1172-Pos Board B16

Effects of Removal and Reconstitution of Troponin and Tropomyosin on Ca^{2+}-Activated Tension Generation in Cardiac Myofibrils

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Tropomyosin is an essential component in the regulation of muscle contraction. A method has been described to remove endogenous tropomyosin (Tm) and troponin (Tn) and replace them with exogenous proteins into rabbit psoas myofibrils (Siththanandan et al., *Biophys. J.*, 2007, suppl. 626a). In the present study the endogenous Tm and Tn are replaced into cardiac mouse myofibrils by a modification of that method. Endogenous Tm and Tn were extracted by washing cardiac mouse myofibrils in a low ionic strength solution at pH 8.0. The wash was repeated several times to increase the amount of extracted Tm. The reconstitution of thin filament regulatory proteins was made in a rigor solution in two steps, first adding Tm and then Tn. To measure the effects of this procedure, samples of myofibrils were analyzed at different stages of the protocol (control, extracted, reconstituted) both functionally (by loss and regain of Ca^{2+} -dependent regulation) and by SDS-PAGE. The success of the exchange was determined by mechanical measurements of calcium dependent force activation on the reconstituted myofibrils. Small bundles of cardiac myofibrils were mounted in a force recording apparatus and activated by rapidly switching between low and high Ca^{2+} solutions (Tesi et al., *Biophys. J.*, 2002, 83, 2142-2151). Maximal isometric tension was 30-35% lower in the reconstituted myofibrils than in control myofibrils but the rate of force activation (k_{ACT}) and that of force redevelopment (k_{TR}) was the same in the two myofibril groups. The effectiveness of Tm replacement in human cardiac myofibrils is under investigation. This approach can be used to test the functional impact of Tm mutations responsible for human cardiomyopathies. Supported by Telethon-Italy GGP07133.

1173-Pos Board B17

Length-Tension Relationships Are Similar Between Cardiac Myocytes And Skeletal Muscle Fibers

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According to the Frank-Starling relationship increased ventricular volume increases ventricular output, which helps match peripheral energy supply with demand. The cellular basis for this relationship is, in large part, the myofilament length-tension relationship. The length-tension relationship in maximally activated skinned preparations is relatively shallow and similar between cardiac myocytes and skeletal muscle fibers. During twitch activations the length tension relationship becomes steeper in both cardiac and skeletal muscle; however, it remains unclear whether length dependence of tension differs between striated muscle cell types during submaximal activations. The purpose of this study was to compare myofilament length-tension relationships between cardiac myocytes, fast-twitch and slow twitch skeletal muscle fibers during half-maximal calcium activations. For these experiments, passive and active sarcomere length-tension relationships were characterized in skinned rat left ventricular cardiac myocytes, fast-twitch and slow-twitch skeletal muscle fibers. For active length-tension relationships, cells were mounted between a force transducer and motor and activated to yield 50-65% maximal force after which isometric force was measured over a range of ascending limb sarcomere lengths monitored by IonOptix SarLen system. Passive tension was considerably greater in cardiac myocytes (3.5 ± 2.5 kN/m^2 at 2.30 ± 0.04μm) compared to both fast-twitch (1.1 ± 0.8 kN/m^2 at 2.70 ± 0.07μm) and slow-twitch (1.4 ± 0.5 kN/m^2 at 2.70 ± 0.07μm) muscle fibers. Active myofilament length-tension relationships were considerably shallower in slow-twitch fibers compared to fast-twitch fibers. Interestingly, cardiac myocytes exhibited two distinct populations of length-tension relationships, one nearly identical to fast-twitch fibers and the other similar to slow-twitch fibers. These results indicate that cardiac myocytes exhibit length-tension relationships very similar to skeletal muscle fibers. The finding of two populations of cardiac myocyte length-tension relationships suggests a mode for adjusting Frank-Starling relationships following acute and/or chronic ventricular adaptations.

1174-Pos Board B18

Effect of Varying Mutant Troponin C Content on Contractile Properties of Striated Muscle

