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Cardiac Troponin I Tyrosine 26 Phosphorylation Decreases Myofilament Ca²⁺ Sensitivity and Accelerates Deactivation

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Abstract

Troponin I (TnI), the inhibitory subunit of the troponin complex, can be phosphorylated as a key regulatory mechanism to alter the calcium regulation of contraction. Recent work has identified phosphorylation of TnI Tyr-26 in the human heart with unknown functional effects. We hypothesized that TnI Tyr-26 N-terminal phosphorylation decreases calcium sensitivity of the thin filament, similar to the desensitizing effects of TnI Ser-23/24 phosphorylation. Our results demonstrate Tyr-26 phosphorylation and pseudo-phosphorylation decrease calcium binding to Troponin C (TnC) on the thin filament and calcium sensitivity of force development to a similar magnitude as TnI Ser-23/24 pseudo-phosphorylation. To investigate the effects of TnI Tyr-26 phosphorylation on myofilament deactivation, we measured the rate of calcium dissociation from TnC. Results demonstrate filaments containing Tyr-26 pseudo-phosphorylated TnI accelerate the rate of calcium dissociation from TnC similar to that of TnI Ser-23/24. Finally, to assess functional integration of TnI Tyr-26 with Ser-23/24 phosphorylation, we generated recombinant TnI phospho-mimetic substitutions at all three residues. Our biochemical analyses demonstrated no additive effect on calcium sensitivity or calcium-sensitive force development imposed by Tyr-26 and Ser-23/24 phosphorylation integration. However, integration of Tyr-26 phosphorylation with pseudo-phosphorylated Ser-23/24 further accelerated thin filament deactivation. Our findings suggest that TnI Tyr-26 phosphorylation functions similarly to Ser-23/24 N-terminal phosphorylation to decrease myofilament calcium sensitivity and accelerate myofilament relaxation. Furthermore, Tyr-26 phosphorylation can buffer the desensitization of Ser-23/24 phosphorylation while further accelerating thin filament deactivation. Therefore, the

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Disclosures

None.

functional integration of TnI phosphorylation may be a common mechanism to modulate Ser-23/24 phosphorylation function.

Keywords

Cardiac troponin I; tyrosine phosphorylation; thin filament deactivation; calcium sensitivity

1. Introduction

The modulation of cardiac muscle contraction by phosphorylation represents a primary mechanism to alter myocardial function to meet demand. At the level of the myofilament, phosphorylation of the troponin (Tn) inhibitory subunit (TnI) represents a critical mechanism to modulate Ca^{2+} -dependent thin filament regulation of myosin's interaction with actin, and therefore cardiac muscle contraction. Recently, a novel phosphorylation of TnI at tyrosine 26 (TnI Tyr-26) was identified [1]. In this finding Zhang *et al.* demonstrated TnI Tyr-26 phosphorylation was present in the non-diseased human heart and that this TnI Tyr-26 phosphorylation was decreased in the failing human heart [1]. These findings suggest a functional role for TnI Tyr-26 in both the normal and pathological heart, however the effect of TnI Tyr-26 phosphorylation on cardiac muscle contractile regulation is unknown.

Contrary to TnI Tyr-26 phosphorylation, the dual phosphorylation of TnI at serine residues 23 and 24 (TnI Ser-23/24) is well described as a direct modulator of cardiac contraction in response to both physiological and pathological stress. Like TnI Tyr-26, TnI Ser-23/24 phosphorylation is also present in the non-diseased heart and decreased in the failing human heart [1–4]. We have demonstrated the functional response of TnI Ser-23/24 phosphorylation is dependent, however, upon its functional integration with other TnI phosphorylation events [5, 6]. Since both TnI Tyr-26 and Ser-23/24 phosphorylations are present in the human heart [1], Tyr-26 may crosstalk on Ser-23/24 phosphorylation function (eg. Tyr-26 phosphorylation may alter the functional effect of TnI Ser-23/24 phosphorylation). The role of TnI Tyr-26 phosphorylation in combination with Ser23/24 has yet to be determined.

To investigate TnI Tyr-26 phosphorylation on cardiac contractile function we determined the functional effects of TnI Tyr-26 phosphorylation in isolation and upon its combined integration with Ser-23/24 phosphorylation on myofilament regulation. TnI Tyr-26 phosphorylation function was assessed for TnI containing Tyr-26 with actual phosphate, pseudo-phosphorylation and phosphorylation null mutations. Our results demonstrate the incorporation of actual phosphate and pseudo-phosphorylation at TnI Tyr-26 both similarly decrease thin filament Ca^{2+} sensitivity, Ca^{2+} -dependent force generation and accelerate Ca^{2+} dissociation from TnC on the thin filament with a magnitude similar to that of TnI Ser-23/24 pseudo-phosphorylation. While the integration of TnI Tyr-26 with Ser-23/24 pseudo-phosphorylation did not further decrease thin filament Ca^{2+} sensitivity compared to either TnI pseudo-phosphorylation alone, their integration did further accelerate thin filament deactivation. Thus while in isolation TnI Tyr-26 phosphorylation functions biochemically identically to Ser-23/24 phosphorylation, its integration with Ser-23/24 to

further accelerate Ca^{2+} dissociation without further decreasing Ca^{2+} sensitivity demonstrates its functional integration, or crosstalk, on Ser-23/24 function. TnI Tyr-26 phosphorylation therefore represents a novel phosphorylation pathway to differentially modulate cardiac contractile function through Ca^{2+} sensitivity and thin filament deactivation when in isolation compared to when integrated with TnI Ser-23/24 phosphorylation.

