differential PKCdelta targeting to multiple myofibrillar proteins other than TnI. Further elucidation of alternative PKCdelta signaling to the myofilments is ongoing. Our results suggest Src-dependent phosphorylation of PKCdelta constitutes an alternative mechanism that allows for stimulus-specific PKCdelta ezymology and myofibrillar targeting within the myocardium.

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Protein Kinase A-based Modulation Of Ca^{2+} Sensitivity In Skinned Skeletal Muscle Fibers Reconstituted With Cardiac Troponin

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It is well known that protein kinase A (PKA) decreases Ca^{2+} sensitivity in cardiac muscle via phosphorylation of troponin I (TnI). In the present study, we directly tested whether PKA-based phosphorylation of cardiac TnI universally modulates Ca^{2+} sensitivity regardless of the type of muscle, by taking advantage of our Tn exchange technique (Terui et al., J Gen. Physiol. 131;275-283:2008). Troponins were extracted from porcine ventricular and rabbit fast skeletal muscles (Ca^{2+} sensitivity: former < latter). Without Tn exchange, PKA decreased Ca^{2+} sensitivity in cardiac (porcine ventricular) muscle, associated with enhanced phosphorylation of TnI. Reconstitution of cardiac muscle with the skeletal Tn complex (sTn) not only increased Ca^{2+} sensitivity but abolished the PKA effect, suggesting that phosphorylation of TnI, but not of myosin-binding protein C, is primarily responsible for the PKA-based reduction in Ca^{2+} sensitivity. Reconstitution of rabbit psoas muscle with the cardiac Tn complex (cTn) decreased Ca^{2+} sensitivity, as previously reported by us (Terui et al., J Gen. Physiol. 131;275-283:2008). PKA decreased Ca^{2+} sensitivity in cTn-reconstituted skeletal muscle, and subsequent exchange for sTn restored Ca^{2+} sensitivity to the original level. A similar result was obtained when skeletal muscle was reconstituted with the hybrid Tn complex (i.e., cTnI-cTnC-sTnT), suggesting that the troponin I-C complex, but not TnT, is essential for PKA-based modulation of Ca^{2+} sensitivity. These findings support the notion that PKA-based phosphorylation of TnI universally modulates Ca^{2+} sensitivity regardless of the type of muscle.

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Cardiac Troponin I Threonine 144 phosphorylation: impact on myofilament function

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Threonine 144 (T144) in the inhibitory region of cardiac troponin I (cTnI) is an important site for PKC mediated phosphorylation in the heart. In addition, presence of this residue is sufficient to impart length dependent activation (LDA) properties onto the cardiac sarcomere (Tachampa Circ. Res., 2008). Here we studied the functional impact of a charge mutation so as to mimic phosphorylation of this residue (T144E). Wild-type (WT) or cTnI-T144E containing recombinant troponin (cTn) complexes were exchanged for endogenous cTn in skinned rat cardiac trabeculae. Force and ATPase activity were measured as function of [Ca2+] at short (2.0 μm) or long (2.2 μm) sarcomere length. T144E induced decreased maximum force development (Fmax) and Calcium sensitivity (EC50), increased cross-bridge cycling rate (tension cost) but, in contrast, did not affect LDA (ΔEC50). We conclude that T144E affects cross-bridge cycling and recruitment independent of sarcomere length.

	WT cTn	T144E cTn
EC50 2.0 (μM)	4.0 ± 0.3	5.9 ± 0.4
EC50 2.2 (μM)	3.5 ± 0.2	5.2 ± 0.3
Fmax 2.0 (mN/mm2)	25.1 ± 4.7	13.0 ± 1.7
Fmax 2.2 (mN/mm2)	37.2 ± 4.7	24.5 ± 2.7
Tension cost 2.0	7.7 ± 0.5	12.0 ± 1.3
Tension cost 2.2	6.3 ± 0.5	9.4 ± 1.2
ΔEC50	0.6 ± 0.1	0.7 ± 0.2

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Effects of Cardiac Troponin C Mutants on TnC-TnI interaction and its modulation by PKA phosphorylation

