The effect of myosin RLC phosphorylation in normal and cardiomyopathic mouse hearts

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Abstract

Phosphorylation of the myosin regulatory light chain (RLC) by Ca^{2+}-calmodulin–activated myosin light chain kinase (MLCK) is known to be essential for the inotropic function of the heart. In this study, we have examined the effects of MLCK-phosphorylation of transgenic (Tg) mouse cardiac muscle preparations expressing the D166V (aspartic acid to valine)–RLC mutation, identified to cause familial hypertrophic cardiomyopathy with malignant outcomes. Our previous work with Tg-D166V mice demonstrated a large increase in the Ca^{2+} sensitivity of contraction, reduced maximal ATPase and force and a decreased level of endogenous RLC phosphorylation. Based on studies demonstrating the beneficial and/or protective effects of cardiac myosin phosphorylation for heart function, we hypothesized that an *ex vivo* phosphorylation of Tg-D166V cardiac muscle may rescue the detrimental contractile phenotypes observed earlier at the level of single myosin molecules and in Tg-D166V papillary muscle fibres. We showed that MLCK-induced phosphorylation of Tg-D166V cardiac myofibrils and muscle fibres was able to increase the reduced myofibrillar ATPase and reverse an abnormally increased Ca^{2+} sensitivity of force to the level observed for Tg-wild-type (WT) muscle. However, in contrast to Tg-WT, which displayed a phosphorylation-induced increase in steady-state force, the maximal tension in Tg-D166V papillary muscle fibres decreased upon phosphorylation. With the exception of force generation data, our results support the notion that RLC phosphorylation works as a rescue mechanism alleviating detrimental functional effects of a disease causing mutation. Further studies are necessary to elucidate the mechanism of this unexpected phosphorylation-induced decrease in maximal tension in Tg-D166V–skinned muscle fibres.

Keywords: cardiac hypertrophy ● mutation ● myosin regulatory light chain ● muscle fibres ● myosin light chain kinase ● phosphorylation ● transgenic mice

Introduction

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant disorder of the heart characterized by increased growth of myocytes (hypertrophy), myofilament disarray, fibrosis and a high incidence of sudden cardiac death (SCD) [1]. FHC was found to originate from mutations in cardiac sarcomeric proteins, such as β-myosin heavy chain, myosin essential (ELC) and regulatory (RLC) light chains, myosin-binding protein-C, actin, α-tropomyosin, troponin T, troponin I, troponin C and titin [1].

Mutations in the *MYL2* gene encoding the human ventricular RLC constitute about 2% of the total sarcomeric protein mutations and are associated with a wide spectrum of morphological and functional abnormalities in the affected patients [1, 2]. To date, 10 RLC mutations have been identified by population studies to cause hypertrophic cardiomyopathy, eight of which are single amino acid substitutions and two are intronic splice site mutations [3–7].

In this report, we focus on one of the most malignant RLC FHC mutations, which is brought about by an aspartic acid to valine substitution at the last amino acid residue of RLC (D166V) [4, 8]. The resulting valine residue of the RLC is positioned in the hinge region ('hook') of the myosin heavy chain (MHC) that serves as a major source of cross-bridge compliance during the contractile cycle [9, 10]. Given the critical position of the D166V mutation in the RLC molecule and its location in relation to the myosin cross-bridge, it is evident why this one amino acid substitution could affect myosin function and transmission of force leading to

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abnormal cardiac contractility and FHC. Our previous work with transgenic (Tg) D166V mouse cardiac muscle preparations suggested that the manifestation of the mutation-induced phenotype could be correlated with a significantly decreased myosin RLC phosphorylation [11, 12]. Tg-D166V papillary muscle fibres demonstrated an increased Ca^{2+} sensitivity of contractile force, reduced maximal ATPase and force; functional changes that coincided with the significantly reduced *in situ* phosphorylation of RLC observed in rapidly frozen Tg-D166V samples compared to Tg-wild type (WT) hearts [11]. Similarly, single molecule detection studies with fluorescently labelled cardiac myofibrils from Tg-D166V mice showed decreased rates of cross-bridge cycling and a much lower RLC phosphorylation compared with Tg-WT myofibrils [12]. Based on these data, we hypothesized that the phenotype associated with the D166V mutation could be alleviated or partially reversed by an *ex vivo* Ca^{2+}-calmodulin (CaM)–activated myosin light chain kinase (MLCK)-phosphorylation of the mutated myocardium. The hypothesis was supported by a large body of basic science and clinical findings demonstrating the beneficial role of RLC phosphorylation on cardiac muscle performance [13–15].