2. Materials and Methods

2.1 cDNA constructs

All cardiac TnI amino acid residue numbers presented in this manuscript are presented according to the human sequence including the first methionine. Site-directed mutagenesis (QuickChange Lightning, Agilent, Santa Clara, CA) was conducted according to manufacturer's instructions to generate cDNA constructs encoding human TnI pseudo-phosphorylations and null mutations: Tyr-26 to Glu (Y26E), Tyr-26 to Asp (Y26D), Tyr-26 to Phe (Y26F), Tyr-29/112 to Phe (Y29/112F), Ser-23/24 to Asp (S23/24D), Ser-23/24 to Asp with Tyr-26 to Glu (S23/24D+Y26E). All resultant constructs were verified by DNA sequencing.

2.2 Protein expression, purification, and TnI Tyr-26 Phosphorylation

Plasmids encoding the individual recombinant human cardiac Tn subunits were transformed and expressed in *Escherichia coli* and purified to homogeneity as previously described [6–9]. TnC^{C35S,T53C,C84S} was labeled with 2-(4'-iodoacetamidoanilo)naphthalene-6-sulfonic acid (IAANS) as previously described [10, 11]. Cardiac Tn complexes were prepared and reconstituted by sequential dialysis as previously described [5, 8, 12]. Rabbit skeletal actin and bovine cardiac tropomyosin were prepared from acetone powder as previously described [13–16]. Thin filaments were reconstituted as previously [6, 11]. TnI Y29/112F was phosphorylated by incubation with LynA or Src (SignalChem, Richmond, BC, Canada) tyrosine kinase in buffer (in mM; 150 KCl, 3 MgCl₂, 10 MOPS, pH 7.0) containing 10 mM ATP for 48 hours to generate TnI containing phosphate at Tyr-26. LynA phosphorylated TnI was affinity purified to remove LynA on a 2 mL Troponin C (TnC) affinity column similar to that previously described [5, 8] and used to prepare purified, recombinant Tn complex for biochemical experiments.

2.3 Thin filament steady-state Ca^{2+} binding to TnC

Steady-state fluorescence measurements were conducted using a Perkin-Elmer LS55 spectrofluorimeter. IAANS fluorescence changes of labeled TnC in reconstituted thin filaments upon addition of various Ca^{2+} were monitored as previously described [10, 11, 13, 17]. Briefly, fluorescence was monitored from reconstituted thin filaments containing various TnI mutations in buffer (in mM; 150 KCl, 3 MgCl₂, 2 EGTA, 200 MOPS, pH 7.0) at 15°C with constant stirring. Calcium sensitivities of conformational changes are reported as the dissociation constant (K_d), representing a mean of three to four titrations.

2.4 Ca^{2+} dissociation from TnC in the thin filament

Ca^{2+} dissociation from TnC in thin filaments containing wild type (WT) or mutant TnI was measured in calcium saturated buffer (in mM; 150 KCl, 3 MgCl₂, 0.2 Ca^{2+} , 10 MOPS, pH

7.0) upon rapid mixing with EGTA buffer at 15°C in a stopped flow instrument (Applied Photophysics Ltd. model SX.18 MV) with a dead time of 1.4 ms as previously described [10, 11, 13, 17] and kinetic values obtained.

2.5 Myocyte Contraction

Cardiac myocyte preparations were mechanically isolated from rat left ventricular tissue that had been snap frozen in liquid nitrogen and stored at -80°C modified from that previously described [18]. Briefly, approximately 40 mg of rat left ventricular tissue was briefly thawed on ice and minced into 1 mm cube pieces before homogenization in 4 mL relaxing solution (in mM; 97.92 KOH, 6.24 ATP, 10 EGTA, 10 Na₂CrP, 47.58 Kprop, 6.54 MgCl₂, 100 BES, pH 7.0) with a 12 mm Polytron homogenizer probe (Kinematica Inc, Bohemia, NY) for 1–2 s at 10,000 RPM. Resultant homogenate was passed through a 70 µm cell strainer. The cell strainer was then washed with 2 mL cold relaxing buffer and centrifuged at 120 g for 1 min at 4°C. Following centrifugation supernatant was removed and resultant cells were skinned by resuspension in relaxing solution containing 1% peroxide-free Triton X-100 (Anapoe-X-100, Anatrace, Maumee, OH) and incubated at room temperature for 10 minutes rocking. Following skinning, myocytes were centrifuged as before and immediately resuspended for Tn exchange.

