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The multifunctional Ca²⁺/calmodulin-dependent protein kinase II delta (CaMKII δ) phosphorylates cardiac titin's spring elements

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Abstract

Titin-based passive stiffness is post-translationally regulated by several kinases that phosphorylate specific spring elements located within titin's elastic I-band region. Whether titin is phosphorylated by calcium/calmodulin dependent protein kinase II (CaMKII), an important regulator of cardiac function and disease, has not been addressed. The aim of this work was to determine whether CaMKII δ , the predominant CaMKII isoform in the heart, phosphorylates titin, and to use phosphorylation assays and mass spectrometry to study which of titin's spring elements might be targeted by CaMKII δ . It was found that CaMKII δ phosphorylates titin in mouse LV skinned fibers, that the CaMKII δ sites can be dephosphorylated by protein phosphatase 1 (PP1), and that under baseline conditions, in both intact isolated hearts and skinned myocardium, about half of the CaMKII δ sites are phosphorylated. Mass spectrometry revealed that both the N2B and PEVK segments are targeted by CaMKII δ at several conserved serine residues. Whether phosphorylation of titin by CaMKII δ occurs *in vivo*, was tested in several conditions using back phosphorylation assays and phospho-specific antibodies to CaMKII δ sites. Reperfusion following global ischemia increased the phosphorylation level of CaMKII δ sites on titin and this effect was abolished by the CaMKII inhibitor KN-93. No changes in the phosphorylation level of the PEVK element were found suggesting that the increased phosphorylation level of titin in IR might be due to phosphorylation of the N2B element. The findings of these studies show for the first time that titin can be phosphorylated by CaMKII δ , both *in vitro* and *in vivo*, and that titin's molecular spring region that determines diastolic stiffness is a target of CaMKII δ .

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Introduction

Abnormal cardiac filling caused by increased diastolic chamber stiffness is an important factor in the pathophysiology of a range of cardiac diseases, including the highly prevalent heart failure with preserved ejection fraction (HFpEF) syndrome [1]. However, the mechanisms that determine the level of diastolic stiffness and its various tuning mechanisms are not completely understood. An important determinant of diastolic stiffness is the giant protein titin that spans from Z-disk to M-band of the cardiac sarcomere and has an elastic I-band region that functions as a diastolic stiffness generating molecular spring [2]. Titin's molecular spring region can be restructured through alternative splicing that gives rise to titin isoforms with spring elements that vary in length and generate different levels of passive stiffness [3]. However, changes in splicing are slow processes that require days to weeks to be accomplished and in recent years it has become apparent that phosphorylation-based mechanisms exist that rapidly tune stiffness. The two main spring elements of cardiac titin, the so-called N2B and PEVK elements, are both targeted by kinases. The PEVK spring element has been shown to be phosphorylated by protein kinase C α (PKC α), a key player in contractile dysfunction and heart failure [4, 5]; single molecule, single cell and myocardial tissue experiments in wildtype and PEVK KO mice have shown that PKC α phosphorylation of the PEVK element *increases* passive stiffness [6–8]. The N2B element of titin is also a kinase substrate whose mechanical properties change following phosphorylation. Protein kinase A (PKA), which is stimulated by the β -adrenergic pathway, phosphorylates the large unique sequence of the N2B element which *reduces* passive stiffness [9, 10]. Similar to PKA, protein kinase G (PKG), a cGMP-dependent kinase that is part of signaling cascades initiated by nitric oxide (NO) and natriuretic peptides (NPs), phosphorylates the unique sequence of the N2B element and reduces passive stiffness; the PKG phosphorylation site in humans (S469) is also a residue targeted by PKA [11].

In this study we focused on the Ca²⁺ and calmodulin dependent serine/threonine kinase (CaMKII) that is activated by increases in cellular Ca²⁺. Four isoforms have been described (α , β , δ , γ) of which CaMKII δ is the predominant isoform in the heart [12]. CaMKII δ phosphorylates several Ca²⁺-handling proteins including phospholamban (PLB) [13, 14], ryanodine receptor (RyR2) [15–17], and L-type Ca²⁺ channel (LTCC) [18], as well as myofilament proteins including TnT [19] and MyBP-C [20]. Our aim was to determine whether CaMKII δ also phosphorylates titin, and to use phosphorylation assays and mass spectrometry to study which of titin's spring elements might be phosphorylated by CaMKII δ . We found that CaMKII δ phosphorylates titin in mouse LV skinned fibers, that the CaMKII δ sites can be dephosphorylated by protein phosphatase 1 (PP1), and that about half of the CaMKII δ sites are phosphorylated under baseline conditions in isolated hearts and skinned myocardium. We also found that the N2B and PEVK segments are targeted by CaMKII δ at several conserved sites. Whether phosphorylation of titin by CaMKII δ occurs *in vivo* was tested in several conditions, including ischemia reperfusion (IR), that is known to activate CaMKII δ [21]. IR induced an increase in the phosphorylation level of CaMKII δ sites on titin and this was abolished by the CaMKII inhibitor KN-93. Findings of this work have been presented previously in abstract format [22].

Material and Methods

In vitro phosphorylation assay of skinned myocardium

All experiments were performed on 3 month old male C57BL/6J mice and were approved by the University of Arizona IACUC and followed the U.S. National Institutes of Health "Using Animals in Intramural Research" guidelines for animal use. Skinned fibers isolated from the left ventricular (LV) wall [23] were incubated for 2h at 30°C with 0.05 U/ μ l human CaMKII δ (expressed in insect cells, Invitrogen, CA USA), in kinase buffer (25 mM BES, 1

mM CaCl₂, 5 μM calmodulin, 4 mM NaATP, 4 mM MgCl₂, 1 mM DTT, 50 μM protein kinase A inhibitor (Sigma), 5 mM NaF, 1 mM Na₃VO₄ pH 7.0), and 10 μCi of [γ -³²P]ATP (specific activity 3,000 Ci/mmol, Perkin-Elmer). Some fibers were dephosphorylated by incubation with 0.75 U/μl of protein phosphatase 1 (PP1; recombinant rabbit muscle α -isoform, Calbiochem), 2h at 30°C, followed by extensive washing and then incubation with CaMKII δ . The reaction was stopped by adding solubilization buffer (6 M urea, 1.5 M thiourea, 2.25% SDS, 56.25 mM DTT, 0.0225% bromophenol blue, 35% glycerol, 0.0825 mg/ml leupeptin, 1 mM E-64, 0.015 mM PMSF, 37.5 mM Tris-HCl pH 6.8) and the proteins were separated on 2–7% SDS-PAGE gel gradient. The gels were stained with Coomassie blue, dried, scanned, and exposed to X-ray film and analyzed. The titin optical density (OD) of the autoradiograph was normalized to that of the Coomassie blue-stained gel, to normalize for protein loading. In a subset of experiments Western Blots were used to detect phosphorylation of S26 and S170 in the PEVK region of the N2B cardiac titin isoform. Skinned fibers were solubilized and proteins were separated on 0.8% agarose gel and transferred to PVDF membrane (Millipore). The membranes were stained with Ponceau S (Sigma) to determine the level of transferred proteins. The membranes were probed with phospho-specific rabbit polyclonal antibodies against titin's p-S26 and p-S170[24]. Secondary antibodies conjugated with fluorescent dyes (Biotium, Hayward, Ca, USA) with infrared excitation spectra were used for detection and membranes scanned and analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences).

