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CARDIOMYOPATHY-CAUSING DELETION K210 IN CARDIAC TROPONIN T ALTERS PHOSPHORYLATION PROPENSITY OF SARCOMERIC PROTEINS

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Abstract

Ca²⁺ desensitization of myofilaments is indicated as a primary mechanism for the pathogenesis of familial dilated cardiomyopathy (DCM) associated with the deletion of lysine 210 (Δ K210) in cardiac troponin T (cTnT). Δ K210 knock-in mice closely recapitulate the clinical phenotypes documented in patients with this mutation. Considerable evidence supports the proposition that phosphorylation of cardiac sarcomeric proteins is a key modulator of function and may exacerbate the effect of the deletion. In this study we investigate the impact of K²¹⁰ deletion on phosphorylation propensity of sarcomeric proteins. Analysis of cardiac myofibrils isolated from Δ K210 hearts identified a decrease in phosphorylation of cTnI (46%), cTnT (30%) and MyBP-C (32%) compared with wild type controls. Interestingly, immunoblot analyses with phospho-specific antibodies show augmented phosphorylation of cTnT-Thr²⁰³ (28%) and decreased phosphorylation of cTnI-Ser^{23/24} (41%) in mutant myocardium. *In vitro* kinase assays indicate that Δ K210 increases phosphorylation propensity of cTnT-Thr²⁰³ three fold, without changing cTnI-Ser^{23/24} phosphorylation. Molecular modeling of cTnT- Δ K210 structure reveals changes in the electrostatic environment of cTnT helix (residues 203–224) that lead to a more basic environment around Thr²⁰³, which may explain the enhanced PKC-dependent phosphorylation. In addition, yeast two-hybrid assays indicate that cTnT- Δ K210 binds stronger to cTnI compared with cTnT-wt. Collectively, our observations suggest that cardiomyopathy-causing Δ K210 has far-reaching effects influencing cTnI-cTnT binding and posttranslational modifications of key sarcomeric proteins.

Keywords

cardiomyopathy; sarcomeric; cardiac troponin T; K210; phosphorylation

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INTRODUCTION

Cardiac myofilaments' response to fluctuating intracellular Ca^{2+} conditions forms a mechanistic basis for contractile dysfunction associated with a variety of pathological conditions. Cardiac troponin (cTn) is the Ca^{2+} sensor of the cardiac contractile apparatus. As a heterotrimeric complex, it consists of a calcium-binding protein, troponin C (cTnC), an actomyosin MgATPase-inhibiting protein, troponin I (cTnI) and a tropomyosin-binding protein, troponin T (cTnT) [1]. At low $[\text{Ca}^{2+}]_i$, cTnI is anchored to the actin filament and through the action of cTnT holds tropomyosin (Tm) in a position that inhibits myosin binding to actin thus preventing force-generating cross-bridge formation. At high $[\text{Ca}^{2+}]_i$, cTnC undergoes a Ca^{2+} -induced conformational change that enhances its affinity for cTnI, relieving its binding to actin. This movement is transmitted through cTnT to Tm, which then slides deeper into the groove formed by the actin filament, facilitating myosin-actin cross-bridge formation [2].

Advances in molecular genetic studies during the past decade have identified 37 mutations in cTnT associated with different forms of cardiomyopathies, either dilated (DCM) or hypertrophic (HCM) (www.cardiogenomics.med.harvard.edu). The underlying molecular mechanism by which missense or deletion mutations in cTnT lead to the pathogenesis of distinct and often lethal forms of cardiomyopathy remains undetermined [3]. Functional analyses indicate that HCM- and DCM-causing mutations in cTnT have distinct and divergent effects on the Ca^{2+} sensitivity of myofilaments [4]. It was found that at least 80% of HCM mutations increased Ca^{2+} sensitivity of force development. In contrast, a reduced Ca^{2+} sensitivity seems to be an essential property of the DCM-causing mutations [5].

Deletion of lysine 210 (ΔK210) was the first mutation in the human cTnT gene that had been linked to familial primary DCM [6]. Du *et al* [7], generated a knock-in mouse model in which the three base pairs coding for the residue K210 were deleted from endogenous *TNNI2* genes by gene-targeting technology. The knock-in mice closely recapitulated the clinical phenotypes documented in patients with this mutation, *i.e.* enlarged hearts, heart failure, and a high incidence of premature death. Yet, the precise molecular mechanism by which ΔK210 leads to DCM remains unresolved. Mechanical experiments with permeabilized (skinned) fibers from mutant hearts demonstrated that ΔK210 has a Ca^{2+} -desensitizing effect on cardiac myofilaments without affecting maximum force [7]. Unlike permeabilized fibers, intact cardiomyocytes isolated from the same hearts showed a depressed contractile response to Ca^{2+} that could not be adequately explained by the deletion of K210 residue [7]. We propose that phosphorylation of cardiac sarcomeric proteins, a key modulator of function, may be responsible for the discrepant results obtained with skinned fibers and intact cardiomyocytes. In general, mechanical experiments with cardiac fibers containing cardiomyopathy causing cTnT mutants (e.g. R92W, R92L) show a surprisingly mild effect that by itself may not account for the full clinical phenotype associated with those mutations [3]. Phosphorylation moieties being labile, it is no surprising that experiments with permeabilized fibers, where no special precaution is taken to preserve the endogenous phosphorylation state, miss this important functional effect observed with intact cardiomyocytes.

