probe cantilever, while the other probe was attached to a rapid displacement generator; the Ca2+ pulse was applied by rapid translation of a double-barreled perfusion pipette (de Tombe, AJP, 2007). Activations were performed at long (SL=2.06 ± 0.03 μm) and short (SL=1.85 ± 0.01; n=5) length. As expected, SL modulated maximum Ca2+ saturated force ~20%. Both the rate of force redevelopment following a rapid release-restretch maneuver (kTr; ~26%) and Ca2+ activated force development (kCa;~14%) were faster at the long SL. In contrast, SL did not modulate any parameters of force relaxation following rapid removal of activator Ca2+. Our data suggest that length dependent myofilament activation in the heart may be the result of differential modulation of activation dynamics in response to changes in sarcomere length.

1162-Pos Board B6

Tropomyosin: Long Range Perturbations In The Hydrophobic Interface

John P. Sumida, Sherwin S. Lehrer.
Boston Biomedical Research Institute, Watertown, MA, USA.

Skeletal tropomyosin, (Tm), is an α-helical coiled-coil which binds to actin, and with troponin, regulates muscle contraction. We previously demonstrated that a conserved Asp 137 in the hydrophobic interface produces a dynamic region in the middle of Tm, and that this region is involved in the myosin dependent activation of the thin filament at high Ca^{2+}, (Sumida et. al. JBC 283 2008). The current work characterizes a long-range interaction between positions 137 and 190. The thermodynamic properties of wild type (WT) Tm and two single mutants, C190A and D137L, are compared with those of the double mutant, D137L/C190A, using differential scanning calorimetry, (DSC), and circular dichroism, (CD). CD measurements show that Ala 190 increases the fraction of helix unfolding in the 40°C pre-transition, before the main transition. DSC measurements support this finding, indicating a large enthalpic pre-transition, (ΔH=150kcal/mol), for the C190A mutant relative to D137L, D137L/C190A, or WT, (average ΔH=20kcal/mol). Additionally, Ala 190 increases the ΔCp, (heat capacity), of Tm ~5 fold, reflecting an increase in solvent exposure of hydrophobic residues in the pre-transition during unfolding.

Since the D137L/C190A and D137L mutants do not exhibit the large enthalpic pre-transition observed for C190A, the Leu mutation at 137 must stabilize the alanine effect observed for C190A mutation 77Å away. This demonstrates how a locally dynamic region near 137 is able to produce global effects along the thin filament, and in this manner provide the proper regulation of the myosin dependent activation of the thin filament. This observation may also contribute to our understanding about the manner in which single point mutations significantly affect function in cardio-myopathies such as FHC and DCM.

1163-Pos Board B7

Localization of The Tropomyosin-Binding Sites in Troponin T And Functional Suppression of An Error Splicing of Its C-Terminal Variable Region

Stephen M. Chong, Hui Wang, J.-P. Jin.
Northwestern University, Evanston, IL, USA.

The interaction between troponin T (TnT) and tropomyosin (Tm) is pivotal in the Ca2+-regulation of muscle contraction. It has been known for three decades that TnT has two binding sites for Tm. The conserved middle and the C-terminal regions of TnT each contain a Tm-binding site and both sites are critical for the function of muscle thin filament. However, the precise locations of the Tm-binding sites have not been identified. By mAb competition assays, we located the middle region Tm-binding site of TnT in the beginning of the conserved sequence. Previous data showed that deletion of the C-terminal 14 amino acids in TnT did not reduce Tm-binding. Cardiac TnT with a longer deletion of the C-terminal 28 amino acids also retained the C-terminal Tm-binding site as shown by its dominant cardiomyopathy phenotype that indicates effective myofilament incorporation. In contrast, a truncation of slow TnT deleting the C-terminal 83 amino acids due to a nonsense mutation significantly lowered Tm-binding affinity and causes a recessive form of nemaline myopathy. Considering the known crystal structure of partial troponin, we further tested additional deletions to locate the C-terminal region Tm-binding site of TnT in the beginning of the T2 segment. Different from the dominant C-terminal truncation mutation of cardiac TnT, an error-splicing of the mutually exclusive exon 16 and exon 17 in fast skeletal muscle TnT significantly lowered Tm-binding affinity by deleting the C-terminal 28 amino acids and replacing it with a long non-sense peptide. The suppression of potentially harmful effects of the splicing error provides a mechanism to protect muscle function.

