contractility through modulating molecular conformation and function of the core structure of cTnl.

1997-Plat
Analysis of Tropomyosin (Tm) Isoforms in Heart Muscle by LC/MS and Western Blotting Demonstrates Previously Uncharacterised High-Level Expression of γ-Tm

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Heart muscle has been considered to contain predominantly α-Tm (Tm1 gene) however this has not been investigated with tools that can clearly distinguish all tropomyosin isoforms. We initially used LC/MS to characterise the isoforms present in Tm purified from human heart muscle. Mass spectroscopy of Tm purified from human donor heart showed two dominant species, a 32754 Da peak, corresponding to acetylated α-Tm and a 32867 Da peak. The second peak was putatively identified by its mass as γ-Tm. These identities were confirmed by comparison with pure recombinant α and γ-skeletal tropomyosin expressed in the baculovirus/sf9 system (supplied by Dr Kristen Nowak, University Western Australia). The novel Tm is slow-skeletal Tm from the TPM3 gene, also known as plicated by Dr Kristen Nowak, University Western Australia). The novel Tm is isoform-specific antibodies. Using the pure recombinant Tm’s, we demonstrated urea SDS-PAGE, which can separate the two species, and the use of Tm levels.

Western blotting with these specific antibodies confirmed significant quantities heart muscle and in myectomy samples showed significantly reduced α-Tm (Tm1 gene). However this has not been investigated with tools that can clearly distinguish all tropomyosin isoforms. We initially used LC/MS to characterise the isoforms present in Tm purified from human heart muscle. Mass spectroscopy of Tm purified from human donor heart showed two dominant species, a 32754 Da peak, corresponding to acetylated α-Tm and a 32867 Da peak. The second peak was putatively identified by its mass as γ-Tm. These identities were confirmed by comparison with pure recombinant α and γ-skeletal tropomyosin expressed in the baculovirus/sf9 system (supplied by Dr Kristen Nowak, University Western Australia). The novel Tm is slow-skeletal Tm from the TPM3 gene, also known as γ-skeletal tropomyosin, (theoretical mass 32859.0 Da). Only very low levels (<5%) of α-Tm or of phosphorylated Tm were detected. Analysis of samples from failing heart muscle and in myocarditis samples showed significantly reduced γ-Tm levels.

The identity of the isoforms characterised in human heart was confirmed by urea SDS-PAGE, which can separate the two species, and the use of isoform-specific antibodies. Using the pure recombinant Tms, we demonstrated that monoclonal antibody CG3 was specific for the γ isoform, CH291 was specific for the α isoform and CH1 did not distinguish between the two isoforms. Western blotting with these specific antibodies confirmed significant quantities of γ-Tm and α-Tm in both human and mouse hearts. In mice, the level of γ-Tm depends on the strain, being around 50% in many strains, but low in FVB/N mice. This discovery is functionally significant as in vitro motility assays using pure γ-Tm and α-Tm shows significant differences between the isoforms.

1998-Plat
Protein Kinase Cα Mediated Phosphorylation of Cardiac Troponin Reduces Maximal Force and Exerts Dual Effects on Ca2+-Sensitivity in Human Cardiomyocytes
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Alpha-adrenergic receptor activated protein kinase Cα (PKCα) modifies cardiac contractile function and is implicated in heart failure. Aim of the study: To determine the specific effects of PKCα-mediated phosphorylation of cardiac troponin (cTn) on myofilament function in failing cardiomyocytes. Methods: Endogenous cTn in Triton-permeabilized cardiomyocytes from patients with end-stage idiopathic dilated cardiomyopathy was partially exchanged (~69%) with PKCα-treated recombinant human cTn(TD), in which the PKA-sites Ser23/24 on cTn were mutated into aspartic acids (D), cTn(TDD) was used to rule out in-vitro cross-phosphorylation of the PKA-sites by PKCα. A comparison was made between the effects of PKCα-treated and untreated cTn(TD). Isometric force was measured at various [Ca2+]i to determine the maximum isometric force per cross-sectional area (Fmax) and the calcium sensitivity of force (pCa50).