Phosphorylation of cardiac troponin has been shown to influence the myofilaments response to Ca^{2+} [8,9]. cTnI has three main phosphorylation clusters (Ser²³/Ser²⁴, Ser⁴³/Ser⁴⁵ and Thr¹⁴⁴) that exert distinct effects on function [8]. Phosphorylation of Ser²³/Ser²⁴, desensitizes the myofilaments to Ca^{2+} , without changing maximal force. On the other hand, Thr¹⁴⁴ phosphorylation seems to attenuate the desensitizing effect of Ser²³/Ser²⁴ phosphorylation [10]. Phosphorylation of Ser⁴³/Ser⁴⁵ cluster has a depressing effect on maximal actomyosin Mg-ATPase rate and Ca^{2+} -activated force [11]. cTnT also contains three phosphorylation clusters: Ser¹, Thr¹⁹⁴/Ser¹⁹⁸/Thr²⁰³, and Ser²⁷⁵/Thr²⁸⁴ – human sequence notation [2]. T²⁰³

was identified as the functionally significant phosphorylation site of cTnT; the other sites do not seem to have a considerable functional effect by themselves [12]. Phosphorylation of T²⁰³ leads to a significant myofilament Ca²⁺ desensitization and a decrease in maximal force, actomyosin Mg-ATPase rate and tension cost.

The aim of the present study is to understand better the mechanism by which Δ K210 exerts its detrimental effect on cardiac myofilament function. Using isolated myofibrils from the hearts of WT and Δ K210 mutant mice, we ask whether the presence of cTnT- Δ K210 in the myofilament milieu alters the phosphorylation propensity of key sarcomeric proteins. We find a decrease in phosphorylation of cTnI-Ser²³/Ser²⁴ and MyBP-C, and an increase in cTnT-Thr²⁰³ phosphorylation. *In vitro* kinase assays also show augmentation of Thr²⁰³ phosphorylation in cTn- Δ K210 compared with WT. In addition, using a yeast two-hybrid assay we show that cTnT- Δ K210 has a higher affinity for cTnI compared with cTnT-WT. Molecular modeling indicates that Δ K210 may lead to changes in the electrostatic environment of cTnT helix 203-224 that: i) alter intra-troponin I-T binding dynamics, and ii) directly or indirectly affect phosphorylation propensity of cTnT, cTnI and MyBP-C.

MATERIALS AND METHODS

Generation of cTnT- Δ K210 construct

To produce the cTnT- Δ K210, QuickChange site-directed mutagenesis (Stratagene) was first carried out according to the manufacturer's recommendations, using mouse cardiac cTnT-wt cDNA as template and following primers: 5'-GAGAGAGAAGAAGAAGATCCTGGCAGAGAGG-3' and 5'-CCTCTCTGCCAGGATCTTC TTCTTCTCTCTC-3'. The identity of construct was verified by DNA sequencing.

Troponin Expression and Purification

Recombinant mouse cardiac cTnT, cTnI and cTnC were expressed and purified as previously described [11,12]. Briefly, cTnT- Δ K210 was expressed in BL21(DE3) cells grown overnight in Luria Broth supplemented with 30 μ g/ml kanamycin. The cell pellet was collected by centrifugation at 6000 $\times$ g for 10 min at 4 °C and then resuspended in TnT-Buffer A containing 25 mM Tris (pH 8.0), 6 M urea, 1 mM EDTA, 0.1 mM AEBSF, 1 mM benzamidine, 1 mM DTT and 0.5% Triton X-100. The cells were lysed by sonication on ice, followed by 60 min of centrifugation at 48,000 $\times$ g at 4°C. The supernatant fraction was subjected to ammonium sulfate fractionation. The obtained pellet was solubilized in TnT-Buffer A, and then dialyzed at 4°C against the same buffer. The dialyzed sample was applied on a DEAE Fast Flow Sepharose column (GE Healthcare) connected to a FPLC System (Amersham Biosciences). cTnT- Δ K210 was eluted with a 0.0–0.5 M KCl gradient in TnT-Buffer A. The fractions containing cTnT were analyzed by SDS-PAGE and those containing more than 90% pure cTnT were pooled, extensively dialyzed against 0.4% formic acid in H₂O, lyophilized and stored in powder form at -80°C.

Recombinant Troponin Complex Reconstitution

Recombinant heterotrimeric troponin complexes were prepared according to stringent conditions designed to avoid the presence of any contaminants, as previously described [12]. Recombinant heterotrimeric cTn complexes were reconstituted by mixing equimolar amounts of cTnT (either wt or Δ K210), cTnI and cTnC in a solubilization buffer containing 6 M Urea, 50 mM Tris pH 8.0, 1 M NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM DTT. The solution was subjected to sequential dialysis using 0.7 M NaCl, 0.4 M NaCl, and finally 0.05 M NaCl in the above buffer minus urea (CaCl₂ was omitted from the final dialysis step). A high performance Resource Q column (GE Healthcare) was then used to further purify the cTn complex from

any contaminants. Troponin complex elutes as a sharp peak well separated from any unincorporated monomeric TnI or TnC subunits.