1164-Pos Board B8

Identification And Characterization Of Cardiac Troponin I From The Trout Heart

Kelly P. Kirkpatrick, Andrew S. Robertson, **Todd E. Gillis**.
University of Guelph, Guelph, ON, Canada.

Trout cardiac myofibrils are ~10-fold more sensitive to Ca^{+2} than those from mammalian hearts when measured at the same temperature. It has been demonstrated that trout cardiac troponin C (ScTnC) has 2.3 fold the Ca^{2+} affinity of human cTnC and is responsible for a 2-fold increase in cardiac myofibril Ca^{+2} sensitivity. The contributions of trout cardiac troponin I (ScTnI) to the Ca^{+2} sensitivity of the trout heart is currently unknown. The cDNA for ScTnI has been cloned using RACE-PCR. Sequencing results indicate that ScTnI is 59% and 56% identical to human cTnI and human skeletal troponin I, respectively, at the amino acid level. Interestingly, ScTnI lacks the ~30-residue N-terminal sequence present in mammalian cTnI that contains two protein kinase A (PKA) target residues at positions 23 and 24. ScTnI has been expressed and the influence of it on the Ca^{2+} activation of human cardiac troponin is currently being characterized using steady state Ca^{2+} binding assays and stopped flow kinetic analysis. This work is supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Foundation for Innovation (CFI) to TEG.

1165-Pos Board B9

PI3-Kinase Controls Smooth Muscle Contraction Via Regulation Of MLCP Activity

Hiroyasu Sakai, **Mitsuo Ikebe**.
Department of Physiology, University of Massachusetts Medical School, Worcester, MA, USA.

We demonstrated for the first time that PI3-kinase plays a role in the regulation of smooth muscle contraction by controlling MLCP. The inhibition of PI3-kinase markedly inhibited Ca2+-induced contraction and GTPγS induced Ca2+ sensitization of α-toxin permeabilized vascular smooth muscle as well as K+-induced contraction of intact vascular smooth muscle. The contractile inhibition was accompanied by the decrease in MLC phosphorylation and MBS phosphorylation at Thr696 and Thr853, which are responsible for the inhibition of MLCP activity. On the other hand, the inhibition of PI3-kinase had no effect on MLCK activity. These results suggest that PI3-kinase is involved in the regulation of MLCP, thus regulating MLC phosphorylation. An Akt specific inhibitor, SH-6, had no effect on the contraction, suggesting that Akt, one of the major down-stream effecter of the PI3-kinase pathway is not involved in this mechanism. MBS phosphorylation at Thr853, a Rho kinase specific site, was decreased by the inhibition of PI3-kinase even at rest, when Rho kinase is not activated. These results suggest that PI3-kinase does not influence the MBS kinases, such as Rho kinase. In fact, we found that the PI3-kinase inhibition activated MBS phosphatase activity. Furthermore, we found that PI3-kinase inhibition increased MBS phsophorylation at the PKG site, suggesting the activation of PKG pathway. Since the activation of the cGMP/PKG pathway decreases MLC phosphorylation by activating MBS phosphatase (Nakamura et al., 2007), our results suggest that PI3-kinase regulates smooth muscle contraction by modulating the PKG pathway.

1166-Pos Board B10

Crossbridge-mediated Activation of Rabbit Skeletal Muscle Myofibrillar ATPase: a Role for the Calcium Binding Domains of Troponin C

Franklin Fuchs, Zenon Grabarek.
Boston Biomedical Research Institute, Watertown, MA, USA.