Here, we show that MLCK-induced phosphorylation of cardiac muscle preparation from Tg-D166V mice was able to increase the mutation-induced decreased levels of myofibrillar ATPase activity. In addition, a D166V-mediated increase in Ca^{2+} sensitivity of force was fully restored to the level of Tg-WT muscle fibres. Surprisingly, in contrast to Tg-WT muscle, which demonstrated a phosphorylation-induced increase in steady-state force, the maximal level of tension in phosphorylated Tg-D166V muscle fibres was decreased. Our results suggest that RLC phosphorylation may operate through different mechanisms in healthy and diseased hearts.

Materials and methods

Animals

All animal studies were conducted in accordance with institutional guidelines. The University of Miami has an Animal Welfare Assurance (A-3224-01, effective July 11, 2007) on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. Tg mouse models expressing the WT or D166V mutant of the human ventricular myosin RLC were generated and characterized as described earlier [11]. As reported earlier, Tg-WT mice expressed ~100% of human ventricular RLC protein (*i.e.* totally replaced the endogenous mouse ventricular RLC) and were compared to Tg-D166V mice that expressed ~95% of the human RLC carrying the D166V mutation [11]. The animals used in this study were ~6 months of age.

Phosphorylation of RLC in mouse cardiac myofibrils

Skinned cardiac myofibrils (CMF) from the right and left ventricles of Tg-WT and Tg-D166V mice were prepared as described earlier [16]. MLC kinase (MLCK) from rabbit skeletal muscle, isolated and purified as described in [17], was used to phosphorylate RLC. CMF were phosphorylated with Ca^{2+}-CaM–activated MLCK in a solution containing: 250 μg myofibrils suspended in 20 mM phosphate buffer (pH 8), 30 mM KCl and 0.5 μM MLCK, 5.0 μM CaM, 0.1 mM $CaCl_2$, 12.5 mM $MgCl_2$ and 5 mM ATP. The phosphorylation reaction was carried out overnight on ice. These conditions were determined to result in fully phosphorylated CMF (Fig. 1, inset). After phosphorylation, CMF were washed thoroughly with buffer containing 30 mM MOPS, 60 mM KCl, 1 mM DTT, pH 7.0. The levels of RLC phosphorylation in Tg-WT and Tg-D166V CMF were determined using Urea/SDS-PAGE as described later.
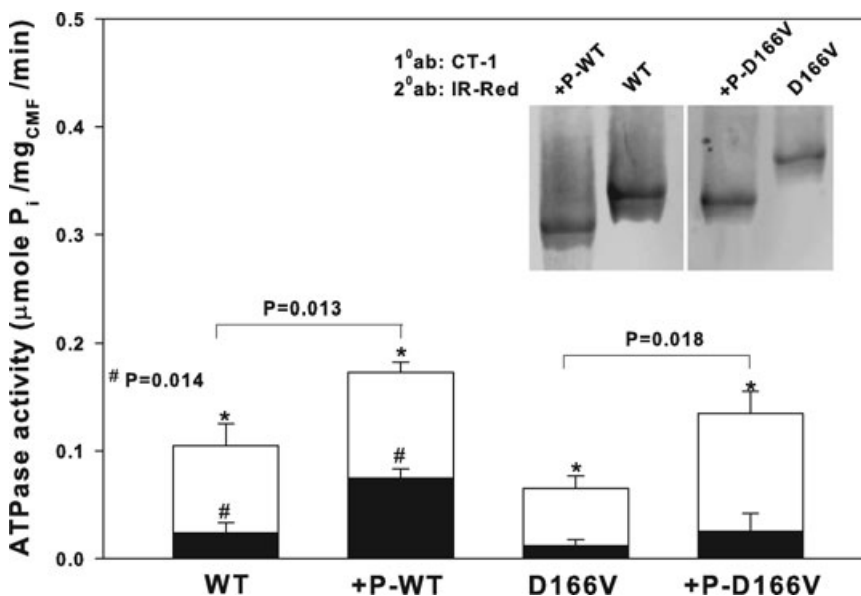
Fig. 1 Myofibrillar ATPase activity measured in Tg-WT and Tg-D166V preparations before and after Ca^{2+}-CaM MLCK treatment. Maximal ATPase activity was measured in *p*Ca 4 (white bars), whereas basal ATPase activity was measured in *p*Ca 8 (black bars). Inset: Urea/SDS-PAGE of phosphorylated and non-phosphorylated cardiac myofibrils (CMF). CMF were run on 8% polyacrylamide gel in the presence of 8 M urea and the RLC protein was visualized with CT-1 antibodies recognizing the total RLC protein content (Materials and Methods). Under these conditions, the phosphorylated form of RLC in +P-WT or +P-D166V CMF migrated faster than the non-phosphorylated WT or D166V. Note, that due to the D166V mutation, the non-phosphorylated D166V migrates slower than the non-phosphorylated WT. 1[0] ab: Primary CT-1 antibodies; 2[0] ab: secondary IR-Red antibodies.