PKC expression, purification and specific activity determination

Human PKC isoforms α , β II, ϵ , δ cDNAs were purchased from American Type Culture Collection and subcloned into pVL1392 or pVL1393 baculovirus vectors (BD Biosciences). The recombinant proteins were produced using the BaculoGold expression system (BD Biosciences) in Sf9 cells, as previously reported [12]. Specific activity of each purified PKC was determined using myelin basic protein (MBP), an equally preferred substrate to all 4 isozymes. The specific activities determined were 6,015 units/mg PKC- α , 873 units/mg PKC- β II, 2,454 units/mg PKC- ϵ , 3,844 units/mg PKC- δ . Units of PKC activity were defined as 1nmol of 32 P incorporated into MBP per minute. *In vitro* kinase assays with cTnT-WT (various concentrations) as substrate were carried for 30 min at 30°C using 0.15 units PKC/reaction.

Phosphorylation of cTnT and cTn by PKC- α

In vitro kinase assays were performed using 1ng/reaction recombinant PKC- α [12] in the presence of cTnT-wt, cTnT- Δ K210, cTn-wt or cTn- Δ K210. Various amounts of proteins (1–6 μ M) were incubated with PKC- α in the presence of 20 mM Hepes pH 7.5, 150 mM NaCl (or 300mM NaCl for cTnT samples), 5mM MgCl₂, 0.1 mM CaCl₂, 20 mM NaF, 0.5 mM AEBSF, 2 μ g/ml Pepstatin, 10 μ g/ml Leupeptin, 2mM DTT, 0.3 mM phosphatidylserine, 0.02 mM diacylglycerol, and 0.4 mM ATP (Sigma) in a VorTemp 56™ Shaking Incubator (Alpco Diagnostics). All the reactions were carried out at 30°C for 15min (20 μ l final volume). Phosphorylation was shown to occur at a constant rate over the first 30 min of the reaction for cTnT sites Thr203 and Thr284, but not cTnI sites Ser23/Ser24.

Myofibrillar isolation

Myofibrils were isolated from left ventricular tissue of 4 and 8 week-old wild type (WT), 8 and 24 week-old TNNT2^{+/ΔK210} heterozygous (HT), and 4 week-old TNNT2^{ΔK210/ΔK210} homozygous (HM) mice hearts as previously reported [13]. Briefly, left ventricular tissue was homogenized with a dounce homogenizer in an ice cold buffer containing 75 mM KCl, 10 mM Imidazole (pH 7.2), 2mM MgCl₂, 2 mM EGTA, 1 mM NaN₃, 1% Triton X-100, 1mM DTT and a cocktail of protease (0.25mM AEBSF, 1.25 μ g/ml Leupeptin, 1.25 μ g/ml Pepstatin A, 1.25 μ g/ml Aprotinin) and phosphatase inhibitors (50mM NaF, 0.25mM Na₃VO₄). After a series of centrifugations and washes in the same buffer, the pure myofibrillar pellet was resuspended in 50% glycerol, 30 mM KCl, 10 mM MOPS (pH 7.0), 1 mM MgCl₂, 1 mM DTT and a cocktail of protease and phosphatase inhibitors (see above), and were either used for analysis or stored at –20°C. There were no differences in phosphorylation pattern between fresh or –20°C stored samples. It is worth mentioning that due to the high glycerol content the myofibrils do not freeze during storage at –20°C.

Sarcomeric proteins' phosphorylation

For analysis, myofibrils were resuspended in loading buffer (8M Urea, 2M thiourea, 50 mM Tris-HCl (pH 6.8), 75 mM DTT and 0.05% bromophenol blue). Note that SDS was omitted from the sample-loading buffer because it was found to interfere with ProQ Diamond staining, giving false positives (personal observation). The RC DC Kit (BioRad) was used according to the manufacturer's instructions to determine protein concentrations of isolated mouse cardiac myofibrils. 8 μ g of denatured myofibrils were then separated on a homemade 12.5% Tri-Gly gel. For phosphoproteins detection, the ProQ Diamond staining protocol (Molecular Probes) was used with minor modifications. Phosphoproteins were visualized in gel using a Typhoon-9410 (GE Healthcare). Same gels were then incubated overnight with SYPRO Ruby stain (Molecular Probes) and rescanned for the determination of total proteins optical densities.

Phosphorylation levels of sarcomeric proteins were determined from the optical density ratio of phosphorylated to total protein using ImageQuant TL software (GE Healthcare). Actin levels were used to verify equal loading of each sample and if necessary to correct for small variations. All samples were run simultaneously on the same gel.