Activation of the thin filament in striated muscle is a cooperative process requiring both the binding of Ca^{2+} to troponin C (TnC) and the binding of myosin crossbridges to actin. The aim of this study was to assess the role of TnC domains in the crossbridge-mediated activation of rabbit skeletal muscle myofibrils in the absence of Ca^{2+}. Activation of myofibrillar ATPase was produced by addition of varying concentrations of myosin S1 modified by N-ethylmaleimide (NEM-S1), which facilitates crossbridge cycling by forcing tropomyosin into the open position on the filament. Comparisons were made of native myofibrils, myofibrils from which TnC was extracted, and myofibrils reconstituted with either a TnC mutant (TnC_{48-82}) in which Ca^{2+} activation was blocked by a disulfide bond in the N-terminal domain (Grabarek, et al, Nature, 345:132,1990) or a proteolytic fragment of TnC (TR2C) lacking the N-terminal Ca^{2+}- binding domain (Grabarek, et al, J. Biol. Chem. 265:13121, 1981). The ATPase activity of native myofibrils was increased ~170% by the addition of NEM-S1 (2-4μM). Following extraction of TnC the addition of the same concentrations of NEM-S1 produced ~60% activation. In both cases higher concentrations of NEM-S1 produced no further increase in activation. With the addition of either TnC_{48-82} or TR2C the degree of activation was higher (70-100%), but required higher NEM-S1 concentrations (4-8μM). These results suggest that both domains of TnC play a role in facilitating optimal crossbridge-mediated activation of the thin filament, presumably by providing alternative binding sites for troponin I.

1167-Pos Board B11

Structurally Unstable Regions in the Tropomyosin-Troponin Complex from Bovine Heart Muscle

Zenon Grabarek.
Boston Biomedical Research Institute, Watertown, MA, USA.

Regulation of contraction in striated muscles requires a semi-independent movement of various domains of the tropomyosin-troponin (TmTn) complex

on the actin filament. Such movement is facilitated by the presence of flexible or structurally unstable regions in these proteins. In particular, it is well documented that the central part of tropomyosin comprises a region that unfolds readily at physiological temperatures. To identify flexible regions in the native TmTn complex isolated from bovine heart muscle, we have used limited proteolysis with Staphylococcus aureus V8 protease that cleaves specifically the peptide bond on the C-terminal side of Glu. At 4°C only troponin T and troponin I are digested at positions 59 and 166, respectively. At 35°C Tm is also digested, initially at position 145, followed by a rapid digestion at positions 142,131 and 150. The resulting two fragments of Tm spanning residues 1-131 (2x14.9 kDa) and 151-284 (2x15.5 kDa) are resistant to further digestion in the presence of troponin, which indicates that they retain their coiled-coil structure. However, in the absence of Tn the C-terminal fragment of Tm undergoes further proteolysis. The troponin C component of the complex is resistant to proteolysis under the conditions used. The two Tm fragments, together with TnC, the N-terminal fragment of TnI (residues 1-166) and the C-terminal fragment of TnT (residues 60-284) form a 125 kDa complex that is stable at low salt concentrations, binds to F-actin irrespective of calcium, and imparts significant calcium-dependent regulation to actomyosin ATPase. These results demonstrate that the central segment of Tm spanning residues 131-151 is structurally unstable irrespective of the presence of troponin and it structure stabilizing effect on the C-terminal part of Tm.

1168-Pos Board B12

Human Cardiac Troponin Cross-Linking and the Functional Consequences

Chad M. Warren, Tomoyoshi Kobayashi, R. John Solaro.
University of Illinois at Chicago, Chicago, IL, USA.