Results: Fmax after exchange with PKCα-treated cTn(TDD) (17.1 ± 1.9 kN/m2) was significantly reduced in comparison with untreated cTn(TD) (26.1 ± 1.9 kN/m2). Subsequent incubation of the cardiomyocytes with activated PKCα in the PKCα-treated cTn(TDD) group did not restore Fmax. Cardiomyocytes exchanged with PKCα-treated cTn(TD) were more sensitive to Ca2+ (pCa50=5.59 ± 0.02) than cells exchanged with untreated cTn(TDD) (pCa50=5.51 ± 0.02). Surprisingly, subsequent PKCα- incubation of cTn(DD) caused a reduction in pCa50 to 5.45 ± 0.02.

Western blot analysis of PKCα-treated cTn showed phosphorylation of Ser42 and Thr143 on cTn. Mass spectrometry revealed two novel phosphorylation sites: Ser198 on cTn and Ser179 on cTn. Conclusions: Specific in vitro PKCα-mediated phosphorylation of cTn increases Ca2+-sensitivity and decreases maximal force. Subsequent PKCα-mediated phosphorylation of the myofilaments in situ decreases Ca2+-sensitivity, without affecting Fmax. These results demonstrate the dual, site-specific effects of PKCα-mediated phosphorylation in human myocardium.

Platform AV: Protein Conformation

1999-Plat
Mapping Foldome Changes with Cysteine Labeling Kinetics Measured by Mass Spectrometry
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Protein unfolding, disassembly, and aggregation underlie many diseases, but detailed study of these processes in intact cells has been limited. Cysteine Shotgun labeling utilizes cell-permeable fluorescent dyes to label exposed cysteine residues and was initially applied to study protein structure changes in response to mechanical stress on cells. We have re-purposed the technique to identify protein changes in whole-cell lysates in native versus urea-denaturing conditions and in live cells all as a function of time and temperature (20-45 deg-C). Labeling rate constants are calculated for any given Cys site by normalizing the protein labeling kinetics to the rapid labeling under denaturing conditions. Proteins can be identified and further analyzed by mass spectrometry to pinpoint specific, susceptible domains involved. A number of proteins contain cysts with a wide variety of rate constants. These proteins include structural proteins such as Filamin A and B, Talin 1, Myosin 9 and alpha-actinin-4, heat shock protein 1 and elongation factor 2. Some of these various proteins contain many cysteine-rich domains and thus appear especially amenable to studying by this new in-cell technique.

2000-Plat
Investigating the Folded State of Type III Fibronectin Domains in Stretched Fibrils
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Fibronectin is an extracellular matrix protein that is assembled by fibroblasts into fibrils. Previous studies have shown that fibronectin fibrils are extremely elastic: cells can stretch them up to four times their resting length. The mechanism of this elasticity has been debated. Some have hypothesized that unfolding of the 15 Type III domains, each of which are a 7-beta strand sandwich, drives fibril elasticity. Others have hypothesized that the elasticity is driven by a conformational change of the entire fibronectin molecule from a compact to an extended form, without unfolding of Type III domains. Here we have investigated the unfolding of Type III domains in stretched fibrils by fluorescent labeling of buried free thiols. We generated a library of recombinant fibronectins, each of which had a single buried free thiol. Multiple studies have shown that fibronectin fibrils are extremely elastic: cells can stretch them up to four times their resting length. The mechanism of this elasticity has been debated. Some have hypothesized that unfolding of the 15 Type III domains, each of which are a 7-beta strand sandwich, drives fibril elasticity. Others have hypothesized that the elasticity is driven by a conformational change of the entire fibronectin molecule from a compact to an extended form, without unfolding of Type III domains. Here we have investigated the unfolding of Type III domains in stretched fibrils by fluorescent labeling of buried free thiols. We generated a library of recombinant fibronectins, each of which had a single buried free thiol in a different Type III domain. Fibronectin matrices containing these recombiant fibronectins were labeled with a thiol reactive fluorescent probe; successful labeling indicated that the domain was unfolded to expose the buried free thiol, while no labeling suggested that the domain remained folded. Fibril labeling experiments were also conducted on fibronectin in solution, as some Type III domains have previously been shown to unfold and refold in the absence of applied tension. Data indicates that Type III domains can be categorized into three groups based on their unfolding properties: nine (9) Type III domains showed no evidence of unfolding in either fibrils or solution; four (4) domains showed evidence of unfolding in both fibrils and in solution; and two (2) domains showed evidence of unfolding exclusively in fibrils. These results suggest that there is a subset of Type III domains that unfold within fibronectin that may play a role in fibronectin fibril elasticity.