Exchange of exogenous recombinant human cardiac Tn into skinned rat myocytes was performed similar to that previously described [6, 9, 12]. Myocytes were resuspended in 100 µL of 15 µM cold recombinant Tn (in mM; 200 KCl, 5 MgCl₂, 5 EGTA, 0.5 DTT, 20 MOPS, pH 6.5) followed by incubation overnight at 4°C. The next day myocytes were pelleted by centrifugation at 120 × g for 1 min at 4°C and washed twice by resuspension in 1 mL cold relaxing solution. Resultant myocytes were resuspended in 1 mL relaxing solution and a sample was collected from every exchange to determine Tn exchange percentage by Western blot as described below. Myocytes were stored on ice and used within 8 hrs.

Calcium regulated force development in skinned Tn exchanged rat ventricular myocyte preparations was performed as previously described [19]. Briefly, Tn exchanged myocytes were transferred to a culture dish coated with 0.1% BSA and attached to two micro-needles using UV-sensitive glue (Norland, Cranbury, NJ) under visualization on an inverted microscope (Nikon) at 60X magnification. Micro-needles were attached to a movable post and a force transducer (Aurora Scientific Inc. 403A transducer, Aurora, ON, Canada) and sarcomere length was adjusted to 2.2 µm by visualization on a calibrated monitor. The perfusion pipette of a constant perfusion control system (VC-8M Eight Channel Mini-Valve Perfusion System, Warner Instruments, Hamden, CT) was then placed closely to the myocyte such that the outflow perfused the entire myocyte. Cells were perfused with maximally activating Ca²⁺ solution to ensure proper cell attachment prior to the start of the experiment. Experiments were performed by perfusing the myocyte with a series of activating mixtures consisting of relaxing and activating solution. Activating solution was identical in composition to relaxing solution but containing varied free Ca²⁺ concentration (pCa 10.0 to 4.5) [20]. Developed force was measured at each activating perfusion followed by perfusion with relaxing solution. The activating developed force was subtracted from the subsequent relax perfused force measure. Time-dependent force rundown was determined

by comparison of the first developed maximal force to the force developed upon final maximal activation at the end of the experiment. Any cell exhibiting greater than 20% force rundown was discarded. Cell cross-sectional area was calculated after the force experiment on a calibrated monitor as previously described [19]. Force-pCa curves were fit using a modified Hill-equation [6, 21]. Experiments were conducted at room temperature. Data were acquired with custom-made LabView software and analyzed using Igor Pro.

2.6 Protein electrophoresis and Western blot

Isolated skinned myocytes or Tn were solubilized in denaturing buffer (2% SDS, 0.1% bromophenol blue, 10% glycerol and 50 mM Tris-HCl, pH 6.8), heated for five minutes at 80°C and clarified by centrifugation for five minutes. SDS-PAGE and Western blot were carried out as previously described [22, 23]. Briefly, proteins were separated on 12% (29:1) Laemmli gel and transferred to PVDF. Following blocking with 1% BSA in TBS resultant membranes were incubated with a mouse anti-cardiac TnI antibody (Fitzgerald; clone C5). Following washes, membranes were incubated with a Dylight labeled fluorescent secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) and visualized on a Typhoon 9410 imager (GE Healthcare, Piscataway, NJ). Differential migration of endogenous mouse TnI vs. exogenous exchanged human TnI allowed for quantification by densitometry analysis. Phos-tag (Wako Pure Chemical Industries, LTD, Richmond, VA) gel electrophoresis and immunoblot to quantify TnI Tyr-26 phosphorylation was conducted in the same manner except with the inclusion of 0.1 mM Phos-tag acrylamide and 0.1 mM MnCl_2 in casted gels. Phos-tag separation and transfer were conducted according to the manufacturer's protocol.

2.7 Data processing and statistical analysis

Data are presented as mean $\pm$ the standard error of the mean. Steady-state Ca^{2+} binding values were fit with a logistic sigmoid function mathematically equivalent to the Hill equation to determine 50% maximal binding as previously described [5, 11]. Kinetic values of Ca^{2+} dissociation were fit using a program (by P. J. King, Applied Photophysics Ltd.) utilizing the nonlinear Levenberg-Marquardt algorithm [17]. Developed force was plotted against Ca^{2+} and fit to a modified Hill equation to determine the pCa_{50} and Hill values as described above. Results of steady-state Ca^{2+} binding, Ca^{2+} dissociation, Tn exchange and force development experiments were compared by 2-way ANOVA with Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1 TnI Tyr-26 phosphorylation decreases Ca^{2+} -dependent thin filament activation

While recent work has demonstrated TnI Tyr-26 phosphorylation is present in the normal human heart and is decreased during heart failure, the functional effects of this post-translational modification remained to be elucidated [1]. To investigate the functional properties elicited by TnI Tyr-26 phosphorylation, we generated TnI containing actual phosphate at tyrosine residue 26. Human recombinant TnI containing native Tyr-26, but Tyr-29 and 112 mutated to the non-phosphorylatable phenylalanine (TnI Y29/112F), was treated with Src or Lyn tyrosine kinase to induce Tyr-26 phosphate incorporation. Phos-tag