Previous structural studies suggested that the cardiac unique N-terminal extension of troponin I interacts with the basic regions near the inhibitory peptide (Howarth et al. (2007) *J Mol Biol* 373, 706-722). To determine possible interactions, we cross-linked single Cys mutant TnI at either position 5 or 19 in the troponin complex with the hetero-bifunctional cross-linker benzophenone-4-maleimide. Data from SDS-PAGE and reversed phase HPLC indicated intramolecular and inter-molecular cross-linking with troponin C and T. Digested peptides separated by reverse phase capillary HPLC were analyzed by Edman sequencing and MALDI-TOF to determine the cross-linked peptides. Moreover, using tandem mass spectrometry, specific sites of interaction were determined intra-molecularly at Met-154 and Met-155 of TnI and inter-molecularly at Met-47 and Met-80 of the TnC. In addition, we measured calcium dependent ATPase rate of reconstituted thin filament-myosin-S1 preparation regulated by either non-labeled or cross-linked troponin. The ATPase rate indicated calcium regulation was lost when the position 5 TnI mutant was cross-linked in the absence of calcium, but was only blunted with position 19 cross-linking. The specific inter-molecular cross-linked sites did not differ between TnI and TnC. However, the intra-molecular cross-linked sites in the two mutants interacted with different sides of the TnI switch peptide, which may allosterically modulate the affinity of calcium to TnC. These data provide novel evidence that the thin filament function may be modified by the N-terminus of TnI interacting intra-molecularly, and inter-molecularly.

1169-Pos Board B13

Troponin I Ser-150 pseudo-phosphorylation alters cardiac contractile mechanics

Brandon John Biesiadecki, Ariyaporn Thawornkaiwong, Janel Jin, R. John Solaro.
University of Illinois at Chicago, Chicago, IL, USA.

The cardiac troponin I (cTnI) subunit of the troponin complex is central in the calcium regulation of cardiac muscle contraction. The functional effects of beta-adrenergic induced cTnI phosphorylation at Ser-23/24 are well established, yet other cTnI residues can also be phosphorylated with unknown effects. Cardiac troponin I is phosphorylated by p21-activated kinase and AMP-activated Protein kinase at Ser-150. To determine the effects of cTnI Ser-150 phosphorylation on calcium regulated steady state force development we exchanged skinned cardiac fiber bundles with either wild type or troponin containing cTnI pseudo-phosphorylated at Ser-150 (S150D). Force vs. calcium measurements demonstrate cTnI S150D significantly increases myofilament calcium sensitivity (pCa_{50} WT=5.90±0.01; S150D=6.12±0.01; $p<0.001$) in the absence of an effect on maximal force, Hill coefficient or length dependent activation. Troponin structure and biochemical data indicate the cTnI Ser-150 region is localized in close proximity to the cTnI N-terminal region containing the Ser-23/24 phosphorylation sites. To investigate if Ser-150 phosphorylation alters the effect of phosphorylation at Ser-23/24 we measured force development in fibers exchanged with troponin containing cTnI pseudo-phosphorylated at Ser-23/24 (S23/24D) or cTnI pseudo-phosphorylated at Ser-23/24/150 (S23/24/150D). Results demonstrate cTnI S150D similarly increased myofilament calcium sensitivity independent of the Ser-23/24 phosphorylation state (pCa_{50} S23/24D=5.67±0.03; S23/24/150D=5.85±0.03; $p<0.001$) resulting in a S23/24/150D calcium sensitivity similar to wild type. This occurs in the absence of an effect on maximal force, Hill coefficient or length dependent activation. Biochemical investigations into the mechanism of cTnI Ser-150 phosphorylation to effect force development are in progress. These data suggest that the increased calcium sensitivity resulting from cTnI phosphorylation at Ser-150 effectively eliminates the desensitizing effect of beta-adrenergic induced cTnI phosphorylation at Ser-23/24 such that in the presence of Ser-23/24/150 phosphorylation myofilament calcium sensitivity is similar to that of un-phosphorylated cTnI.

1170-Pos Board B14

Contractile Response to Endothelin in Myocytes Expressing Troponin I Ser43/45 Substitution Mutants

Dustin Robinson, **Margaret Westfall**.
University of Michigan, Ann Arbor, MI, USA.