In vitro kinase assay of titin recombinant proteins

The human titin recombinant fragments N2B, PEVK (from cardiac N2B isoform), Ig8-15, Ig84-91 and the murine recombinant N2B were expressed in *E coli* as describe [25]. The purified recombinant proteins were incubated with 0.05 U/μl human recombinant CaMKII δ (Invitrogen, CA USA), kinase buffer (see above), and 10 μCi of [γ -³²P]ATP (Perkin-Elmer), 2h at 30°C. The proteins were solubilized (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1 mM 2-mercaptoethanol, 0.01% bromophenol blue) and separated on 4–20% gradient or 12% SDS-PAGE. The gels were Coomassie blue stained, dried, scanned, exposed to X-ray film, and analyzed as described above. We also probed the phosphorylation levels of PEVK's S26 and S170 in recombinant protein using Western blots. The human titin recombinant fragment PEVK expressed in *E coli* including WT, mutants S26A, S170A, and S26A/S170A[24] were incubated for 2h at 30°C with 0.05 U/μl human recombinant CaMKII δ (Invitrogen, CA USA) in kinase buffer (see above), and 10 μCi of [γ -³²P]ATP (Perkin-Elmer). The proteins were solubilized (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1 mM 2-mercaptoethanol, 0.01% bromophenol blue) and separated on 10% SDS-PAGE. The gels were Coomassie blue stained, dried, scanned, exposed to X-ray film, and analyzed as described above. Additionally some gels were also transferred to PVDF membrane (Millipore) and blotted with phospho-specific antibodies against p-S26 and p-S170 (for details, see above).

Tandem mass spectrometry coupled to liquid chromatography

Nonphosphorylated and CaMKII δ -phosphorylated human and murine titin N2B recombinant proteins (see above) were electrophoresed and Coomassie blue stained. The N2B bands were excised from the gels and digested with trypsin or chymotrypsin. Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) analyses were carried out (LTQ Orbitrap Velos Thermo Scientific Inc., MA USA) and tandem MS spectra of peptides were analyzed with TurboSEQUENT. Various possible modifications such as alkylation of cysteine residues and of methionine residues and phosphorylation were included in the search parameters.

Isoproterenol stimulation of intact cardiac myocytes

Cells were isolated as described previously [26, 27]. In brief, the heart was cannulated via the aorta and perfused for 4 min with perfusion buffer (in mM: 113 NaCl, 4.7 KCl, 0.6 KH_2PO_4 , 0.6 Na_2HPO_4 , 1.2 MgSO_4 , 12 NaHCO_3 , 10 KHCO_3 , 10 HEPES, 5.5 glucose, 5 BDM, 10 taurine, 20 Creatine, 5 Adenosine, 5 Inosine adjust pH to 7.4 at 37°C), followed by perfusion with digestion buffer (perfusion buffer plus 0.06 mg/ml of TM liberase [Liberase TM research grade medium thermolysin concentration; Roche Applied Science, IN USA], and 12.5 μM CaCl_2) for 8–10 min. The left ventricle was cut into small pieces that were triturated several times with a transfer pipette and then filtered through a 300- μm nylon mesh filter. The cells were gravity pelleted and Ca^{2+} was reintroduced to a final concentration of 1.8 mM. Some of the cells were solubilized and electrophoresed [28, 29] to determine titin content. Activation of CaMKII δ was determined indirectly by the measurement by Western Blot of the phosphorylation level of the specific CaMKII δ target Thr17 on phospholamban, PLB [30, 31]. The cardiomyocytes were stimulated as described by Erickson et al [32] with some modifications. Freshly prepared calcium tolerant cardiomyocytes in minimum essential medium α (MEM α) (Invitrogen) supplemented with 1.8 mM calcium chloride were transferred to Petri dish coated with 0.01% poly-L-lysine (Sigma). The myocytes were allowed to adhere for 30 min at 37°C, 5% CO_2 in a CO_2 incubator (Thermo Scientific). The cell suspension was paced (1 Hz) and 1 μM Okadaic acid (Sigma) was added and the cells incubated for 10 min. The cells were electrically stimulated (paced) using a 6 well C-Dish electrode assembly in conjunction with a C-Pace EP cell culture stimulator (IonOptix). 1 μM isoproterenol (Sigma) was added to the cardiomyocytes and the cells paced at 1 Hz for 20 min. One set of cells were pre-incubated with 1 μM of the CaMKII δ inhibitor KN93 (Calbiochem) for 10 min before the isoproterenol was added. Then the frequency of stimulation was raised to 4 Hz and the cells were paced for an additional 20 min. The medium was removed and the cells were solubilized by adding solubilization buffer (see above) and heated at 65°C for 5 min. The proteins were separated by SDS-PAGE (5%) and transferred to PDVF membrane. The membranes were blotted with rabbit anti-p-Thr17 (Badrilla, Leeds, UK) and mouse anti-PLB (Badrilla, Leeds, UK) as a loading control (in select experiments mouse anti-GAPDH (Abcam) was used as a second loading control. Secondary antibodies conjugated with fluorescent dyes (Biotium, Hayward, Ca, USA) with infrared excitation spectra were used for detection and membranes scanned and analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences).