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Genetic therapies show much potential as novel, long-term treatments for skeletal and cardiac muscle diseases. Troponin C (TnC), a subunit of troponin, is a particularly promising target for genetic therapies, as it is involved in thin filament activation, Ca^{2+} signaling and Ca^{2+} handling. Specifically, expression of engineered TnC mutants with altered Ca^{2+} binding properties has the potential to restore normal contractile function in diseased/damaged muscle with abnormal Ca^{2+} properties. We are, therefore, studying skeletal TnC (sTnC) and cardiac TnC (cTnC) mutants that we, and others, have demonstrated affect Ca^{2+} signaling: F27W, M80Q sTnC and I60Q sTnC, and cTnC L48Q and I61Q. Since various amounts of mutant proteins are incorporated into the myofilaments following viral vector administration of mutant TnC cDNA, the goal of this study was to determine the minimal amount of protein expression necessary to alter contractility in striated muscle. In preliminary studies, different ratios of F27W, M80Q to WT sTnC (100:1, N=4; 50:50, N=4; 25:75, N=4; 1:100, N=6; C_0=1mg/mL for all TnCs) were reconstituted into demembranated skeletal muscle fibers. Following reconstitution, the maximal Ca^{2+} activated force (F_{max}) was $90 \pm 0.02\%$ (WT), $83 \pm 0.03\%$ (100:1), $92 \pm 0.04\%$ (50:50) and $90 \pm 0.01\%$ (25:75) of pre-extracted F_{max} (216 ± 15 mN/mm^2). Ca^{2+} sensitivity was significantly enhanced for all ratios (100:1, $pCa_{50}=6.17 \pm 0.02$, $p<0.001$; 50:50, $pCa_{50}=5.90 \pm 0.03$, $p<0.05$; 25:75, $pCa_{50}=6.01 \pm 0.01$, $p<0.01$) as compared to WT ($pCa_{50}=5.76 \pm 0.04$). These preliminary data indicate that <25% of the mutant protein is required to alter contractility without changing the maximal Ca^{2+} activated force. Continuing work will identify the minimal level of mutant TnC incorporation in striated muscle that significantly alters pCa_{50} and the rate force develops.

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Local Detection Of Intracellular Reactive Oxygen Species In Single Intact Contracting Skeletal Muscle Fibers

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Skeletal muscles produce low levels of reactive oxygen species (ROS) under resting conditions while during contractile activity the rate of ROS production increases. Low levels of ROS production may act to stimulate adaptive responses in skeletal muscle, while increased ROS dependent oxidation at sarcoplasmic reticulum, myofilaments and other EC coupling components likely lead to decrements in contractile function. Multiple sites exist for ROS production, including mitochondria and NADPH oxidase; however, the contribution of each of these and the factors that regulate the increased production of ROS during contractile activity remains to be determined. Here we use repetitive field stimulation of single FDB myofibers as a model of ROS secondary to repetitive activity. In FDB's loaded with the cytosolic, non-specific ROS probe DCFH, we have imposed intermittent (0.5 Hz) trains (150msec, 0.5msec sq. pulse @, 50Hz) of tetanic stimulation to establish a reliable *in vitro* model for activity dependent ROS production. In recent studies with this model, we have begun to explore site dependent generation of ROS with a redox sensitive variant of green fluorescent protein (roGFP) that is targeted to the mitochondria (mito-roGFP). Following cDNA electroporation *in vivo,* expression of mito-roGFP, and FDB isolation, we report evidence of the fidelity and specificity of this probe in mitochondria and the activity dependent mitochondrial redox status during our stimulation paradigm.

1176-Pos Board B20

Comparison of W7 binding to the Regulatory N-domain of Human Cardiac Troponin C in the absence and presence of the Switch Region of Human Cardiac Troponin I

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cTnC-cTnI interaction plays a critical role in transmitting the Ca^{2+}-signal to the other myofilament proteins in heart muscle contraction. As such, the cTnC-cTnI interface constitutes a logical target for cardiotonic drugs that can modulate the Ca^{2+}-sensitivity in cardiomyocytes. We have shown that W7, a calmodulin antagonist, binds specifically to the N-domain of cTnC, and this binding can occur concurrently with the switch region of cTnI. Moreover, we have shown that that the affinity of W7 for cNTnC•Ca^{2+} in the cTnC•3Ca^{2+}•$cTnI_{34-71}$•$cTnI_{128-163}$ complex is ~2-fold weaker than for the isolated cNTnC•Ca^{2+}. This suggests that W7 plays role in inhibiting the cTnC-cTnI interaction in the activation of muscle contraction. In this study, we determined the dissociation constants for W7 binding to cNTnC•Ca^{2+}in complex with two versions of the switch region of cTnI ($cTnI_{144-163}$ and $cTnI_{147-163}$). The results showed that the N-terminal three residue extension in $cTnI_{144-163}$ over $cTnI_{147-163}$ decreases the W7 affinity by ~3.5-fold ($K_D = 0.14 \pm 0.04$ mM for W7 binding cNTnC•Ca^{2+}•$cTnI_{147-163}$ and $K_D = 0.52 \pm 0.16$ mM for W7 binding cNTnC•Ca^{2+}•$cTnI_{144-163}$). This observation suggests competitive binding between W7 and the N-terminus of the switch region of cTnI to the cNTnC•Ca^{2+}. We have determined the NMR structures of cNTnC•Ca^{2+}•W7 and cNTnC•Ca^{2+}•$cTnI_{147-163}$•W7 complexes. This pair of structures provides a detailed understanding of the mechanism underlying the mode of action of W7 on the cTnC-cTnI interface and generates structural insights into the features that are important for the design of cardiotonic drugs.