ATPase assays

Myofibrillar ATPase activity assays using Tg-WT and Tg-D166V CMF were performed in a solution of 20 mM MOPS (pH 7.0), 40 mM KCl, 2.5 mM MgCl$_2$, 2 mM EGTA and calculated CaCl$_2$ concentration to produce 10^{-8} M (basal activity) or 10^{-4} M (maximal activity) of free [Ca^{2+}] [18]. After 5 min. of incubation at 30°C, the reaction was initiated with 2.5 mM ATP and terminated after 10 min. with 5% trichloroacetic acid. Released inorganic phosphate was measured according to the method of Fiske and Subbarow [19].

Phosphorylation of RLC in mouse cardiac papillary muscle fibres

Glycerinated-skinned papillary muscle fibres from Tg-WT and Tg-D166V mice were isolated from mouse left ventricles and prepared using established protocols [20]. Fibre bundles of three to five single muscle fibres were phosphorylated with Ca^{2+}-CaM–activated MLCK in a solution containing 0.5 μM MLCK, 5 μM CaM in pCa 6 buffer [18]: 10^{-6} M free [Ca^{2+}] (total calcium propionate, CaPr = 4.997 mM), 1 mM free [Mg^{2+}] (total MgPr = 3.88 mM), 7 mM EGTA, 20 mM MOPS, 2.5 mM [Mg-ATP^{2-}] (total Na$_2$ATP = 3.056 mM), 15 mM creatine phosphate, 15 units/ml phosphocreatine kinase, pH 7.0 (adjusted with KOH), ionic strength 150 mM (adjusted with KPr = 38.41 mM), for 1 hr at room temperature. Alternatively, phosphorylation of fibre bundles was performed while mounted on a Guth force transducer. The fibres were skinned before phosphorylation with pCa 8 relaxing solution (same composition as pCa 6 except 10^{-8} M free [Ca^{2+}]) containing 1% Triton X-100 for 30 min. Phosphorylation of the latter was performed in the solution of 0.5 μM MLCK, 5 μM CaM in pCa 6 for 30 min. at room temperature. Both treatments were equally efficient in achieving the same level of RLC phosphorylation (Fig. 3, insets). After phosphorylation, the fibres were washed in pCa 8 buffer and subjected to force measurements.

Steady-state force measurements and myofilament Ca^{2+} sensitivity in phosphorylated and non- phosphorylated fibres

The fibres were tested for steady-state force development in a pCa 4 solution (composition is the same as pCa 8 buffer except the [Ca^{2+}] = 10^{-4} M) and relaxed in pCa 8 solution. Steady-state force development was monitored for phosphorylated Tg-D166V and Tg-WT papillary muscle fibres as well as for their non-phosphorylated counterparts. To account for potential decreases in force due to fibre exposure to sub-maximal calcium concentrations during phosphorylation, control Tg-WT and Tg-D166V fibres were tested in parallel to those being phosphorylated after their 30-min. incubation in the pCa 6 solution with no added CaM–MLCK complex. Maximal force (in Newtons) was calculated per cross-section of muscle fibre and expressed in kN/m^2. The cross-sectional area was calculated based on measurement of the fibre width using an SZ6045 Olympus microscope (zoom ratio of 6.3:1, up to 189 × maximum magnification) and the assumption that the fibre is circular in diameter. The measurement was taken at ~3 points along the bundles and averaged. The average length and diameter of small fibre bundles selected for the experiments were ~1.2–1.4 and 90–120 μm, respectively. To determine the force–pCa dependence, the fibres were exposed to solutions of increasing Ca^{2+} concentrations (from pCa 8 to pCa 4) and force development was monitored. The data were analysed using the Hill equation yielding the pCa$_{50}$ and n_H (Hill coefficient) [21].