Ischemia-reperfusion (IR) experiments

Isolated hearts were perfused according to the Langendorff technique, at constant temperature (37°C), at 90mmHg constant pressure (~3 mL/min), and using a pacing rate of 450 beats/min [33]. The mechanical activity of the heart was assessed by passing into the left ventricle a latex balloon connected to a pressure transducer. After stabilization the hearts were perfused for 20 min (pre-ischemia), global ischemia was produced by interruption of the coronary flow for 20 min. after which perfusion was restored (reperfusion). The hearts were flash frozen after different periods of reperfusion. To evaluate the influence of CaMKII we used the cell permeable CaMKII inhibitor KN93 (IR-KN) (Calbiochem). For the IR-KN group, 5 μM KN93 was added in all solutions. After stabilization, the IR and IR-KN hearts were subject to global no-flow ischemia for 20 minutes; control hearts continued to be perfused. After ischemia, hearts were reperfused for 3 minutes and hearts were then rapidly removed and flash frozen in liquid nitrogen or rapidly dissected and permeabilized for back-phosphorylation assays (see above). For titin protein analysis, 0.8% agarose electrophoresis was performed. The muscle samples were solubilized (see above) and electrophoresed. The 0.8% agarose gels were run at 15 mA, 3 h 20 min at 4°C. The proteins were transferred to PVDF membrane. The membranes were stained with Ponceau S (Sigma) to visualize total transferred proteins. The membranes were probed with phospho-specific rabbit polyclonal

antibodies against titin's p-S26 or p-S170[24]. The activation level of CaMKII δ was determined indirectly by the measurement of the phosphorylation level of the specific target Thr17 on phospholamban (PLB) by Western Blot, as stated above. Proteins were separated by SDS-PAGE (15%) and transferred to PVDF membrane (Millipore). The membranes were blotted with rabbit anti-p-Thr17 and mouse anti-PLB (for details, see above).

Statistical Analysis

Data are presented as mean \pm SE. Statistical tests for significance were *t*-tests and ANOVA, as appropriate. Probability values <0.05 were taken as significant.

Results

CaMKII δ phosphorylates cardiac titin

To determine whether titin is phosphorylated by CaMKII δ a back-phosphorylation assay was performed on mouse LV skinned cardiac fibers. The fibers were incubated with CaMKII δ and Ca²⁺/calmodulin in the presence of [γ -³²P]ATP. Results showed phosphorylation of MyBP-C, a well-known CaMKII δ substrate, a weak signal from TnI, and a strong CaMKII δ auto-phosphorylation signal[34] (Fig. 1A). Importantly, titin was also phosphorylated by CaMKII δ . Phosphorylation of titin was mainly on T1, the full length titin molecule, and not on T2 (Fig. 1B). (T2 is a degradation product that is likely to form during sample preparation and that corresponds largely to the A-band portion of the molecule[35].) We also pre-treated skinned muscle with protein phosphatase 1 (PP1) and found that this significantly enhanced CaMKII δ phosphorylation by nearly 3- fold (Fig. 1B and Fig. 1C, left). From the results of the back-phosphorylation assay, the baseline phosphorylation level of the CaMKII δ accessible sites was estimated ((PP1- Ctrl)/PP1) at 60.3 \pm 6.0% (Fig. 1C, right). In summary, the results showed that 1) CaMKII δ phosphorylates titin, 2) CaMKII δ sites on titin can be dephosphorylated by PP1, and 3) the baseline phosphorylation level of CaMKII δ accessible sites in mouse skinned myocardium is relatively high.

CaMKII δ phosphorylates titin's N2B and PEVK elements

The finding that phosphorylation of titin takes place on T1 suggests that CaMKII δ phosphorylates sites in the I-band region of the titin molecule. It was therefore tested whether titin's spring elements are CaMKII δ targets. In vitro phosphorylation assays were performed with recombinant proteins that comprise titin's I-band segments: tandem immunoglobulin segments representing both the proximal and distal tandem Ig segment (Ig8-15 and Ig27-34), the N2B element (both the human and mouse versions), including its flanking Ig domains (Ig24, 25 and 26), and the human PEVK and its flanking Ig domains (see Fig. 2A and Fig. 3A). It was found that CaMKII δ does not phosphorylate the tandem Ig segments (Fig. 2B), but that it does phosphorylate the N2B (Fig. 2B) and PEVK elements (Figs. 3 and 4).

In order to identify the amino acid residues of the N2B element that are phosphorylated by CaMKII δ , nano-LC tandem MS/MS was used. All residues in the N2B unique sequence (except one, see Supplemental Figure 1, residue in grey) were covered in the digests and mass spectrometry detected 9 serine and threonine sites that had been phosphorylated by CaMKII δ (Supplemental Figure 1). A sequence alignment using the N2B sequence of a wide range of species revealed that only two serine residues (S34 and S493 in human N2B unique sequence) are highly conserved (Fig. 2C).

An in vitro kinase assay was also performed on the PEVK element, using the 180 residue PEVK sequence of the human cardiac N2B isoform and its flanking Ig domains I27 and I84 (Fig. 3A). (Note that preliminary phosphorylation studies revealed that reproducible results

devoid of spurious phosphorylation events are only obtained when the flanking Ig domains are present.) The recombinant protein was phosphorylated by CaMKII δ and was then analyzed by nano-LC tandem MS/MS. Five sites were found to be phosphorylated (S26, T70, T80, T117, and S170, see Fig. 3B) of which only S26 and S170 are highly conserved (Fig. 3C). Of these sites S170 (LRPGSG) is a consensus sequence for CaMKII δ (X-R-X-X-S/T-X)[12]. Intriguingly, both S26 and S170 have previously been found to be phosphorylated by PKC α [6], suggesting that both CaMKII δ and PKC α can phosphorylate overlapping titin residues.

Using phospho-specific antibodies to the PEVK's S26 and S170 sites we confirmed that these two sites are phosphorylated by CaMKII δ in wildtype PEVK (Fig. 4A top and bottom, left two lanes) whereas mutation of serine to alanine abolished this effect (Fig. 4A, top and bottom). We also studied the phosphorylation level of the two sites in skinned muscle fibers and found that CaMKII δ increased the level of S26 by $28 \pm 11.3\%$ (n=4) and S170 by $31.9 \pm 12.3\%$ (n=4), respectively.

Mass spectrometry on the PEVK had revealed that in addition to S26 and S170, CaMKII δ also phosphorylates T70, T80 and T117. We addressed the importance of these additional sites in phosphorylation assays with [γ - ^{32}P]ATP and using PEVK WT and S26A, S170A and S26A/S170A PEVK mutants, reasoning that their importance would be revealed by ^{32}P incorporation in the single and double mutants. Mutating S26 and S170 individually to alanine abolished $\sim 50\%$ of the ^{32}P incorporation. Because only S170 is part of a CaMKII δ consensus sequence, we anticipated that it might be a better substrate and would incorporate a larger amount of ^{32}P than S26. However, the obtained results do not support this notion. Interestingly, mutating the S26 and S170 sites simultaneously abolished $\sim 75\%$ of the ^{32}P incorporation (Fig. 4B). This suggests that some or all of T70, T80, and T117 residues are phosphorylated by CaMKII δ and that mutating the two serines alters the likelihood that they become phosphorylated (see also Discussion). However, S26 and S170 are the dominant CaMKII δ targets in the PEVK element.