Activation of protein kinase C (PKC) by the neurohormone, endothelin increases peak shortening and accelerates relaxation in adult rat cardiac myocytes. Endothelin-activated PKC also phosphorylates cardiac troponin I (cTnI), and our laboratory previously demonstrated a temporal role for the cTnIThr144 and cTnISer23/24 phosphorylation sites in the accelerated relaxation response to endothelin. The goal of the present study is to determine whether cTnISer43/45, a third potential target for PKC-mediated phosphorylation, plays a significant role in the contractile response to endothelin. Contractile function in adult rat cardiac myocytes expressing either cardiac troponin I with Ser43/45Ala (cTnIS43/45A) or Ser43/45Asp (cTnIS43/45D) substitutions was measured 4 days after gene transfer. Western analysis demonstrated 85-90% replacement of endogenous cTnI with cTnIFLAG, cTnIS43/45AFLAG, or cTnIS43/45DFLAG by 4 days post-gene transfer. The amplitude of sarcomere shortening increased 13.2+4.6% and the return velocity, a measure of relaxation rate increased 14.4+6.3% (n=14) in response to 10 nM endothelin over 15 min. In myocytes expressing cTnIS43/45A or cTnIS43/45D, the relaxation response to endothelin was similar to myocytes expressing cTnI or cTnI-FLAG over the same time interval. The substitution mutant, cTnISer43/45Ala/Thr144Pro (cTnIA2P) was then studied to further investigate the role of individual cTnI phosphorylation sites in the endothelin response. Gene transfer of cTnIA2P produced significant cTnI replacement in preliminary studies of adult myocytes. However, in contrast to cTnIS43/45A or cTnIS43/45D, the accelerated relaxation response to 15 min of 10 nM endothelin was reduced in myocytes expressing cTnIA2P compared to cTnI-expressing myocytes (-2.3+ 4.7%, n=3). These preliminary results suggest Thr144, but not Ser43/45 plays a role in accelerating relaxation rate during the initial response to PKC activation by endothelin.

1171-Pos Board B15

Functional Impact of TnT Mutations Responsible for Hypertrophic Cardiomyopathy on Tn-Exchanged Single Myofibrils

Fumiko Matsumoto[1], Nicoletta Piroddi[2], Alexandra Belus[2], Beatrice Scellini[2], Kayo Maeda[3], Chiara Tesi[2], Yuichiro Maeda[3,4], Satoru Fujiwara[1], Corrado Poggesi[2].
[1]Japan Atomic Energy Agency 3191195 Tokai, Japan, [2]Università di Firenze 50134 Firenze, Italy, [3]ERATO Actin Filament Dynamics Project, SPring-8 Center RIKEN 6795148 Sayo-cho, Japan, [4]Nagoya University 4648601 Nagoya, Japan.

Hypertrophic Cardiomyopathy (HCM) is an autosomal dominant cardiac disease resulting from mutations in genes encoding contractile proteins, including troponin (Tn; the complex of TnT, TnI and TnC). From measurements of ATPase activity of myofibrils, into which TnT mutants were exchanged, we showed that two HCM-associated mutations of TnT, E244D and K247R, increase the maximum ATPase activity without any change in Ca^{2+} sensitivity (Matsumoto et al., BSJ 2007, 1P150). It is, however, still not clear if the mutations have direct effects on the mechanical properties of muscle because ATPase activity and tension development are not necessarily coupled. We thus examined the effects of the mutations on maximal isometric tension and kinetics of Tn-exchanged myofibrils. Measurements were made in single myofibrils activated and relaxed by a fast solution switching device, with a dead time < 10ms (Tesi et al., Biophys. J., 2002, 83, 2142). Maximal isometric tension values of the mutant TnT exchanged myofibrils were significantly higher (E244D) or tended to be higher (K247R) than those of the wild type TnT exchanged myofibrils while Ca^{2+} sensitivity was unchanged. These results indicate that the effects of the mutations on myofibril mechanics are directly coupled with the effects on the ATPase activity. Measurements of the rate constants of force generation showed that the mutant TnT exchanged myofibrils had similar kinetics as the wild type TnT exchanged myofibrils. The result suggests that kinetics of cross bridge transitions are not influenced by these mutants.