Immunoblot analysis

Site-specific phosphorylation of cTnT at Thr²⁰³ and Thr²⁸⁴ (control) was followed using custom made phospho-specific antibodies purified by peptide-affinity chromatography, as previously described [14]. Phospho-specific antibodies were thoroughly characterized for specificity by peptide competition assays as described [14]. The phosphor-antibodies are specific to phosphorylated residues and have no reactivity with dephosphorylated (following phosphatase treatment) or non-phosphorylated cTnT. Moreover, the antibodies are inhibited by the phospho-peptide used as immunogen but not the non-phospho-peptide [14]. Total cTnT was determined by immunoblotting with either CT3 antibody (provided by the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa) or JLT12 antibody (Sigma). cTnI-S^{23/24} phospho-antibody was from Cell Signaling Technology and pan-cTnI-C5 antibody was from Fitzgerald Industries. Anti-actin (Sigma) antibody was used to verify and correct for small variations in loading. Unless otherwise noted in the text, proteins were separated by 12.5% SDS-PAGE, transferred to a Hybond-LF membrane (GE Healthcare), blocked for 1–2 hr at room temperature in 5% ECF blocking agent (GE Healthcare) and incubated overnight in primary antibodies. After respective secondary antibody incubation, reactivity was detected using the ECF kit (GE Healthcare) according to the manufacturer's recommendations. Immunoblots were analyzed with the ImageQuant TL software. The efficiency of protein transfer from the gel to the membrane was determined by staining the gel with Coomassie brilliant blue R-250.

Yeast two-hybrid assays

cTnI and cTnT (WT or Δ K210) cDNA were subcloned into the Matchmaker Gal4 two-hybrid system 3 (Clontech) vectors pGADT7 and pGBKT7 [15]. pGAD-cTnI and pGBK-cTnT were co-transformed into the *lacZ* phenotype yeast strain AH109, using a lithium acetate method. Only yeast cells carrying both plasmids grew on the selective plates lacking nutritional markers Leu/Trp. For the β -gal (or colony-lift) assay, freshly transformed yeast cells that grew on selective media were replica-plated to filter papers, immersed into liquid nitrogen to lyse the cells, placed on filters pre-soaked in 49 mM X- β -gal solution and incubated at 30°C for up to 4 hr. In the α -gal assays yeast transformants were spread on plates containing X- α -gal (40mg/mL, Sigma), incubated at 30°C and then checked for color development.

Protein Modeling

Three dimensional modeling of cTnT helix 1 (residues 202–224) containing phosphorylation residue Thr203 and lacking Lys210, was performed with the 3D-JIGSAW program [16–18] based on the homologous structure of the partial cardiac troponin solved by x-ray diffraction [19]. cTnT-H1-WT and modeled cTnT-H1- Δ K210 graphical models were constructed with the WebLab ViewerPro molecular simulations software.

Statistical Analysis

All data are presented as mean $\pm$ SEM, and were analyzed for statistical significance using a Student's t-test (KaleidaGraph Software). Values of $p < 0.05$ were considered significant.

RESULTS

Differential phosphorylation of sarcomeric proteins

To determine whether K210 deletion induces alterations in phosphorylation of troponin and other sarcomeric proteins, we investigated total and site-specific phosphorylation of myofilament proteins in myofibrils isolated from wild-type (WT), heterozygous *TNNT2*^{+/ΔK210} (HT) and homozygous *TNNT2*^{ΔK210/ΔK210} (HM) mice hearts [7]. Cardiac myofibrils were prepared under conditions shown to preserve phosphorylation status of the sarcomeric proteins (in the presence of protease and phosphatase inhibitors). Total phosphorylation levels were determined for myosin light chain 2 (MLC2), troponin I (cTnI), troponin T (cTnT), desmin and myosin binding protein-C (MyBP-C). Tropomyosin (Tm) and α -actinin were below the detection limit of ProQ Diamond phosphostain in these samples. However, increasing the amount of myofibrils to 15 μ g/lane allowed clear detection of the two phospho-proteins. There were no differences in Tm or α -actinin phosphorylation levels in wt and mutant hearts. Data presented in Figure 1 (and Table 1) show significant decrease of cTnT (by 29%), cTnI (by 46%) and MyBP-C (by 31%) basal phosphorylation levels in HM hearts, but not HT either 8-wk or 24-wk, compared with WT hearts. MLC₂ phosphorylation levels were unchanged. Interestingly, interfilament protein desmin shows a 33% phosphorylation decrease in the 24-wk HT hearts but no changes in the HM hearts. Table 1 indicates that 4-wk and 8-wk wild type hearts have similar levels of myofilament protein phosphorylation.