In vivo CaMKII δ phosphorylation

Whether CaMKII δ phosphorylates titin in vivo was addressed in mouse isolated hearts that underwent an ischemia-reperfusion (IR) protocol, a condition that is wellknown to activate CaMKII δ [12, 31, 36]. It has been shown in the mouse that in IR CaMKII δ is highly activated within minutes of the start of reperfusion and that its activity peaks when reperusing for ~ 3 min [31]. We used back-phosphorylation assays with CaMKII δ on mouse isolated hearts and found 1) an increase in titin phosphorylation following 3 min of reperfusion and 2) that this increase was absent when the experiments were carried out in the presence of the CaMKII inhibitor KN-93 (Fig. 5A). A WB analysis with the PEVK phospho-S26 and phospho-S170 antibodies showed no change in the phosphorylation level of S26 and S170 (Fig. 5B, left and right). We also studied intact cardiac myocytes that were twitch activated and stimulated with isoproterenol (ISO), a condition that has been shown in rabbit cardiac myocytes to cause CaMKII δ activation [32, 37]. Whether this also occurs in mouse myocytes was tested by using the phosphorylation status of phospholamban (PLB) Thr17, a well-known target of CaMKII δ [30]. Indeed we found that ISO increased the phosphorylation level of Thr17 and that this could be inhibited by KN-93 (Fig. 5C). We then probed the phosphorylation level of titin's PEVK S26 and S170 and found that these sites were unaffected by ISO (Fig. 5D, left and right). Thus the experiments show that CaMKII δ sites on titin are accessible in vivo but they do not support that S26 and S170 of the PEVK element are involved.

Discussion

By phosphorylating a wide range of calcium handling and myofilament proteins CaMKII δ regulates systolic and diastolic function and plays important roles in the physiology and pathology of the heart[12, 38, 39]. Using phosphorylation assays and mass spectrometry the present study investigated whether CaMKII δ phosphorylates also titin and we focused on titin's PEVK and the N2B elements, two important spring elements within titin's extensible I-band region. In both skinned fibers and in intact hearts CaMKII δ phosphorylates titin and about half of the CaMKII δ accessible sites are in their phosphorylated state under normal conditions. In vitro kinase assays showed that N2B and PEVK elements are both phosphorylated by CaMKII δ , including at several wellconserved serine residues. Below we discuss these results in detail.

N2B element phosphorylation

Back-phosphorylation assays on skinned fibers showed that cMyBP-C and titin are strong CaMKII δ substrates among the myofilament proteins, with titin ^{32}P incorporation on T1 (full length titin molecule) but not T2 (A-band region of titin). This preferential phosphorylation behavior of titin suggests that the phosphorylation sites are in the Iband region of the sarcomere, which is consistent with previous work that has shown that titin's spring elements are 'phosphorylation hotspots'[6, 9, 11, 40]. The computer program GPS 2.1.2 of kinase specific phosphorylation sites [41] predicts multiple CaMKII phospho-sites in the N2B elements (results not shown) with S34 as a high confidence target. We therefore performed *in vitro* CaMKII δ phosphorylation experiments on the N2B element. This revealed 9 phospho-sites in the N2B element including S34. None of these phospho-sites overlap with the N2B sites that are known to be targeted by other kinases (PKA/PKG: S469[11] and ERK2: S294, S244 and S202). Of the CaMKII δ sites, only S34 and S493 are highly conserved (Fig. 2C) suggesting that these two sites might perform important functions. Currently we can only speculate what these functions might be. The N2B element is known to interact with the small heat shock protein α Bcrystallin[42, 43] and with four-and-a-half LIM protein, FHL[44] and CaMKII δ phosphorylation of the N2B element might regulate protein-protein interactions. It is also worth considering that phosphorylation of the N2B element by CaMKII δ alters passive tension, analogous to the effect of PKA/PKG phosphorylation of the N2B element. The N2B element is a cardiac specific spring element that dominates the elasticity of titin at intermediate to long sarcomere lengths[45]. Extension of the N2B element follows wormlike-chain entropic behavior and is characterized by a persistence length (a measure of the bending rigidity) of ~ 0.65 nm[25, 46]. PKA/PKG phosphorylation induces structural transitions in the N2B element that increase the persistence length and thereby lower passive tension[47]. Regulation of passive tension through PKA/PKG phosphorylation is clinically important as hypo-phosphorylation of the PKA/PKG site contributes to elevated passive tension in HFpEF patients[1]. In summary, multiple residues in the N2B element are phosphorylated including two highly conserved sites (S34 might be most important). We speculate that CaMKII δ phosphorylation triggers local structural transitions that, analogous to the changes that follow PKA/PKG phosphorylation, lower passive tension.

PEVK element phosphorylation

In vitro kinase assays, mass spectrometry, and skinned fiber studies also revealed that CaMKII δ targets the PEVK region of titin and CaMKII δ is the first known kinase that phosphorylates more than one spring element. Mass spectrometry revealed 5 PEVK sites, distributed more or less evenly along the PEVK sequence (Fig. 3B). Of these identified sites only S26 and S170 are predicted by 'GPS 2.1.2 phosphosite [41]' to be high confidence CaMKII sites (S170 is a consensus sequence for CaMKII δ (X-R-X-X-S/TX)[12]) and both

sites are highly conserved in a wide range of species (Fig. 3C). Site directed mutagenesis showed that individually mutating these serines to alanines abolished ~50% of WT ^{32}P incorporation (Fig. 4B). Surprisingly, mutating both serines simultaneously did not fully abolish ^{32}P incorporation, but resulted in ~25% ^{32}P incorporation that remained. A possible explanation is provided by the previous work that showed that mutating S26 and S170 to alanines causes structural changes in the PEVK[48] and such changes might permit phosphorylation of one or more of the three threonines that mass spectrometry analysis had uncovered (T70, T80 and T177). Regardless, the phosphorylation assays on mutant PEVK showed that S26 and S170 are the most important CaMKII δ sites. Interestingly, S26 and S170 were also previously shown to be phosphorylated by PKC α [6]. [Note that others have also found that the same amino acid can be phosphorylated by several kinases, for example S368 of the neuronal nicotinic receptor $\alpha 4$ subunits is phosphorylated by both PKA and PKC[49].] This interesting finding suggests that there might be crosstalk between CaMKII δ and PKC α signaling on titin and warrants follow-up work, especially in heart failure conditions where both kinases display deranged signaling[4, 5, 38].