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We are considering several cardiac TnC mutants as potential therapeutic strategies for cardiomyopathies. To judge their potential to improve *in situ* function it is important to understand how these mutants affect TnC-TnI interaction and its modulation by PKA-mediated phosphorylation of TnI Ser 23, Ser 24. In this study, we are characterizing two cardiac TnC mutations, Leu48Glu (L48Q) and Ile61Glu (I61Q), with increased and decreased (respectively) Ca^{2+} binding affinity. In previous studies we showed these mutations resulted in increased (L48Q) and decreased (I61Q) Ca^{2+} sensitivity of steady state force in skinned rat trabeculae. To determine if these changes in Ca^{2+} sensitivity were due to altered TnC-TnI interactions we generated a structural marker by attaching IANBD to Cys84 in the N-lobe of cTnC. Half-maximal IANBD fluorescence saturation of Ca^{2+} binding occurred at pCa7.42 for L48Q cTnC, 7.38 for wild-type (WT) cTnC and 7.30 for I61Q cTnC. In both the absence and presence of saturating Ca^{2+} (0.6 μM TnC) IANBD fluorescence increased with increasing TnI and saturated at different [TnI] in the order L48Q, WT, I61Q. Fluorescence half-maximal saturation occurred at 0.26μM(saturating Ca^{2+}) and 0.25 μM (no Ca^{2+})TnI for L48Q cTnC, 0.78 μM and 0.49μM for WT cTnC , and 1.45 μM and 0.69 μM I61Q cTnC according to the exponential function fit of the data. However, preliminary experiments suggest that when PKA phosphorylated [cTnI] was titrated to cTnC, in both the absence and presence of saturating Ca^{2+}, IANBD fluorescence enhancement with L48Q may be impaired. The data thus far suggest that single amino acid mutations that alter Ca^{2+} binding affinity of TnC can influence interaction with TnI and its modulation by PKA mediated phosphorylation of Ser 23, Ser 24. Supported by HL65497 to MR.

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Analysis of Cardiac Myofibrillar Troponin I Phosphorylation in Normal and Failing Human Hearts Using Phos-Tags

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Recently, we have used phosphate-affinity SDS-PAGE gels containing Phos-tag-acrylamide (a phosphate-chelating molecule), to determine the level of cardiac troponin I (cTnI) phosphorylation in human myofibrillar extracts. The Phos-tag moiety binds to, and retards, the mobility of phosphoproteins through the gel and results in the separation of the phosphoprotein bands according to their phosphorylation level.

Samples from time-courses of in-vitro PKA catalytic subunit-treated recombinant human cTnI and myofibrillar extracts from non-failing donor, hypertrophic obstructive cardiomyopathy (HOCM) and end-stage failing human heart tissue were analysed by phosphate-affinity SDS-PAGE. Separate gel bands corresponding to 1P, 2P, 3P, 4P and 5P cTnI were observed for the PKA-treated recombinant cTnI. Western blotting probed with the anti-cTnI antibody 14G5 and several different site specific phospho-cTnI antibodies demonstrated that all five of these phospho-species bound to a Ser24P-specific antibody, while a Thr144P-specific antibody only reacted with the 3P, 4P and 5P phospho-species of cTnI.

We observed 3 phospho-species of cTnI in the human heart tissue extracts, which correspond to 0P, 1P and 2P cTnI. Ratios of 0P cTnI were significantly higher in failing and HOCM (both 63 ± 4%) compared to donor (8 ± 2%) while ratios of 2P were significantly lower (failing = 6 ± 2%, HOCM = 8 ± 2%, donor 73 ± 6%). Western blots demonstrated that in human heart cTnI phosphorylation of Ser23/24 was mainly present in the 2P species (with a very small proportion in the 1P species) and that there was no phosphorylation at Thr144. Calculated levels of total cTnI phosphorylation in both HOCM (0.37 ± 0.03, n=50, $p<0.0001$) and failing heart (0.38 ± 0.03, n=24, $p<0.0001$) were significantly reduced from levels in donor heart (1.65 ± 0.04, n=38) and were comparable to previously determined measurements obtained from Pro-Q Diamond phosphoprotein gel staining.

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Effect of Troponin I Ser23/24 Bis-Phosphorylation on Ca^{2+}-Sensitivity is Dependent on PKA Phosphorylation of Other Contractile Proteins

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Upon β-adrenergic stimulation, protein kinase A (PKA) enhances cardiac Troponin I (cTnI) phosphorylation at ser23/24. PKA treatment leads to a decrease in myofilament Ca^{2+}-sensitivity. However, the specific effect of PKA-mediated phosphorylation of cTnI in human myocardium is unclear since PKA phosphorylates a broader set of contractile proteins, in particular myosin binding protein C (cMyBP-C).