Site-specific phosphorylation of cTnT and cTnI in isolated myofibrils

Next we investigated changes at specific phosphorylation sites (cTnT and cTnI) in isolated myofibrils from mutant and wild type hearts. The functionally critical phosphorylation residue Thr²⁰³ of cTnT showed enhanced phosphorylation in all the mutant hearts when compared with WT (figure 2 A, B). Since there were no differences between 4 and 8 week-old WT hearts, only the 8-wk data is shown. Heterozygous hearts displayed only modest increases compared with a 28% augmentation of Thr²⁰³ phosphorylation in HM myocardium. This is surprising since total cTnT phosphorylation was significantly lower in HM hearts compared with controls (Figure 1, Table 1). Considering that cTnT has 5 other potential phosphorylation sites apart from Thr²⁰³ (see figure 3A) it is possible for one site to increase (i.e Thr²⁰³) while the overall phosphorylation to decrease. This is supported by the fact that phosphorylation of two other cTnT sites, Thr²⁸⁴ (figure 2C) and Ser²⁷⁵ (data not shown), decreased noticeably in HM. In agreement with the trend noted for total cTnI phosphorylation (Table 1), cTnI-Ser^{23/24} phosphorylation dropped significantly in the HM (~40%) but not the heterozygous hearts (figure 2D). Other cTnI phosphorylation sites showed no changes (data not shown).

Phosphorylation of cTnT-WT by PKC isozymes

To determine whether ΔK^{210} has a direct or indirect effect on troponin phosphorylation we performed *in vitro* kinase assays. Comparative specific activities of four recombinant PKC isozymes (α , β II, ϵ , δ) were tested toward an equally preferred substrate, myelin basic protein (MBP). Kinase assays performed under conditions of equal PKC activity (0.15 units/reaction) in the presence of increasing concentration of cTnT-wt (figure 3C) indicate that conventional PKCs ($\alpha > \beta$ II) are better cTnT kinases than the novel PKCs (α and ϵ). Subsequent phosphorylation studies used solely PKC- α .

cTnT and cTnI phosphorylation by PKC- α

In vitro kinase assays, in the presence of low concentrations of PKC (1ng/reaction) and excess substrate, were used to examine whether ΔK^{210} influences PKC- α phosphorylation kinetics of cTnT-T²⁰³ or cTnT-T²⁸⁴ (as control). Experiments where cTnT by itself (WT or ΔK^{210}) served as substrate for PKC- α show that deletion of K²¹⁰ has no effect on T²⁰³ while enhancing

T²⁸⁴ phosphorylation (figure 4A,C). However, when more physiological substrates were used, troponin complexes containing equimolar concentrations of cTnI-wt, cTnC-wt and either cTnT-wt or cTnT-ΔK210 (see figure 3B), ΔK210 augmented T²⁰³ but not T²⁸⁴ phosphorylation (figure 4B,D). On the other hand, cTnI-S^{23/24} phosphorylation was not affected by deletion of K210 in cTnT (figure 4E).

To further examine the differences in T²⁰³ and T²⁸⁴ phosphorylations induced by ΔK210, kinetic parameters were determined under conditions where initial velocity could be determined (Figure 5 and Table 2). The apparent Michaelis-Menten constant (K_m) values for T²⁰³ phosphorylation increased in the order Tn-ΔK210 < Tn-wt < TnT-wt < TnT-ΔK210. Tn-ΔK210 complexes display V_{max} values 2-fold higher than those determined for Tn-wt, indicating faster reaction rates for Tn-ΔK210 compared with Tn-wt. As judged from the ratio of V_{max}/K_m the relative efficacy of T²⁰³ sites of wt or ΔK210 proteins to serve as PKC-α substrates, in descending order, were: Tn-ΔK210 > TnT-wt > TnT-ΔK210 > Tn-wt. Deletion of K210 residue had little effect on the kinetics of phosphorylation of T²⁸⁴ site.

Yeast two-hybrid binding assay

A yeast two-hybrid assay was used to evaluate whether ΔK210 propagates its noxious effect through altered cTnT-cTnI binding. Figure 6A, shows schematically the main steps of the assay. The strength of the interaction between cTnT and cTnI is directly proportional to the color development determined by two different colorimetric assays, α-gal (identical to β-gal, data not shown) and β-gal (i.e. dark blue indicates enhanced interaction between the two proteins, figure 6B, 6C). Our data suggests that cTnT-ΔK210 binds stronger to cTnI compared with the cTnT-WT counterpart.

DISCUSSION

Our study provides novel evidence that deletion of K²¹⁰ in cTnT, linked to familial dilated cardiomyopathy, may propagate its deleterious effect through alterations in cTnI-cTnT interaction and basal phosphorylation levels of key contractile proteins. Isolated cardiac myofibrils from homozygous ΔK210 hearts indicate differential phosphorylation of cTnT (augmented T²⁰³ phosphorylation), cTnI (decrease S^{23/24} phosphorylation) and reduced overall MyBP-C phosphorylation. An important finding of our study is that deletion of K²¹⁰ enhances PKC phosphorylation kinetics for T²⁰³. Our previous analysis of the functional role of each cTnT phosphorylation site identified a distinct region centered on T²⁰³ that when modified by phosphorylation controls force, actomyosin Mg-ATPase rate, Ca²⁺ sensitivity and cross bridge cycling of mouse cardiac fiber bundles [12]. Apparently phosphorylations of the other cTnT sites have no detectable direct functional role (although they probably act indirectly to enhance the effect of Thr²⁰³ phosphorylation) [12]. Our results suggest that augmentation of cTnT-T²⁰³ phosphorylation on a background of decreased overall sarcomeric proteins phosphorylation may exacerbate the myofilaments Ca²⁺ desensitization and may explain the contractile deficit documented in the ΔK210 hearts.