The PEVK structure is not well-defined, but the existence of structural motifs has been proposed[50]. Although the high proline concentration is likely to preclude the formation of sizeable α -helices and β -sheet structures, NMR and circular dichroism (CD) experiments suggest that polyproline helix-coil motifs are structural features of the PEVK[50]. The fact that the PEVK sequence, especially around S26 and S170, is highly conserved in a wide variety of species, also suggests that the PEVK is locally structured. Single molecule force spectroscopy has shown that prior to phosphorylation, the WT PEVK has a persistence length of ~1 nm and that phosphorylation reduces this value to ~0.5 nm[6, 48]. Serine-to-alanine mutations also significantly reduce persistence length in both single mutants and the double mutant[48], suggesting that both S26 and S170 are important in maintaining structural integrity. It has been proposed that the S26A and S170A mutations disrupt the stabilizing interactions involving the native serine residues, which increases the conformational entropy of the PEVK and results in a lower effective persistence length[48]. A reduction in persistence length is predicted to *increase* passive tension, and cellular and tissue studies on wildtype and PEVK deficient mice have indeed provided evidence that PKC α phosphorylation of S26 and S170 increases passive tension[6, 8]. The same effect is expected when the sites are phosphorylated by CaMKII δ . In summary, the PEVK element is phosphorylated by CaMKII δ , S26 and S170 are the main targets, and the effect of phosphorylation is likely to be an increase in passive tension.

In vivo titin phosphorylation

Effects seen *in vitro* with purified proteins or with skinned fibers to which exogenous kinases are added not necessarily translate to *in vivo* conditions where, for example, protein-protein interactions can mask phosphorylation sites or where proximity between kinase and substrate can be a limiting factor. Hence we studied intact mouse hearts that underwent an ischemia reperfusion (IR) insult and intact cells that were stimulated with isoproterenol (ISO). Both conditions are known to activate CaMKII δ [31, 32, 36, 37], and this was confirmed in our study by the increased phosphorylation level of the CaMKII δ site on PLB, Thr17. Titin phosphorylation was also increased, with the increase likely due to CaMKII δ as the effect was abolished by the CaMKII δ inhibitor KN-93 (Fig. 5A). Considering that no change was found in the phosphorylation levels of the PEVK S26 and S170 the *in vivo* increase in titin phosphorylation might be due to phosphorylation of the N2B element. Possible explanations for preferential phosphorylation are differences in substrate affinity and in distance between the phosphorylation sites on titin and CaMKII δ . CaMKII is highly enriched along the Z-bands[12] and from previous immunoelectron microscopy studies[51] it can be estimated that within the physiological sarcomere length range of the heart (in the

mouse ~1.8–2.2 μm [52]) the C-terminus of the PEVK (where S170 is found) is ~150 nm away from the edge of the Z-disk[51]. This does not mean that the PEVK will never become phosphorylated as there is a low amount of cytosolic CaMKII δ [53] and when activated chronically, this might phosphorylate the PEVK. On the other hand, the N-terminus of the N2B element (where S34 is found) is on average 100 nm closer to the Z-band than the PEVK[51]. It seems therefore reasonable to propose that due to its close proximity to the N-terminal end of titin's spring region, CaMKII δ preferentially phosphorylates the N2B element. It is interesting to note that one of the functions attributed to CaMKII δ is the frequency dependent acceleration of relaxation (FDAR)[12, 54], by phosphorylating PLB and promoting Ca²⁺ reuptake from the cytosol by SERCA, maintaining thereby efficient ventricular filling at high heart rates. Preferential phosphorylation of the N2B element by CaMKII δ and an ensuing decrease in titin-based passive tension would further augment ventricular filling. Thus it is possible that phosphorylation of PLB and titin, serves overlapping functions in maintaining diastolic function at high heart rates.

In summary, our work revealed the novel finding that CaMKII δ phosphorylates titin, both in vitro and in vivo. Phosphorylation takes place on titin's spring elements, suggesting a role for CaMKII δ in passive tension modulation. Whether deranged expression and activity of CaMKII δ in patients with structural heart disease and arrhythmias[38] contributes to titin-based diastolic dysfunction is an area that should be addressed next.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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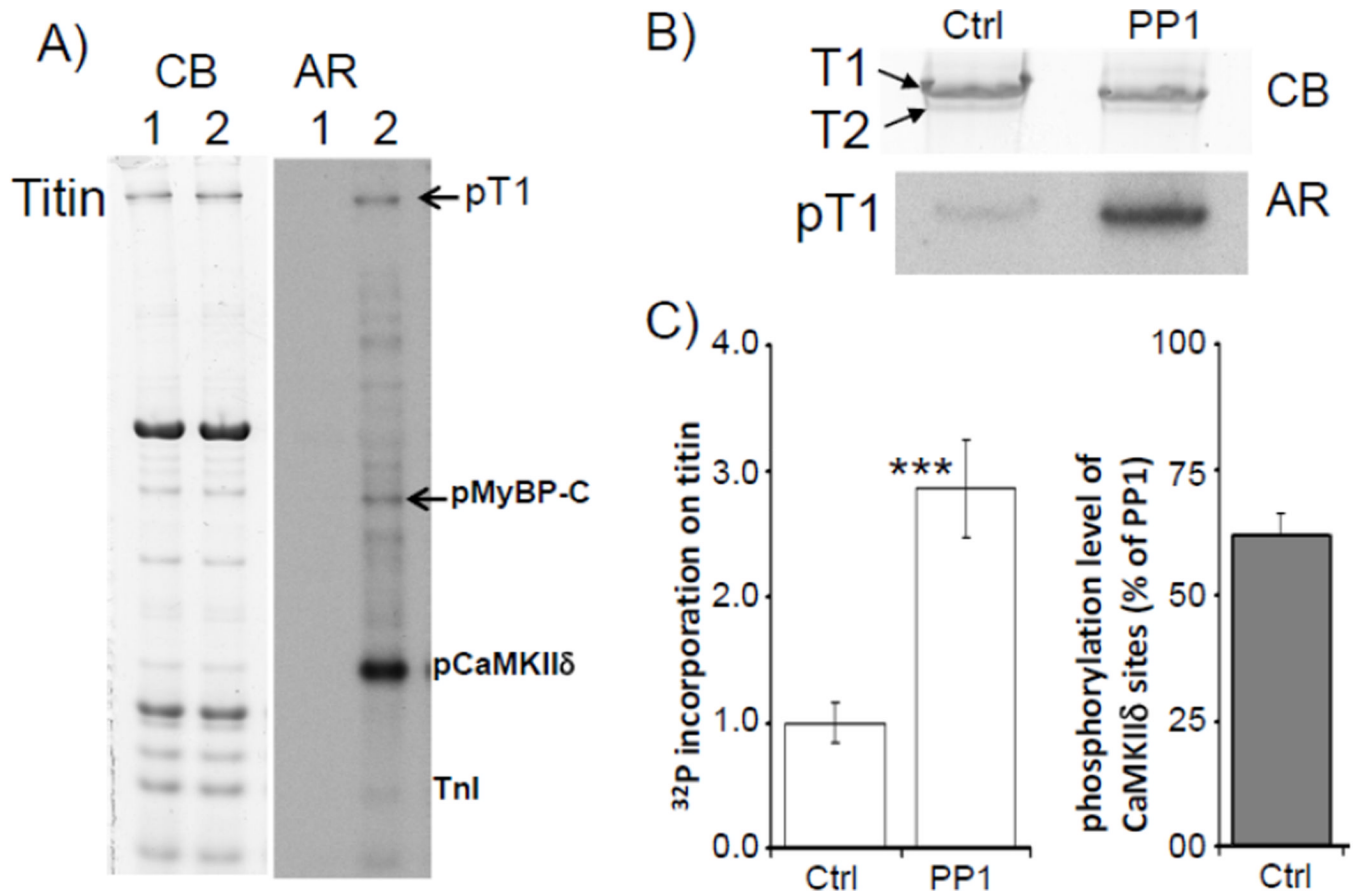