Analysis of RLC phosphorylation

The levels of RLC phosphorylation in rapidly frozen ventricular samples, CMF and papillary muscle fibres were determined using Urea/SDS-PAGE and phospho-RLC–specific antibodies (generously provided by Dr. Neal Epstein, National Institutes of Health). Specifically, to determine RLC phosphorylation *in situ*, after euthanasia the hearts from Tg-D166V and Tg-WT mice were excised and the ventricles were isolated and flash frozen in liquid nitrogen. The tissue was thawed in a buffer consisting of 8M Urea, 10 mM Tris-HCl, pH 7.0, 1% SDS, 1% β-mercaptoethanol (β-ME), 1 mM EDTA, 1 mM PMSF, 5 nM microcystin and protease inhibitor cocktail, and then homogenized in the same buffer for 2 min. at 25 Hz. This step was repeated until the tissue was completely homogeneous. The homogenates were combined with equal amounts of SDS-PAGE sample buffer and run on 15% polyacrylamide-SDS gel as described in [11]. To determine the level of RLC phosphorylation in CMF, ~100 μl CMF suspended in 30 mM MOPS, 60 mM KCl, 1 mM DTT, pH 7.0 were combined with 70 mg of crystalline urea, 10 μl β-ME and 5 μl bromophenol blue and run on 8% polyacrylamide gel in the presence of 8M urea [22]. Under these conditions, the phosphorylated form of RLC in +P-WT and +P-D166V CMF migrated faster than the non-phosphorylated counterpart (Fig. 1, inset) [23]. The extent of RLC phosphorylation in papillary muscle fibres was tested after the fibres were sonicated in a solution of 1% SDS and 10% β-ME for 30 min. on ice, mixed with SDS sample buffer and run on 15% polyacrylamide-SDS gel. All gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Phosphorylated Tg RLC in +P-WT or +P-D166V muscle fibres was detected with the +P-RLC antibodies specific for the phosphorylated form of cardiac RLC [24], followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800 (Rockland, Gilbertsville, PA, USA). The total RLC protein was determined with rabbit polyclonal CT-1 antibodies produced in this laboratory [20, 25]. Total ELC (ELC of myosin), which served as a loading control, was detected with the monoclonal ab680 antibody (Abcam, Cambridge, MA, USA) followed by a secondary goat anti-mouse antibody conjugated with the fluorescent dye, Cy 5.5. Blots were scanned and respective bands were quantified using the Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE, USA).

Histopathology

After euthanasia, hearts from Tg-WT and Tg-D166V mice were excised and immersed in 10% buffered formalin [11, 20]. Slides of whole mouse hearts were prepared by American Histolabs, Inc. (Gaithersburg, MD, USA). The paraffin-embedded longitudinal sections of whole mouse hearts stained with haematoxylin and eosin (H&E) and Masson's trichrome were examined for overall morphology and fibrosis using a Dialux 20 microscope, 40×/0.65 Leitz Wetzlar objective and AxioCam HRc (Zeiss, Thornwood, NY, USA) [11, 20, 25].

Statistical analysis

Data are expressed as the average of *n* experiments ± S.E. (standard error). Student's *t*-test was used for simple comparisons of two groups (*e.g.* non-phosphorylated *versus* phosphorylated). For multiple comparisons between groups, a one-way ANOVA and the Holm-Sidak method (Sigma Plot 11; Systat Software, Inc., San Jose, CA, USA) were used. Statistically significant differences were defined as $P < 0.05$.

Results

The effect of RLC phosphorylation on myofibrillar ATPase activity in Tg-WT and Tg-D166V mice