To understand better the mechanism by which deletion of K²¹⁰ confers a higher phosphorylation propensity to T²⁰³, we modeled the region of cTnT containing these two residues. Since the high-resolution x-ray structure of the core cardiac troponin complex contains the fragment of cTnT spanning residues 202–285 (human sequence), we used this structure for our model building [19]. Figure 7 shows a surface representation of helix 203–224 of cTnT-wt and ΔK210. The native structure shows the protruding T²⁰³ residue on the same face of the helix as K²¹⁰, flanked by two glutamate residues E²⁰⁴ and E²⁰⁶. Interestingly, this structure points to a possible unique feature of K²¹⁰: a helix stabilizing interaction with E²¹⁴. Another helix stabilizing electrostatic bond is shown between E²⁰⁴ and K²⁰⁷. It is well documented that two of the five amino acids (M, A, L, E, K) with the highest helical forming

propensity $K^{(-)}-E^{(+)}$ when located at positions $i, i+4$ (e.g. K^{210} and E^{214}) and $i, i+3$ (e.g. E^{204} and K^{207}) have the tendency to form helix stabilizing salt-bridges (electrostatic bonds) [20]. The molecular model of $\Delta K210$ (generated by the use of 3D-Jigsaw program) shows some interesting local changes, without affecting the helical content. This model is in good agreement with circular dichroism data showing the α -helical content of cTnT- $\Delta K210$ was nearly identical to that of cTnT-wt [21]. In the absence of K210 there is a rearrangement of E/K electrostatic interactions. E214 engages in a new bond with K217, the E204-K207 bond is broken and the two residues are pulled apart from each other. K210 deletion reorganizes the electrostatic microenvironment near T^{203} leading to a clustering of basic charges on the same side of the helix (fig. 7). This may explain the enhanced phosphorylation kinetics by PKC- α , which preferentially phosphorylates sites bordered by highly basic residues [22].

The idea that a cardiomyopathy mutation could influence phosphorylation propensity of a nearby site has been proposed for some time. However, until now, there was no systematic analysis to demonstrate this effect. Equally interesting is the fact that deletion of a residue in cTnT has far reaching effects, influencing PKA-dependent phosphorylation of neighboring contractile proteins cTnI and MyBP-C. Recent advances in MyBP-C structure suggest that N-terminal domains of MyBP-C (including the m-domain containing the cardiac specific PKA phosphorylation sites) bind F-actin [23–25]. In this case, the m-domain of MyBP-C would be anchored on the thin filament in the vicinity of cTnT and cTnI. The question is how could the deletion of K210 lead to alterations in PKA-dependent phosphorylation of cTnI and MyBP-C? Although experiments proposed here do not directly address this question, recent evidence from our laboratory point to a potential scenario. Using a yeast-two hybrid system we have recently identified a novel function for cTnT as an anchoring protein for cAMP-dependent protein kinase A (PKA)[15]. Sequence homology followed by mutagenesis studies confirmed the existence of a cTnT PKA binding site on the same helix harboring T^{203} and K^{210} . Yeast-two hybrid data indicate that PKA affinity for cTnT- $\Delta K210$ is lower than for cTnT-WT [15]. Low PKA-cTnT affinity would diminish thin filament sequestration of PKA, resulting in decrease phosphorylation of its two main substrates cTnI and MyBP-C. Taken together these observations suggest that deletion of K210 on one hand impairs cTnI and MyBP-C phosphorylation by weakening PKA docking to cTnT, and on the other hand enhance PKC-dependent phosphorylation of cTnT- T^{203} . Increased T^{203} phosphorylation [12] and decreased MyBP-C phosphorylation [26] may work in synergy to exacerbate the effect of K^{210} deletion and may explain the defect in contractile dynamics seen in the HM knock-in myocardium.

$\Delta K210$ also enhances cTnT-cTnI binding affinity, as demonstrated by yeast two-hybrid assays (fig. 6). The implication is that a stronger cTnT-cTnI interaction may lead to a more rigid troponin with impaired Ca^{2+} sensing/transmitting properties. As a result the $\Delta K210$ myofilaments would be less reactive to Ca^{2+} . The yeast two-hybrid system (Y2H) is a sensitive eukaryotic assay widely used to detect novel interactions between proteins and to map the specific interacting domains of two proteins known to form a complex [27]. The strength of the interaction between two proteins is associated with the level of expression of an appropriate reporter gene. The work of Estojak, *et al* [28] indicates that there is a good correlation between the strength of interaction of two proteins predicted by Y2H assay with that determined *in vitro* by biochemical assays.