Western blot with an anti-TnI antibody demonstrated both Src and Lyn kinase treatment resulted in the appearance of a single phosphorylated species (Fig 1A). Phos-tag of Lyn kinase treated TnI containing all 3 tyrosine residues mutated to phenylalanine (TnI Y26/29/112F) yielded no phosphorylated TnI species verifying Lyn incorporation of phosphate solely at Tyr-26 (data not shown). Large scale treatment of TnI resulted in greater than 90% actual phosphate incorporation at Tyr-26. This resultant TnI containing phosphate at Tyr-26 was TnC-affinity purified and reconstituted with T53C IAANS labeled TnC and TnT to form human Tn complex. The effect of Tn containing TnI Tyr-26 phosphate (TnI Y26Phos) on Ca^{2+} regulation of the thin filament was determined by measuring the change in IAANS fluorescence upon steady-state Ca^{2+} binding to TnC in the reconstituted thin filament. Consistent with our previous studies, thin filaments containing WT TnI exhibited a pCa_{50} of 5.75 [5]. The binding of Ca^{2+} to TnC in thin filaments containing TnI Y26Phos was significantly decreased compared to filaments containing WT TnI (Fig 1B and Table 1). This decreased Ca^{2+} -sensitive binding was the result of TnI Tyr-26 phosphate incorporation as Ca^{2+} binding in filaments containing TnI Y29/112F was identical to those of WT filaments (Fig 1C). To determine if TnI pseudo-phosphorylation at Tyr-26 was sufficient to mimic the effect of actual phosphate incorporation, we generated TnI with Tyr-26 mutated to aspartic acid (TnI Y26D) or glutamic acid (TnI Y26E). Thin filaments containing either TnI Y26D or TnI Y26E both exhibited decreased Ca^{2+} binding to TnC that was of a similar magnitude to TnI Y26Phos filaments (Fig 1D and Table 1). Finally, to determine if the TnI Y26Phos, Y26E and Y26D effects resulted from the structural effects of altered charge we investigated TnI Tyr-26 mutated to phenylalanine (TnI Y26F) as a conservative, null mutation. Ca^{2+} binding to TnC in thin filaments containing TnI Y26F was not different from that of WT filaments, demonstrating the structural mutation of Tyr-26 to phenylalanine alone is not sufficient to elicit altered Ca^{2+} binding (Fig 1C). These findings demonstrated that both the incorporation of actual phosphate and the pseudo-phosphorylation of TnI at Tyr-26 similarly decrease Ca^{2+} binding to TnC in the reconstituted thin filament.

3.2 TnI Tyr-26 phosphorylation decreases Ca^{2+} -sensitive force production

To assess the effect of TnI Tyr-26 phosphorylation on force generation, we measured Ca^{2+} -dependent force development in left ventricular rat skinned myocytes exchanged with human cardiac Tn containing either WT or Y26E TnI. Since recombinant human cardiac TnI migrates slower by SDS-PAGE than endogenous rat cardiac TnI, the exchange percentage of all measured myocyte preparations was determined by Western blot. Western blot for cardiac TnI demonstrated 53% of the endogenous rat TnI was replaced by exogenous human TnI WT and 51% was replaced by TnI Y26E. (Fig 2A). This replacement was not statistically different between any of the exchanged groups and was previously demonstrated as sufficient to obtain a maximal functional effect for TnI S23/24D [24]. Following exchange, resultant myocytes were subjected to varied concentrations of Ca^{2+} and the developed force was measured. Consistent with our Ca^{2+} binding measurements, force- Ca^{2+} measurements demonstrate the exchange of Tn containing TnI Y26E decreased the pCa_{50} by 0.09 units compared to Tn containing TnI WT (Fig 2B). The exchange of Tn containing TnI Y26E did not differ from Tn containing TnI WT in maximal force production or the Hill coefficient (Table 2). Thus, the TnI Tyr-26 phosphorylation induced decrease in Ca^{2+} binding to TnC results in a decreased Ca^{2+} sensitivity of cardiac muscle force production.

3.3 Troponin I Tyr-26 phosphorylation accelerates thin filament deactivation

The N-terminal phosphorylation of TnI at Ser-23/24 accelerates the dissociation of Ca^{2+} from TnC and accelerates cardiac muscle relaxation kinetics [5, 25, 26]. Considering phosphate incorporation at Tyr-26 is located in the TnI N-terminal extension, we hypothesized Tyr-26 phosphorylation would similarly accelerate Ca^{2+} dissociation from TnC and thin filament deactivation. To assess the rate of Ca^{2+} dissociation from TnC in the thin filament, we utilized stopped-flow fluorimetry to monitor the change in fluorescence of IAANS-labeled TnC in Ca^{2+} saturated thin filaments following rapid mixing with EGTA. Consistent with our previous work [5], thin filaments containing TnI WT exhibited Ca^{2+} dissociation from TnC of 100/s (Fig 3). As shown in Figure 3, TnI Tyr-26 pseudo-phosphorylation significantly accelerated the rate of Ca^{2+} dissociation from TnC in the thin filament by 3.1-fold compared to filaments containing TnI WT. Thus, similar to TnI Ser-23/24, TnI Tyr-26 phosphorylation dramatically accelerates thin filament deactivation.