ATPase activity assays were performed on phosphorylated (+P) or non-phosphorylated–skinned cardiac myofibrils from Tg-WT and Tg-D166V mice. As shown in Figure 1, the maximal ATPase activity (μmole Pi/mg $_{CMF}$/min) determined at pCa 4 was 1.6-fold lower in non-phosphorylated D166V CMF (0.065 ± 0.009, $n = 5$) compared to non-phosphorylated WT CMF (0.105 ± 0.021, $n = 8$). Following phosphorylation, the myofibrillar ATPase activity increased in both +P-WT (0.173 ± 0.009, $n = 7$) and +P-D166V (0.135 ± 0.020, $n = 5$) CMF; however, the phosphorylation-induced increase in Tg-D166V myofibrils was larger than that in Tg-WT (Fig. 1, white bars). Therefore, phosphorylation of Tg-D166V CMF was able to recover low levels of maximal myofibrillar ATPase activity observed for non-phosphorylated–mutated CMF and bring it to an intermediate level, between WT and +P-WT. Phosphorylation-induced changes were also observed in the basal levels of myofibrillar ATPase activity measured at pCa 8.0 (Fig. 1, black bars), but the differences were only significant in WT myofibrils ($P = 0.014$).
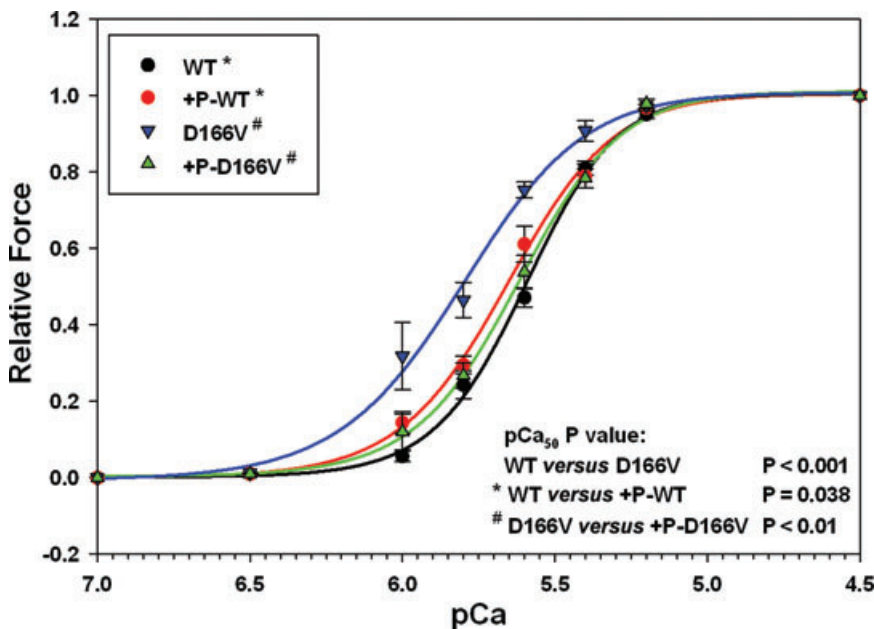
The effect of ex vivo RLC phosphorylation on Ca^{2+} sensitivity of force development in Tg-WT and Tg-D166V–skinned muscle fibres

The effect of Ca^{2+}-CaM MLCK-induced phosphorylation of the RLC on the force–pCa relationship was studied in skinned glyceri-nated fibres from Tg-D166V and Tg-WT mice. As seen in Figure 2, phosphorylation of Tg-WT fibres resulted in a small leftward shift in the force–pCa dependence with ΔpCa$_{50}$ = 0.06, for $n = 7$ individual muscle fibres ($P = 0.038$) demonstrating an increase in Ca^{2+} sensitivity upon phosphorylation. In contrast, phosphorylation of Tg-D166V fibres resulted in a large decrease in myofilament Ca^{2+} sensitivity with ΔpCa$_{50}$ = –0.20, $n = 8$ fibres ($P < 0.01$). Therefore, phosphorylation of D166V-mutated papillary muscle fibres reversed the increased Ca^{2+} sensitivity of force of the diseased D166V muscle and brought it back to the level observed for WT. No significant phosphorylation induced changes in myofilament co-operativity (n_H) were observed in Tg-WT or Tg-D166V papillary muscle fibres (Fig. 2).

The effect of Ca^{2+}-CaM MLCK-induced phosphorylation of RLC on maximal force in Tg-WT and Tg-D166V–skinned muscle fibres

We further studied the effect of RLC phosphorylation on the maximal steady-state force in skinned muscle fibres from Tg-WT and Tg-D166V mice. Phosphorylation of RLC in Tg-WT papillary muscle fibres with Ca^{2+}-CaM MLCK resulted in a small but significant increase in maximal force; from $F_{max} = 57.30 \pm 0.17$ to $F_{max} = 59.19 \pm 0.26$ kN/m^2 (Fig. 3A). Tg-WT fibres that were incubated in pCa 6 solution in the absence of CaM–MLCK and used as a control were indistinguishable from WT muscles ($F_{max} = 56.88 \pm 0.20$ kN/m^2). The P values determined with one-way ANOVA and the Holm–Sidak method for pairwise multiple comparison procedures were: $P < 0.01$ for WT versus +P-WT, $P = 0.01$ for +P-WT versus control fibres and $P > 0.05$ for WT versus control fibres.