Collectively, our observations suggest that cardiomyopathy-causing $\Delta K210$ has far-reaching effects influencing cTnI-cTnT binding and posttranslational modifications of key sarcomeric proteins, which may underlie the dysregulation of myofilament function and DCM progression. This novel effect of $\Delta K210$ is likely to represent a more general trend of cardiomyopathy-inducing mutations positioned near critical phosphorylation sites.

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FIGURE 1.

Differential phosphorylation of sarcomeric proteins in isolated myofibrils from $\Delta K210$ homozygous hearts. A, cardiac myofibrillar proteins (8 μ g/lane) were resolved by 12.5% SDS-PAGE and stained with ProQ Diamond for phospho-proteins detection and Sypro Ruby for total protein detection. Main sarcomeric phospho-proteins are indicated, together with actin, to show equal protein content (Sypro) and disparity in phosphorylation levels (ProQ) in the WT and $\Delta K210$ myofilaments. Data shown are from 4 week-old animals. There are no significant differences in phosphorylation pattern of sarcomeric proteins in 4 and 8 week-old WT hearts. B, Optical densities of phospho/total protein ratio ($n = 5$ hearts/group). The results are normalized by setting the mean value for WT to 100. Data are means $\pm$ SEM. Values marked with asterisk were deemed statistically significant ($p < 0.05$). A.U., arbitrary units.

FIGURE 2.

Analysis of site-specific phosphorylation of troponin T and I in cardiac myofibrils isolated from WT, heterozygous (HT), and homozygous (HM) hearts. A, representative immunoblots show reactivity of anti-phosphorylation specific antibodies cTnT-pT²⁰³, cTnT-pT²⁸⁴ (control) and cTnI-pS^{23/24} with freshly isolated cardiac myofibrils. Actin was used to verify and correct for small variations in loading. B, C, D bar graphs show the phosphorylation profiles of cTnT-T²⁰³, cTnT-T²⁸⁴ and cTnI-S^{23/24}, respectively. cTnT-T²⁰³ phosphorylation increased while T²⁸⁴ and cTnI-S^{23/24} decreased in mutant hearts. Data are means of the values calculated from the optical density of the bands obtained by Western blotting normalized against pan-cTnT or pan-cTnI densities (n = 5 hearts/group). Values marked with asterisk were deemed statistically significant ($p < 0.05$).

FIGURE 3.

Cardiac troponin preparations and concentration dependent phosphorylation by PKC isozymes. A, schematic representation of cTnT and cTnI, showing main phosphorylation sites and the location of K210 relative to Thr203. Amino acid residues near studied phosphorylation sites are shown using the 1 letter abbreviation. cTnT is numbered according to the human sequence. B, Recombinant cardiac troponins were expressed in *E. Coli*, purified to near homogeneity (as shown by SDS-PAGE) and were used to reconstitute troponin complexes. TnT-wt and cTnT-ΔK210 are shown as purified individual proteins or as part of the troponin complexes in the presence of wild type murine cTnI and cTnC. Molecular weight markers (Bio-Rad) are shown on the left. Gel was stained with Coomassie brilliant blue. C, *in vitro* kinase assays with cTnT-WT as substrate were carried out for 30 min at 30°C using 0.15 units PKC/reaction in the presence of phosphatidylserine and diacylglycerol, and Ca²⁺ for PKC-α and β. Phosphorylated proteins were resolved by SDS-PAGE and stained with ProQ Diamond. Data indicate that PKC-α is a better cTnT kinase than the other PKCs.

FIGURE 4.

Time-course analysis of site-specific phosphorylation of WT and Δ K210 cTnT or cTn complexes. *In vitro* kinase assays show time dependent PKC- α phosphorylation of cTnT sites T²⁰³ and T²⁸⁴, and cTnI-S^{23/24}. Δ K210 has no effect on T²⁰³ phosphorylation in cTnT alone (A) but it speeds up the rate of phosphorylation reaction when incorporated in the troponin complex (B). Interestingly, T²⁸⁴ phosphorylation rates are opposite from those observed with T²⁰³ (C, D). Phosphorylations of cTnI sites S23/24 are not affected by Δ K210 (E). Data are means of optical density values ($\pm$ SEM) determined by analyzing the band intensities from immunoblots using ImageQuant TL software and normalized to total cTnT (A–D) or cTnI (E)

detected by an anti-TnT or anti-cTnI pan antibodies. All reactions contained 1 ng PKC- α and 1 μ M substrate, and were carried out at 30°C.

FIGURE 5.

In vitro kinase assays for determination of kinetic constants. Increase in substrate concentrations differentially affect phosphorylation of T²⁰³ in cTnT alone compared with cTn complexes. ΔK210 enhance PKC phosphorylation of T²⁰³ site when present in troponin complex (B) but not when in cTnT alone (A). Phosphorylation of T²⁸⁴ follows the same trend as T²⁰³ for cTnT alone (C) but not cTn (D). Data are means of optical density values ($\pm$ SEM) determined by analyzing the band intensities from immunoblots using ImageQuant TL software and normalized to total cTnT detected by an anti-TnT pan antibody. All reactions contained 1 ng PKC- α in the presence of varying substrate concentrations, and were carried out at 30°C.

FIGURE 6.

