTT, the addition of DTDP and GSH sequentially,