3.4 TnI Tyr-26 differentially modulates Ser-23/24 phosphorylation Ca^{2+} sensitivity and dissociation

The basal levels of both TnI Ser-23/24 and Tyr-26 phosphorylation identified in the non-failing human heart are decreased during heart failure [1]. We have demonstrated that the functional effects of TnI Ser-23/24 phosphorylation are dependent upon its functional integration with other TnI phosphorylation events [5, 6]. We therefore sought to investigate the effect of Tyr-26 functional integration on Ser-23/24 phosphorylation function when integrated as may occur in the human heart. Toward this end, we employed TnI containing dual pseudo-phosphorylation substitutions of Ser-23/24 and Tyr-26 (TnI S23/24D+Y26E). Thin filament Ca^{2+} binding experiments demonstrate TnI Y26E, TnI S23/24D and TnI S23/24D+Y26E all exhibit similarly decreased TnC Ca^{2+} binding compared to filaments containing TnI WT (Fig 4A, Table 1). To investigate the effect of functional integration on force development, endogenous Tn in rat myocytes were exchanged with recombinant human cardiac Tn. Western blot demonstrated Tn exchange resulted in 52% exchange for Tn containing TnI S23/24D and 58% for TnI S23/24D+Y26E (Fig 4B). This replacement was not statistically different between any of the exchanged groups. Similar to Ca^{2+} binding experiments, the Ca^{2+} sensitivity of force development in myocytes exchanged with Tn containing TnI S23/24D+Y26E was not further decreased compared to that of myocytes exchanged with Tn containing TnI S23/24D alone and was similar to that of TnI Y26E exchange alone (Fig 4C, Table 2). Interestingly, the combination of TnI S23/24D+Y26E did further accelerate the rate of thin filament Ca^{2+} dissociation by 1.7 fold compared to filaments containing TnI S23/24D or TnI Y26E alone (Fig 4D). Thus, while the functional integration of TnI S23/24D+Y26E does not impart additional Ca^{2+} desensitization compared to TnI S23/24D or TnI Y26E alone, its integration does further accelerate thin filament deactivation. The integration of Tyr-26 with Ser-23/24 phosphorylation therefore differentially accelerates thin filament deactivation without further decreasing Ca^{2+} sensitivity.

4. Discussion

Post-translational modifications of myofilament proteins represent a significant mechanism that modulates heart function to match supply with demand by affecting cardiac contraction [27, 28]. The current study aimed to assess the functional properties elicited by the novel N-terminal phosphorylation of TnI at Tyr-26 recently identified in the human heart [1] and its functionally integrated effects on TnI Ser-23/24 phosphorylation. Our major findings demonstrate: 1) Phosphate incorporation and pseudo-phosphorylation at TnI Tyr-26 decreases Ca^{2+} -sensitive development of force and thin filament activation with a magnitude similar to that of TnI Ser-23/24 phosphorylation. 2) TnI Tyr-26 pseudo-phosphorylation accelerates thin filament Ca^{2+} dissociation with a magnitude similar to TnI Ser-23/24 pseudo-phosphorylation. 3) The integration of TnI Tyr-26 and Ser-23/24 pseudo-phosphorylation on the same TnI molecule maintains Ca^{2+} desensitization similar to each individual pseudo-phosphorylation by itself but further accelerates thin filament deactivation greater than either pseudo-phosphorylation alone. These findings demonstrate that while TnI Tyr-26 phosphorylation by itself functions similarly to TnI Ser-23/24 phosphorylation, the functional integration of TnI Tyr-26 on Ser-23/24 imparts a novel mechanism to differentially accelerate thin filament deactivation without further decreasing Ca^{2+} sensitivity.

4.1 TnI Tyr-26 phosphorylation regulatory effects

The contractile and thin filament regulatory effects of TnI Tyr-26 represent the first known myofilament protein tyrosine phosphorylation to directly modulate cardiac function. Tyrosine kinase phosphorylation events in the heart are rare, with reports suggesting only about 2% of all cardiac muscle phosphorylation events occur on tyrosine [29]. Therefore, unlike serine or threonine kinase signaling pathways that induce phosphorylation of multiple myocardial proteins, the low number of proteins phosphorylated through tyrosine kinase signaling provides a relatively specific signaling pathway to modulate cardiac contraction. Targeting TnI Tyr-26 phosphorylation through tyrosine kinase signaling may therefore provide a more specific mechanism to directly modulate the thin filament regulation of contraction in the absence of concurrently altering Ca^{2+} cycling associated with Protein Kinase A (PKA) dependent TnI phosphorylation.

The Tyr-26 pseudo-phosphorylation mutation is sufficient to recapitulate Tyr-26 phosphate effects on Ca^{2+} sensitivity (Fig 1B,D) and therefore provides a suitable tool to investigate TnI Tyr-26 phosphorylation function. We further demonstrate that the desensitization effect of TnI Tyr-26 phosphorylation results from the incorporation of the negative charge at this residue as the structural change to phenylalanine did not alter sensitivity (Fig 1C). These data demonstrate TnI Tyr-26 phosphorylation is a significant post-translational modification to modulate cardiac force development and therefore may play a significant role in contractile regulation of the heart.