Figure 1. Titin is phosphorylated by CaMKII δ

A) Mouse skinned LV muscle fibers incubated with CaMKII δ . Left panel: Coomassie blue (CB) stained 2–7% gradient gel. Right panel: corresponding autoradiograph (AR). Lane 1: skinned fibers in kinase buffer (containing Ca^{2+} /Calmodulin, and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$). Lane 2: skinned fibers in kinase buffer plus CaMKII δ . B) Effect of pre-incubation of mouse skinned cardiac fibers with protein phosphatase 1 (PP1). Top: lane 1 (Ctrl) skinned fibers incubated with CaMKII δ . Lane 2 (PP1) skinned fibers pre-treated with PP1 and then incubated with CaMKII δ . Upper panel: Coomassie blue (CB) stained gel; Lower panel: corresponding autoradiograph (AR). C) Left: pre-incubation with PP1 resulted in a significant increase in CaMKII δ induced ^{32}P incorporation on titin. Right: Phosphorylation level of CaMKII δ accessible sites under control conditions (expressed as percentage of ^{32}P incorporation on titin in PP1 pretreated and then CaMKII δ phosphorylated skinned muscle). (N=12, *** $p < 0.001$ in t -test of PP1 treated vs. ctrl muscle. T1: intact titin; T2: titin degradation product.)

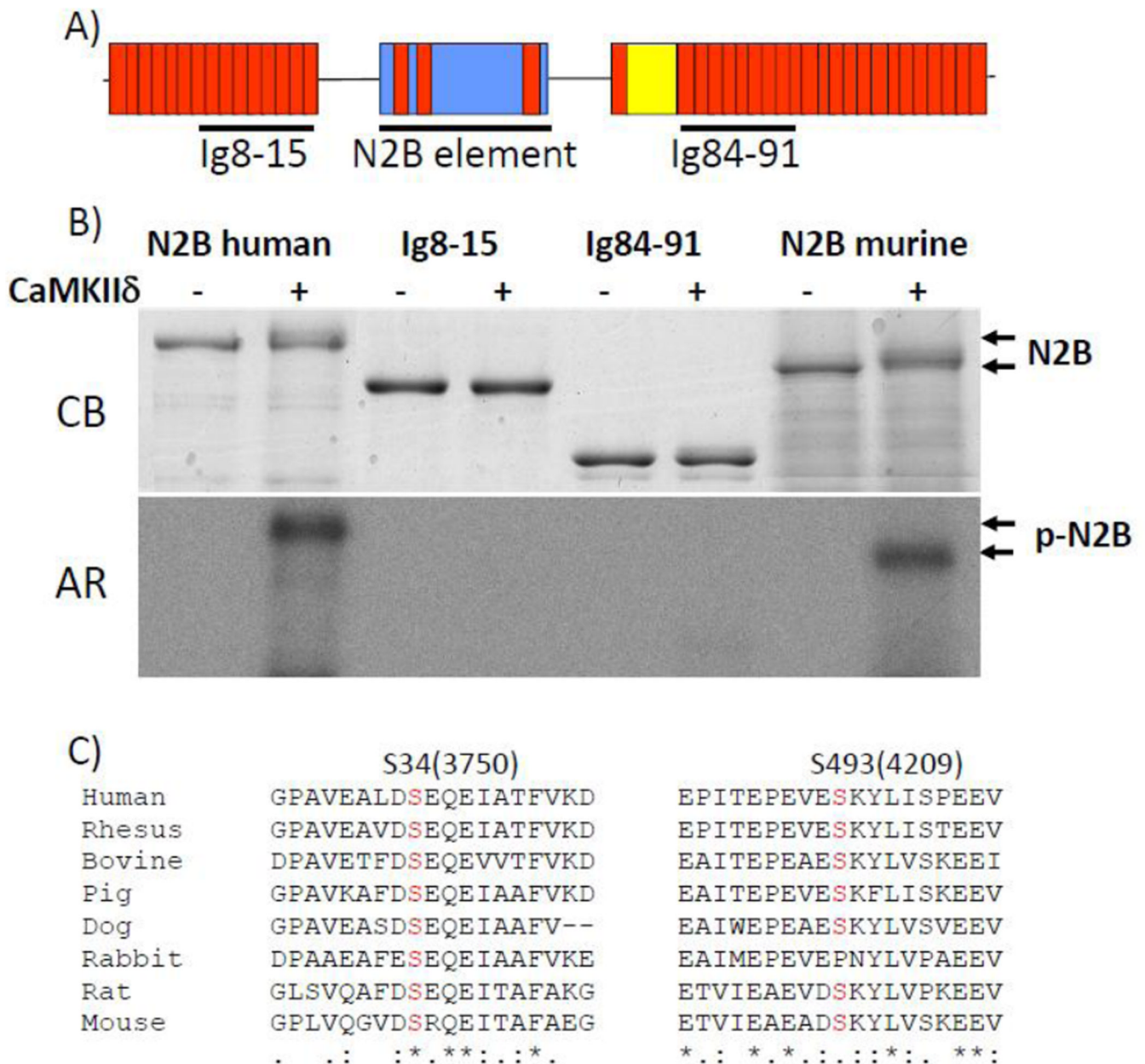


Figure 2. The N2B element of cardiac titin is phosphorylated by CaMKII δ

A) Domain organization of cardiac titin's I-band region (N2B isoform) and location of recombinant proteins used in kinase assay: Ig8-15, Ig84-91, and the N2B element (both the human and murine versions were used). B) Upper panel: Coomassie blue (CB) stained gel. Lower panel: corresponding autoradiograph (AR) of recombinant proteins incubated in kinase buffer (containing Ca²⁺/Calmodulin, and [γ -³²P]ATP) without (-) and with CaMKII δ (+). C) Conserved Serines that are phosphorylated by CaMKII δ (numbering based on N2B unique sequence in human).

