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.

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Human Cardiac Troponin Cross-Linking and the Functional Consequences

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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.

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

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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.

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

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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.

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

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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.