The removal of Ca^{2+} from TnC and subsequent thin filament deactivation is necessary for cardiac relaxation and the rate of this removal is critical to lusitropy [30, 31]. Such increased cardiac contractile relaxation is a significant effect of the classical TnI Ser-23/24 phosphorylation [25, 26]. Based upon the similar effect of TnI Tyr-26 and Ser-23/24

phosphorylation to decrease Ca^{2+} sensitivity, we proposed TnI Tyr-26 phosphorylation would also enhance thin filament deactivation. In support of this hypothesis, we demonstrate TnI Tyr-26 pseudo-phosphorylation accelerates Ca^{2+} dissociation from TnC with a magnitude similar to that of TnI Ser-23/24 alone (Fig 3 and Fig 4C). While the effect of TnI Tyr-26 on *in vivo* muscle relaxation remains to be tested, this finding supports the significance of Tyr-26 phosphorylation to enhance thin filament deactivation in the absence of crossbridges, a property that may be necessary for increased cardiac relaxation to maintain cardiac output during increased heart rate.

While the kinase responsible for the phosphorylation of TnI Tyr-26 in the heart is currently unknown, the Src tyrosine kinase family members are an appealing possibility. Src family members are expressed in the cardiac myocyte cytosol with specific members activated during cardiac ischemia [32]. We demonstrate the Src family kinase members Lyn and cSrc phosphorylate TnI Tyr-26 *in vitro* (Fig 1A). Interestingly, the Src kinase family recognition sequence (EEXY) is similar to the TnI sequence surrounding Tyr-26 (SSNY) [33] that could become a better substrate for Src when TnI Ser-23/24 contains negatively charged phosphate ($\text{S}_{(P)}\text{S}_{(P)}\text{NY}$). It is intriguing to consider a possible coupling of Tyr-26 Src and Ser-23/24 PKA phosphorylation. If this were true, Src family regulation of Tyr-26 phosphorylation could explain the concurrent decrease in both Tyr-26 and Ser-23/24 phosphorylation observed in the failing heart [1]. Tyrosine kinase and phosphatase signaling to the myofilament are significantly understudied and it is currently unknown if TnI Tyr-26 phosphorylation in the heart is modulated by the Src kinase family, tyrosine phosphatase, TnI Ser-23/24 linked phosphorylation or another tyrosine kinase pathway. Further studies are warranted and ongoing to resolve the signaling events responsible for TnI Tyr-26 phosphorylation in the heart.

4.2 TnI Tyr-26 integration on Ser-23/24 phosphorylation function

The functional regulatory effects of the classical TnI Ser-23/24 phosphorylation are dependent upon the myofilament post-translational background [34]. We previously demonstrated TnI Ser-150 phosphorylation as a specific mechanism to alter Ser-23/24 functional effects [5, 6]. TnI Tyr-26 is therefore the second TnI phosphorylation we have identified to exhibit differential functional integration with Ser-23/24 [5] (Fig 4). The molecular mechanism of how isolated TnI phosphorylation can alter Ca^{2+} sensitivity and Ca^{2+} dissociation while integrated phosphorylation combination does not further alter Ca^{2+} sensitivity but does further accelerate Ca^{2+} dissociation is unclear. The cardiac TnI N-terminal extension (residues 1–32), which bears the Tyr-26 residue, has been demonstrated to directly interact with the Ndomain of TnC [35]. NMR studies demonstrate the cardiac TnI extension contacts the N-domain of TnC near the regulatory Ca^{2+} binding site [35–37] and these interactions are weakened when Ser-23/24 are phosphorylated or substituted with phospho-mimetics [36, 38, 39]. Recent data also suggests this weakening of the interaction between the TnI extension and N-domain of TnC invokes a conformational shift in which acidic residues of the TnI extension interact with conserved basic residues of the TnI Cterminal inhibitory peptide [40]. This state is proposed to allow the TnI extension to modulate inhibitory peptide binding to actin. Similar to the proposed effects of TnI Ser-23/24 phosphorylation, we predict TnI Tyr-26 phosphorylation to similarly weaken the

interaction of the TnI extension with the TnC N-domain, extending the TnI N-terminal helix and invoking interaction with the inhibitory peptide. Indeed, the truncation of the cardiac TnI extension has been shown to fully mimic the Ca^{2+} desensitization effect of Ser-23/24 phosphorylation supporting TnI Ser-23/24 phosphate induced repulsion is an all-or-none effect [22, 41]. Our results demonstrating the integration of TnI Tyr-26 with Ser-23/24 phosphorylation does not further decrease Ca^{2+} sensitivity beyond that of either phosphorylation alone (Fig 4A) suggests both TnI Tyr-26 and Ser-23/24 phosphorylation act through similar Tn interactions to alter Ca^{2+} sensitivity. However, the further acceleration of Ca^{2+} dissociation from the thin filament upon TnI Tyr-26 phosphorylation integration (Fig 4D) suggests additional mechanisms are involved in this functional effect. Ca^{2+} association has generally been considered to be diffusion controlled; yet we have demonstrated this is not necessarily the case [14, 42–45]. We propose this differential regulation of Ca^{2+} sensitivity and Ca^{2+} dissociation therefore involves the modulation of both Ca^{2+} association and dissociation. No matter the mechanism, TnI phosphorylation integration on TnI Ser-23/24 function is not solely limited to Ser-150 but also includes Tyr-26. TnI phosphorylation functional integration may therefore be a common mechanism to modulate Ser-23/24 function. Future structural experiments are necessary to elucidate the basis for the Tyr-26 phosphorylation induced functional properties reported here, and whether they are consistent with structural data of Ser-23/24 phosphorylation.