Fig. 2 The effect of RLC phosphorylation on the force–pCa relationship in skinned muscle fibers from Tg-WT mice and Tg-D166V. The pCa$_{50}$ and n_H (Hill coefficient) values were: WT (black): pCa$_{50}$ = 5.60 $\pm$ 0.01, n_H = 2.89 $\pm$ 0.15; +P-WT (red): pCa$_{50}$ = 5.66 $\pm$ 0.02, n_H = 2.47 $\pm$ 0.16; D166V (blue): pCa$_{50}$ = 5.83 $\pm$ 0.04, n_H = 2.16 $\pm$ 0.23; +P-D166V (green): pCa$_{50}$ = 5.63 $\pm$ 0.04, n_H = 2.57 $\pm$ 0.19.
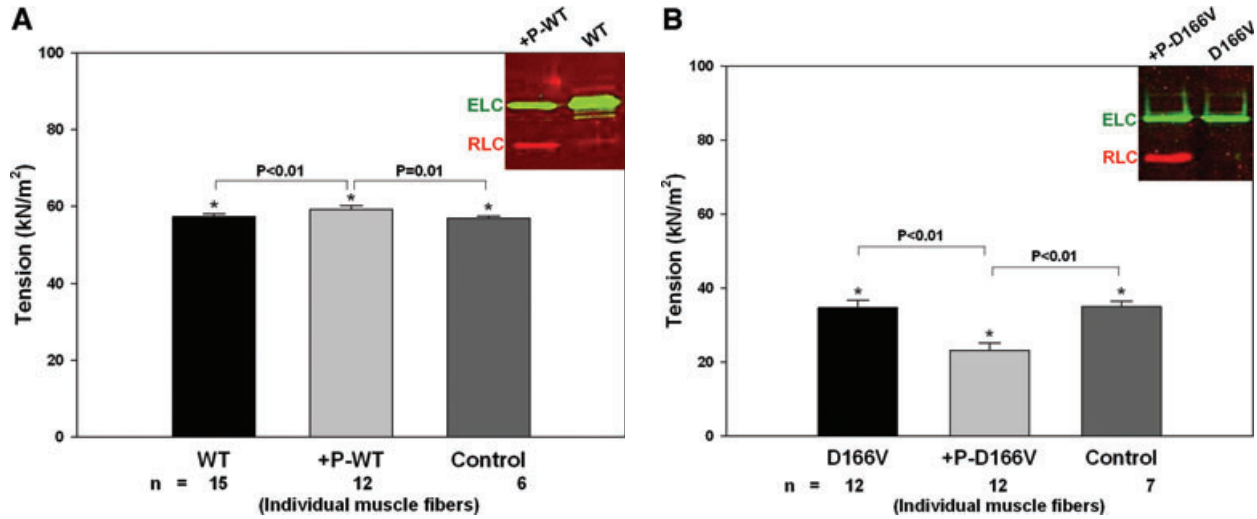
Fig. 3 The effect of RLC phosphorylation on maximal force development in Tg-WT (**A**) and Tg-D166V (**B**) papillary muscle fibres. Maximal force was measured in pCa 4 buffer before and after CaM–MLCK treatment. Control WT and control D166V fibres were incubated in pCa 6 buffer with no CaM–MLCK complex. Insets: Western blot of experimental WT and +P-WT fibres (**A**); D166V and +P-D166V fibres (**B**). Phosphorylation of RLC in +P-WT or +P-D166V muscle fibres was detected with the +P-RLC antibodies specific for the phosphorylated form of cardiac RLC followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800 (red bands). Total ELC, which served as a loading control, was detected with the monoclonal ab680 antibody followed by a secondary goat anti-mouse antibody conjugated with the fluorescent dye, Cy 5.5 (green bands). RLC: regulatory light chain of myosin (red); ELC: essential light chain of myosin (green).
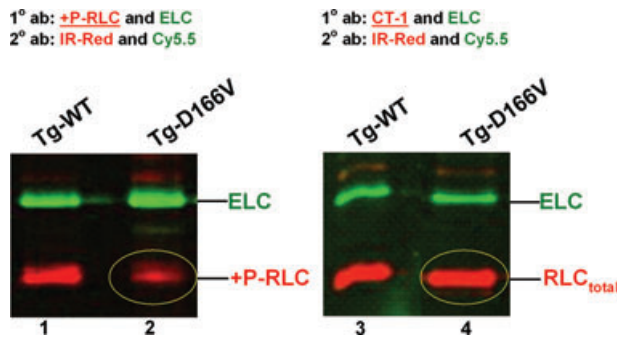

Fig. 4 The effect of D166V mutation on RLC phosphorylation *in situ*. Left ventricles of Tg-WT and Tg-D166V mice were flash frozen in liquid N_2 and subsequently analysed for level of RLC phosphorylation (Materials and Methods). Lanes 1 and 3: Tg-WT; lanes 2 and 4: Tg-D166V. Left: Immunoblotted with +P-RLC (antibody recognizing phosphorylated form of RLC: red). Right: Immunoblotted with CT-1 (antibody recognizing total RLC: red). Circled: D166V protein: phosphorylated (left), and total (right). IR-Red 800 and Cy5.5: secondary fluorescent antibodies.