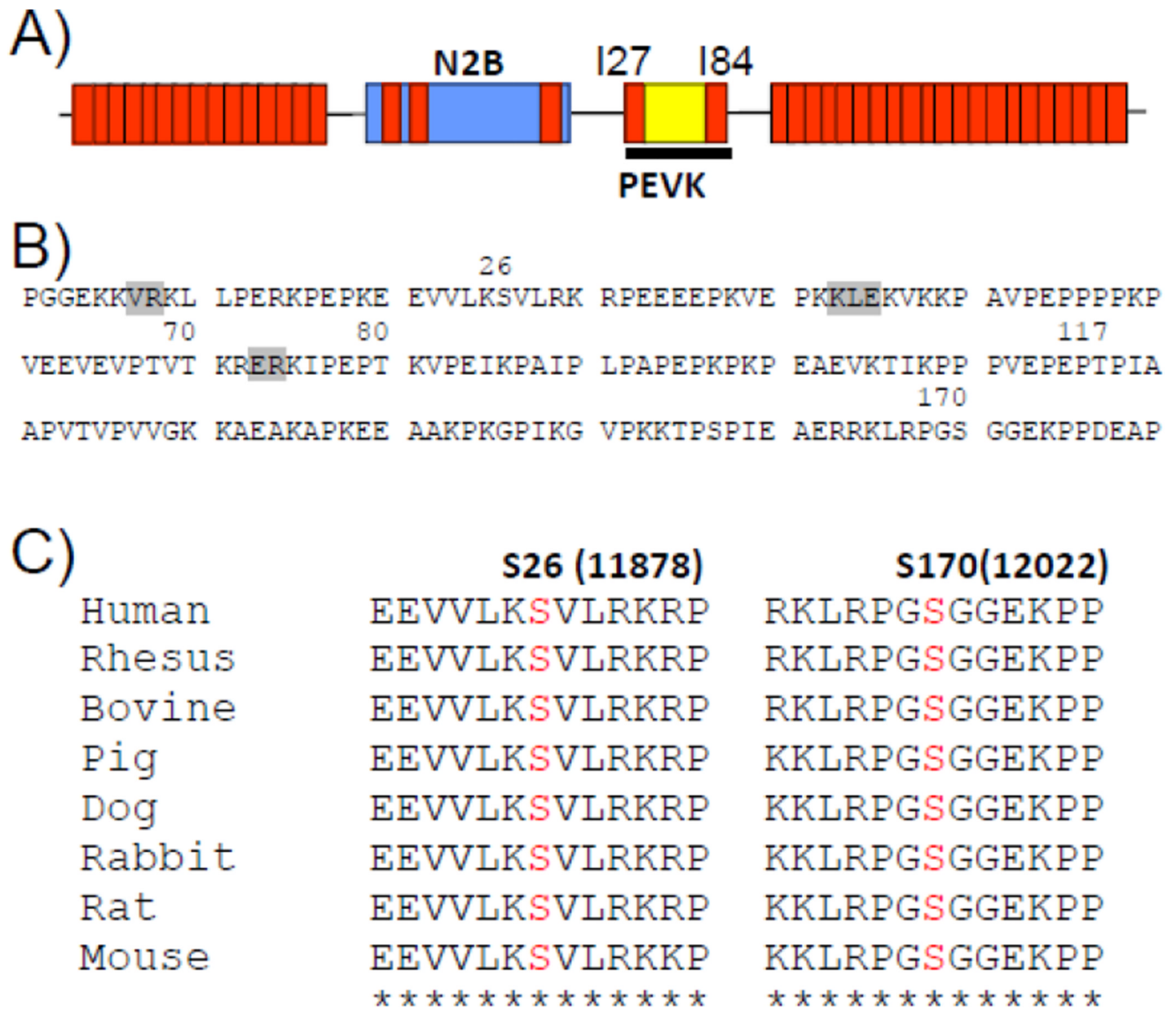


Figure 3. CaMKII δ phosphorylates titin's PEVK spring element

A) Schematic diagram showing location of PEVK element in extensible region of cardiac N2B isoform. B) Amino acid sequence of PEVK element (human) with indicated CaMKII δ phosphorylation sites identified by mass spectrometry. Five phosphorylation sites were identified (S26, T70, T80, T117, S170) of which S26 and S170 are highly conserved (C). (Residues shown in grey were not covered in mass spectrometry analysis.)