4.3 The role of TnI Tyr-26 phosphorylation in heart function

The presence of basally phosphorylated TnI Tyr-26 [1] suggests its role in the maintenance of normal physiological heart function. Our finding that TnI Tyr-26 phosphorylation integration affects Ser-23/24 phosphorylation function (Fig. 4) further implies a sophisticated modulatory role. The addition of Ser-23/24 phosphorylation to a TnI molecule that already contains Tyr-26 phosphorylation does not further decrease myofilament Ca^{2+} sensitivity (Fig 4A). This basal presence of TnI Tyr-26 phosphorylation may therefore buffer the Ser-23/24 phosphorylation desensitization effect but also allows for an exaggerated Ca^{2+} -sensitization response when both phosphorylations are removed. However, since the integration of TnI Ser-23/24 with Tyr-26 phosphorylation does further accelerate thin filament deactivation (Fig 4D) their integration may be more relevant to increase cardiac lusitropy rather than alter contractility. Ultimately these biochemical measurements do not completely recapitulate *in vivo* cardiac function. It will therefore be important in the future to determine how this TnI phosphorylation integration induced differential change in Ca^{2+} sensitivity and Ca^{2+} dissociation will actually translate to affect *in vivo* cardiac contractility and lusitropy.

In addition to its physiological regulatory effects, TnI Tyr-26 phosphorylation is also implicated in contractile modulation during heart failure [1]. Both TnI Ser-23/24 and Tyr-26 phosphorylation are decreased in the failing human heart [1–4]. Similar to that proposed for TnI Ser-23/24, the decrease in Tyr-26 phosphorylation is likely to contribute to the increased Ca^{2+} sensitivity observed during heart failure [46–48]. Whether the functional effects of these concurrent TnI phosphorylation alterations are maladaptive or compensatory to cardiac contractile dysfunction remains to be determined.

5. Conclusions

In the data reported here, we demonstrate TnI Tyr-26 phosphorylation functions similarly to Ser-23/24 in isolation, but differentially alters Ca^{2+} sensitivity and dissociation upon integration with Ser-23/24 phosphorylation. Thus, in addition to our previous report that Ser-150 phosphorylation imparts TnI functional integration [5, 6], we now demonstrate TnI Tyr-26 phosphorylation also exhibits functional integration on the effects of Ser-23/24. This second report of integration, or crosstalk at the functional level, supports a common mechanism of TnI phosphorylation integration to differentially modulate contractility and lusitropy. These effects of the novel TnI Tyr-26 to alter myofilament regulation identifies the first thin filament tyrosine phosphorylation to modulate contractile function and therefore presents a novel pathway to modulate heart function.

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The abbreviations used are

Tn	troponin
TnI	troponin I
TnC	troponin C
IAANS	2-(4'-iodoacetamidoanilo)naphthalene-6-sulfonic acid
WT	wild type
PKA	protein kinase A

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Highlights

- TnI Y26 is the first Tyr residue demonstrated to alter contractile function.
- TnI Y26 phosphorylation decreases calcium sensitivity and accelerates deactivation.
- Combined Y26 and S23/24 phosphorylation does not further decrease sensitivity.
- TnI Y26 and S23/24 phosphorylation further accelerates thin filament deactivation.

Fig. 1. TnI Tyr-26 phosphorylation decreases Ca^{2+} sensitivity of the thin filament

Phos-tag electrophoresis and representative TnI Western blot of TnI Tyr-26 phosphorylation following treatment with Src or Lyn tyrosine kinase (**A**). Change in IAANS fluorescence resulting from Ca^{2+} binding to TnC in thin filaments containing non-phosphorylated wild-type TnI (*TnI WT*, black circle), TnI containing Tyr-26 actual phosphate (*TnI Y26-Phosphate*, gray triangles) (**B**), TnI containing null mutations (*TnI Y26F*, gray triangles and *TnI Y29/112F*, black squares) (**C**), or pseudo-phosphorylated TnI Tyr-26 (*TnI Y26D*, gray triangles and *TnI Y26E*, black squares) (**D**). OP, TnI containing no phosphate; 1P, TnI containing a single phosphate.

Fig. 2. TnI Tyr-26 pseudo-phosphorylation decreases Ca^{2+} -sensitive force development

TnI Western blot determination of Exogenous Human Tn exchange into skinned rat cardiac myocytes. Representative blots of myocytes for each exchange are shown. Quantification for each exchange group was conducted by densitometry analysis and the average is displayed in the graph below. RcTnI; purified rat cardiac TnI, HcTnI; purified human cardiac TnI, Control; sham buffer exchanged, WT; exchanged with Tn containing TnI WT, Y26E; exchanged with Tn containing TnI Y26E (A). Average force-pCa measurements of skinned

rat cardiac myocytes exchanged with Tn containing non-phosphorylated wild-type TnI (*TnI* WT, black circles) or TnI Y26E (*TnI* Y26E, gray triangles) (**B**).