The gel shown in Figure 3A (inset: red bands) illustrates the level of RLC phosphorylation in +P-WT and WT muscles determined by SDS-PAGE and +P-RLC antibodies specific to the phosphorylated form of cardiac RLC (see Materials and Methods). As depicted in Figure 3B, phosphorylation of D166V papillary muscle fibres resulted in a large (~35%) decrease in the maximal

force from $F_{max} = 34.64 \pm 0.56$ to $F_{max} = 23.12 \pm 0.55$ kN/m^2 ($P < 0.01$). Control Tg-D166V fibres treated in parallel in pCa 6 solution in the absence of CaM–MLCK (see Materials and Methods, for details) were indistinguishable from non-phosphorylated D166V preparations ($P > 0.05$) and showed no change in maximal force ($F_{max} = 34.92 \pm 0.55$ kN/m^2; Fig. 3B). This result indicated that the decrease in force observed in MLCK-treated D166V fibres was clearly associated with RLC phosphorylation of Tg-D166V fibres. The gel shown in Figure 3B (inset) depicts RLC phosphorylation in +P-D166V and D166V fibres.

The effect of the D166V mutation on endogenous RLC phosphorylation in Tg-D166V *versus* Tg-WT mouse hearts

Left ventricles of mice tested in functional assays were rapidly frozen in liquid nitrogen and examined for endogenous levels of RLC phosphorylation by SDS-PAGE and immunoblotting with +P-RLC antibody. Similar to what we observed in our earlier study [11, 12], a significant (approx. threefold) decrease in RLC phosphorylation was detected in left ventricular extracts from Tg-D166V compared with Tg-WT mice (Fig. 4, left). The lower level of RLC phosphorylation in Tg-D166V was not due to a lower RLC content, as verified by the total RLC protein detected with CT-1 antibody in Tg-D166V compared to Tg-WT mice (Fig. 4, right).
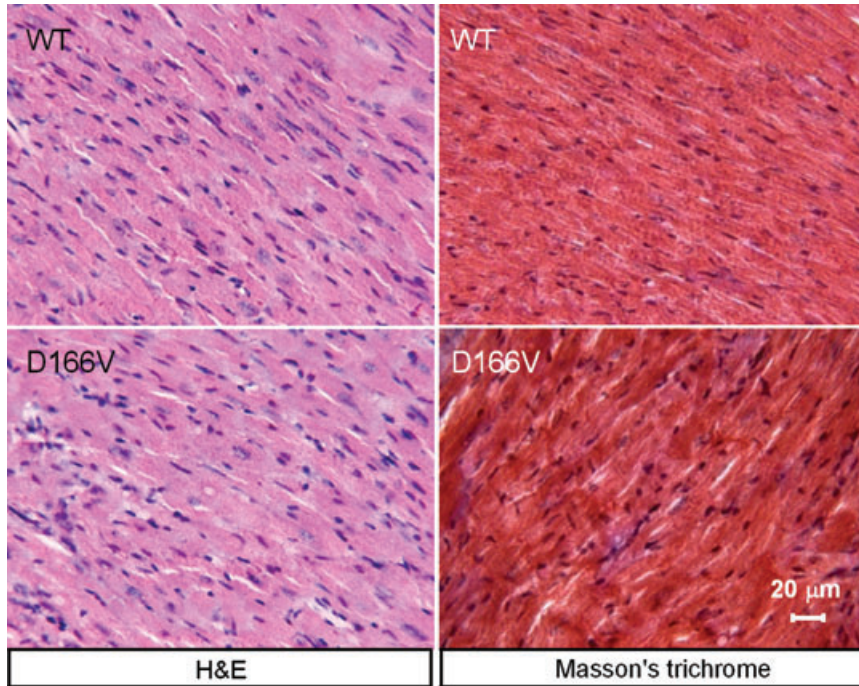
Fig. 5 Representative longitudinal sections from left ventricles of ~6-month-old Tg-WT and of ~6-month-old Tg-D166V mice stained with haematoxylin and eosin (H&E) for overall morphology and Masson's trichrome for signs of fibrosis. Scale bar = 20 μm.

The effect of low D166V phosphorylation on myofilament organization in Tg-D166V mice

To determine whether the decreased levels of RLC phosphorylation in Tg-D166V mouse hearts led to or were caused by the D166V-induced structural defects, the myocardium of Tg-D166V mice was compared with Tg-WT (Fig. 5). No major difference in heart morphology was observed between Tg-D166V and Tg-WT mice, as evidenced by haematoxylin and eosin tissue staining. Similarly, Masson's trichrome staining demonstrated no fibrosis in the hearts of Tg-D166V or Tg-WT mice (Fig. 5). Therefore, lower levels of endogenous RLC phosphorylation observed in left ventricles of Tg-D166V mice did not induce or were not caused by any abnormal tissue morphology (Fig. 5).