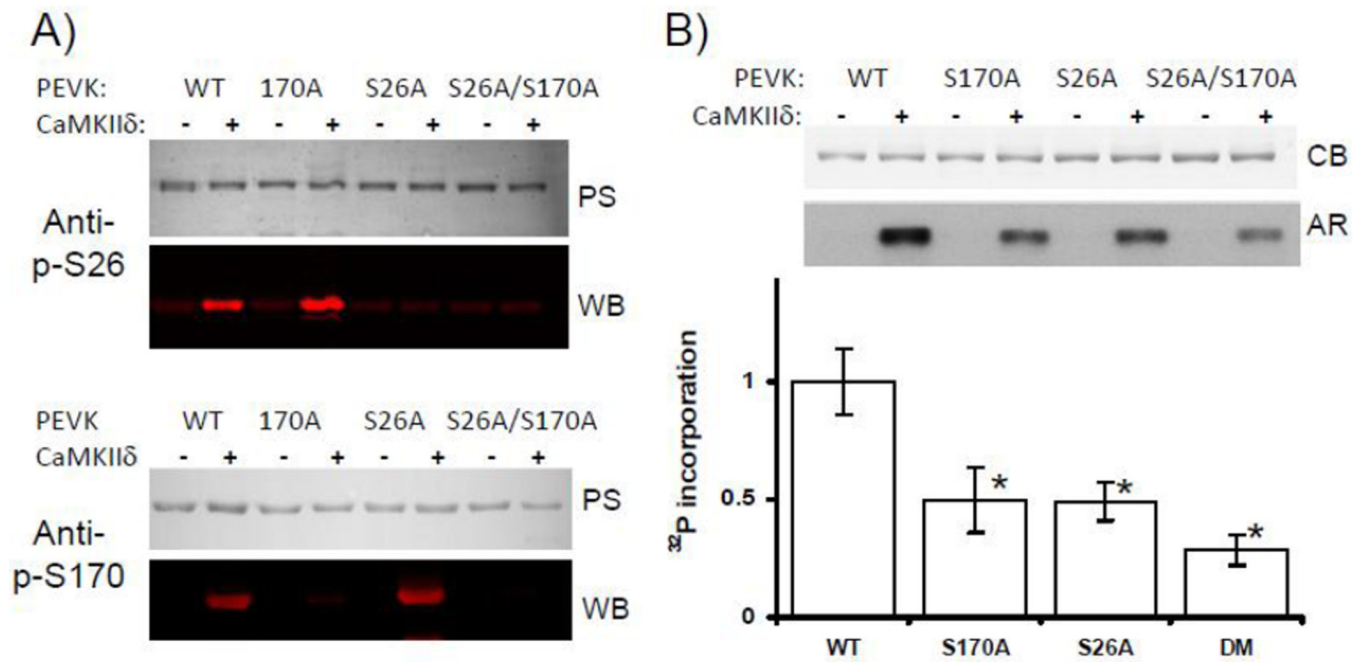


Figure 4. Evaluation of PEVK S26 and PEVK S170 as CaMKIIδ phosphorylation sites

A) PEVK WT, and PEVK mutants S170A, S26A, and S170A/S26A were incubated with CaMKIIδ and the phosphorylation status of S26 and S170 evaluated in WBs with phospho-specific anti-p-S26 (top) and anti-p-S170 (bottom) antibodies[6]. Results show that both sites are phosphorylated by CaMKIIδ. B) Proteins were incubated in kinase buffer (containing Ca²⁺/Calmodulin, and [γ -³²P]ATP) without (-) and with CaMKIIδ (+). Proteins were then electrophoresed, Coomassie blue stained (top), and exposed to autoradiographic film (bottom). Top: phosphorylation results in incorporation of ³²P in all proteins, but the level varies and is highest in the WT protein. Bottom: Quantitative analysis of ³²P incorporation (normalized to protein loading) shows that the single mutants incorporate only about half the WT amount. The phosphorylation level in the double mutant is ~75% less than in WT PEVK. See text for details. (N=5, * p<0.05 in ANOVA vs WT; PS: Ponceau S; WB: western blot; CB: Coomassie blue; AR: Autoradiograph.)

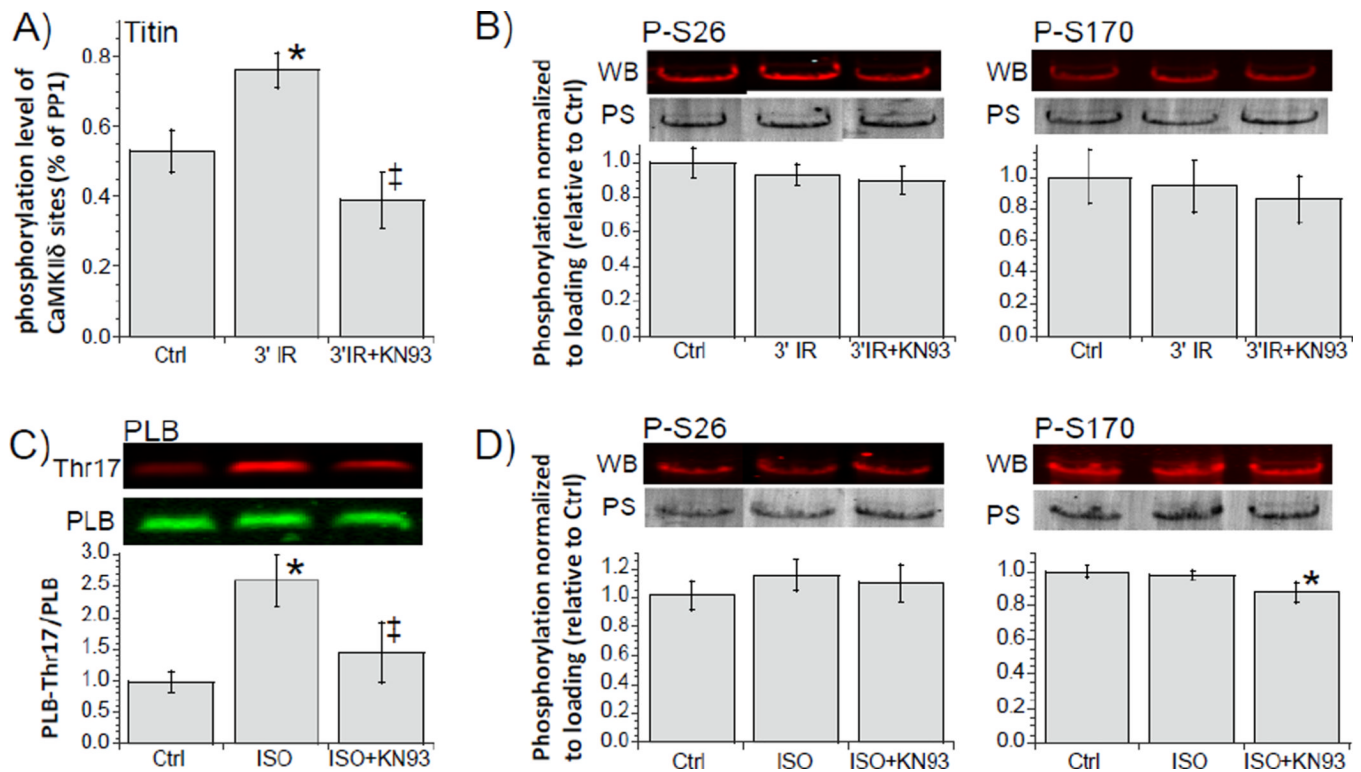


Figure 5. Phosphorylation of titin in intact hearts (A and B) and intact cardiac myocytes (C and D)

A) Isolated hearts underwent a 20 min period of global ischemia followed by reperfusion for 3 min in absence and presence of the CaMKII inhibitor KN93. Left: back phosphorylation assays with CaMKIIδ show an increase in titin phosphorylation of the IR hearts and that this increase was inhibited by KN93. B) left and right: IR hearts analyzed with WB and p-S26 (left) and p-S170 (right) antibodies. C) Mouse intact cardiac myocytes were electrically paced at 4Hz in absence or presence of isoproterenol. p-Thr17 of PLB was increased in ISO treated cells and this effect was inhibited by KN93. D) left and right: pS26 (left) and pS170 (right) levels were not altered by ISO. (N varied between 5 and 8; *p<0.05 (ANOVA) vs. ctrl; ‡ p<0.05 (ANOVA) vs. ISO; PS: Ponceau S; WB: western blot; PLB antibody against unphosphorylated phospholamban that was used for loading control (See Methods).)