Fig. 3. TnI Tyr-26 pseudo-phosphorylation accelerates Ca^{2+} dissociation from TnC on the thin filament

Representative change in IAANS fluorescence of stopped-flow Ca^{2+} dissociation from thin filaments containing non-phosphorylated wild-type TnI (*TnI WT*, black line, $100.3 \pm 1.9/\text{s}$, $n=12$), or pseudo-phosphorylated TnI Tyr-26 (*TnI Y26D*, light grey line, $278.0 \pm 13.1/\text{s}$, $n=18$ and *TnI Y26E*, dark grey line, $305.6 \pm 19.4/\text{s}$, $n=18$). Traces are normalized and staggered for clarity. *a*, significantly different from TnI WT $p < 0.001$.

Fig. 4. TnI Tyr-26 integration with Ser-23/24 phosphorylation differentially modulates Ca^{2+} sensitivity and Ca^{2+} dissociation

Change in IAANS fluorescence resulting from Ca^{2+} binding to TnC in thin filaments containing non-phosphorylated TnI (TnI WT, closed circle), Ser-23/24 pseudo-phosphorylated TnI (TnI S23/24D, gray triangles) or combined Ser-23/24 and Tyr-26 pseudo-phosphorylated TnI (TnI S23/24D+Y26E, black squares) (A). TnI Western blot determination of Exogenous Human Tn exchange into skinned rat cardiac myocytes. Representative blots of myocytes for each exchange are shown. Quantification for each exchange group was conducted by densitometry analysis and the average is displayed in the graph below. RcTnI; purified rat cardiac TnI, HcTnI; purified human cardiac TnI, Control; sham buffer exchanged, S23/24D; exchanged with Tn containing TnI S23/24D, S23/24D+Y26E; exchange with Tn containing TnI S23/24D+Y26E (B). Average force-pCa measurements of skinned rat cardiac myocytes exchanged with Tn containing wild-type TnI (TnI WT, black circles), Ser-23/24 pseudo-phosphorylated TnI (TnI S23/24D, gray triangles), or combined Ser-23/24 and Tyr-26 pseudo-phosphorylated TnI (TnI S23/24D+Y26E, black squares) (C). Representative change in IAANS fluorescence of stopped-flow Ca^{2+} dissociation from thin filaments containing nonphosphorylated wild-type TnI (TnI WT,

*black line, $100.3 \pm 1.9/s$, $n=12$), Ser-23/24 pseudo-phosphorylated TnI (*TnI S23/24D*, *light grey line, $274.1 \pm 14.3/s$, $n=28$*) and combined Ser-23/24 and Tyr-26 pseudo-phosphorylated TnI (*TnI S23/24+Y26E*, *dark grey line, $477.36 \pm 25.9/s$, $n=14$*). Traces are normalized and staggered for clarity. *a*, significantly different from TnI WT $p<0.001$; *b*, significantly different from TnI S23/24D $p<0.001$ (**D**).*

Table 1Thin filament steady-state Ca^{2+} binding.

Tn	Ca^{2+} binding		n
	pCa ₅₀	nHill	
Tn WT	5.75±0.01	2.30±0.20	5
Tn Y26-Phos	5.53±0.02 <i>a, b, c</i>	2.24±0.02	3
Tn Y26E	5.51±0.04 <i>a, b, c</i>	3.79±1.06	3
Tn Y26D	5.50±0.01 <i>a, b, c</i>	2.41±0.35	3
Tn S23/24D	5.49±0.02 <i>a, b, c</i>	3.79±1.06	5
Tn S23/24D+Y26E	5.49±0.02 <i>a, b, c</i>	2.41±0.35	3
Tn Y26F	5.76±0.04	2.39±0.05	3
Tn Y29/112F	5.73±0.02	2.79±0.19	3

Values are mean ± SEM. pCa₅₀, -log[Ca²⁺] at 50% activation; nHill, Hill coefficient; n, number of measurements in each group. One-way ANOVA Tukey post-hoc test demonstrates a significant interaction of TnI WT and null mutants versus TnI pseudo-phosphorylation type.

^a significantly different from WT p<0.001;

^b significantly different from Y26F p<0.001;

^c significantly different from Y29/112F p<0.001.

Table 2

Force development in skinned myocytes.

Tn	Force			n
	pCa ₅₀	nHill	Fmax (mN/mm ²)	
Tn WT	5.72±0.02	2.91±0.14	11.60±0.90	16
Tn Y26E	5.63±0.01 ^a	3.41±0.19	9.64±0.9	13
Tn S23/24D	5.65±0.01 ^a	2.83±0.18	10.30±0.99	10
Tn S23/24D+Y26E	5.65±0.01 ^a	3.04±0.17	11.60±0.81	11

Values are mean ± SEM. pCa₅₀, -log[Ca²⁺] at 50% activation; nHill, Hill coefficient; n, number of skinned myocyte preparations in each group from at least 3 heart preparations. One-way ANOVA Tukey post-hoc test demonstrates a significant interaction of TnI WT versus TnI pseudo-phosphorylation type.

^a p<0.001;