Yeast two-hybrid assay. A, schematic representation of the main steps of the yeast two-hybrid assay. cTnI (subcloned into the pGADT7 – activation domain vector) and cTnT (subcloned into the pGBKT7 – DNA binding vector) were co-transformed into the AH109 yeast strain. B, β -gal assay shows the interaction between cTnT and cTnI as monitored by color blue development. The intense blue color developed by yeast cells containing cTnT- Δ K210 and cTnI indicate a stronger interaction between these two proteins compared with WT control (lighter blue). C, Color intensity of blue colonies was determined using Image J software and normalized to that of control colonies (white). Data shown is from a single experiment and is representative of 4 separate experiments.

FIGURE 7.

Molecular graphics views of cTnT helix 202–224 WT or lacking K²¹⁰. Partial cardiac troponin core structure (1J1E.pdb) solved by x-ray diffraction [19] was used as the starting point for modeling the partial structure of cTn-ΔK210. Modeling was performed with the 3D-JIGSAW program. Solvent-accessible surface representations of cTnT helix 202–224, shows the position and charge of acidic (red color) and basic (blue color) residues in WT (*left*) and ΔK210 model (*right*). While the disappearance of K²¹⁰ does not induce gross structural changes (*right*) it does alter the orientation of basic residues (R²⁰⁵, K²⁰⁷–K²⁰⁹), breaks the electrostatic interactions E²⁰⁴-K²⁰⁷ and K²¹⁰-E²¹⁴, and facilitates the formation of the novel E²¹⁴-K²¹⁷

bond. These microenvironment alterations enhance the basic electrostatic potential near T²⁰³ making it a much better substrate for PKC- α .

TABLE I

Differential phosphorylation of sarcomeric proteins in wild-type (WT), heterozygous ΔK210 (HT), and homozygous ΔK210 (HM) hearts.

Sample	Total phosphorylation (relative units)				
	MLC2	cTnI	cTnT	Desmin	MyBP-C
WT 4-wk	0.40 ± 0.06	2.07 ± 0.18	1.04 ± 0.04	0.48 ± 0.05	1.50 ± 0.03
WT 8-wk	0.36 ± 0.05	2.09 ± 0.21	0.94 ± 0.05	0.53 ± 0.04	1.46 ± 0.02
HT 8-wk	0.45 ± 0.06	2.46 ± 0.19	1.01 ± 0.05	0.48 ± 0.04	1.30 ± 0.06
HT 24-wk	0.39 ± 0.03	1.8 ± 0.08	0.94 ± 0.05	0.36 ± 0.03*	1.35 ± 0.09
HM 4-wk	0.35 ± 0.04	1.12 ± 0.15*	0.67 ± 0.09*	0.48 ± 0.06	1.02 ± 0.06*

* Data are means of at least three separate experiments from five different hearts. Phosphorylation values were determined from the analysis of optical density corresponding to the respective sarcomeric protein, as described in *Experimental procedures*. All data are presented as means ± SEM, and were analyzed for statistical significance using a Student's t-test ($= p < 0.05$).

Table II

Summary of kinetic constants for phosphorylation of cTnT-Thr²⁰³ and cTnT-Thr²⁸⁴ by PKC- α .

Sample	Phosphorylated Residue	$K_{m(app)}$ (μM)	V_{max} (units $\cdot\mu L^{-1}$)	$V_{max}/K_{m(app)}$ (units $\cdot\mu L^{-1}\cdot\mu M^{-1}$)
cTnT-wt	T ²⁰³	4.85 $\pm$ 0.83	1.62 $\pm$ 0.18	0.33 $\pm$ 0.08
cTnT- $\Delta K210$	T ²⁰³	5.09 $\pm$ 0.97	1.31 $\pm$ 0.12	0.26 $\pm$ 0.05
cTn-wt	T ²⁰³	3.74 $\pm$ 0.67	0.615 $\pm$ 0.05	0.16 $\pm$ 0.03
cTn- $\Delta K210$	T ²⁰³	2.49 $\pm$ 0.29	1.22 $\pm$ 0.06	0.49 $\pm$ 0.06
cTnT-wt	T ²⁸⁴	4.43 $\pm$ 0.96	1.59 $\pm$ 0.18	0.36 $\pm$ 0.09
cTnT- $\Delta K210$	T ²⁸⁴	7.40 $\pm$ 0.93	1.66 $\pm$ 0.29	0.22 $\pm$ 0.07
cTn-wt	T ²⁸⁴	9.24 $\pm$ 0.75	1.76 $\pm$ 0.13	0.19 $\pm$ 0.02
cTn- $\Delta K210$	T ²⁸⁴	8.04 $\pm$ 0.88	1.90 $\pm$ 0.26	0.24 $\pm$ 0.06

In vitro kinase assays (Fig 6) were used to estimate the apparent Michaelis-Menten constant $K_{m(app)}$, V_{max} (the maximum rate of reaction at infinite substrate concentration) and the ratio $V_{max}/K_{m(app)}$ which denotes catalytic efficiency. Values are means $\pm$ SEM.