Discussion

This report is the first to demonstrate the physiological effects of RLC phosphorylation in cardiomyopathic Tg mouse hearts. Hypertrophic cardiomyopathy is one of the most common genetic cardiovascular disorders caused by mutations in genes encoding cardiac sarcomeric proteins but the molecular mechanism by which these single amino acid substitutions lead to specific disease phenotypes is yet to be elucidated. The pathways of target-specific interventions that could result in rescue of the pathologi-

cal phenotype also remain undiscovered. In this study, we focus on one such mechanism acting through phosphorylation of myosin RLC and show that it can reverse or partially recover the detrimental consequences of the D166V mutation.

Effect of RLC phosphorylation in cardiac myofibrils and under load in skinned papillary muscle fibres

Based on studies demonstrating the effect of myosin phosphorylation on cardiac function and the observation that Tg FHC-mutated mouse hearts display low levels of RLC phosphorylation [11, 12, 16], we hypothesized that an *ex vivo* phosphorylation of Tg-D166V cardiac muscle may rescue detrimental contractile phenotypes associated with this malignant FHC mutation.

Experiments performed in cardiac muscle myofibrils, under unloaded conditions, clearly showed the beneficial effect of RLC phosphorylation on the ATPase activity in Tg-D166V muscle. Low levels of maximal ATPase activity observed in non-phosphorylated Tg-D166V myofibrils could be recovered by RLC phosphorylation (Fig. 1). Because the heart operates against a load of mean arterial blood pressure and peripheral resistance, the physiologically relevant effects of RLC phosphorylation are those observed under loaded conditions, that is when myosin performs work in isometrically contracting skinned papillary muscle fibres. Consistent with other reports (reviewed in Refs. 13 and 14), a phosphorylation-induced increase in maximal force production and Ca^{2+} sensitivity

of tension were observed in Tg-WT muscle preparations. However, phosphorylation of the muscle carrying the malignant D166V-RLC mutation showed a much more complex pattern of changes that in some cases were strikingly different than those observed in the WT myocardium.

As reported previously [11], we observed an abnormally large increase in the calcium sensitivity of force in non-phosphorylated Tg-D166V muscle fibres compared to non-phosphorylated Tg-WT counterparts (Fig. 2). The MLCK-induced phosphorylation of RLC in Tg-D166V muscle shifted the force–pCa dependence back to that observed in Tg-WT muscle. Therefore, phosphorylation of RLC worked as a rescue mechanism in the mutation-compromised myocardium, reversing its overly sensitive responsiveness to calcium. Unexpectedly, the maximal level of force in phosphorylated Tg-D166V muscles was decreased (Fig. 3B). This effect was in contrast to Tg-WT muscle (Fig. 3A), which demonstrated a phosphorylation-induced increase in steady-state force. No such effect on maximal force was observed previously during cardiac [24, 26, 27] or skeletal [23, 28–31] muscle contraction. A plausible mechanism of this phosphorylation-induced decrease in maximal force is that the substitution of the bulky valine for the negatively charged aspartic acid in Tg-D166V muscle may lead to abnormal alignment of D166V myosin cross-bridges along actin filaments. This in turn would cause weaker binding and decreased force generation in Tg-D166V muscle fibres. The question remains as to whether these phosphorylation-induced changes in force generation in Tg-D166V muscle are beneficial or detrimental to the workings of the mutated hearts.

Attenuation of RLC phosphorylation *in vivo*: relation to heart disease

Our previous and current studies with Tg mouse models of FHC demonstrate a direct relationship between severity of hypertrophic phenotype and the level of RLC phosphorylation. Mouse hearts carrying the malignant R58Q-RLC mutation exhibited significantly lower levels of RLC phosphorylation compared to WT hearts or those of benign disease phenotypes [16, 32, 33]. The recent report from the Stull laboratory also showed that attenuation of RLC phosphorylation in cardiac MLCK knockout mice resulted in cardiomyocyte hypertrophy with histological evidence of necrosis and fibrosis [26]. These findings suggest that the D166V mutation-mediated decrease of RLC phosphorylation in Tg-D166V myocardium (Fig. 4) may initiate hypertrophic remodelling of the heart and stimulate the development of cardiomyopathic phenotype. It is interesting to note that whereas tissue abnormalities are clearly manifested in older Tg-D166V mice [11], they are not present in younger animals (Fig. 5). Most likely, pathological changes follow the functional abnormalities (inability to become phosphorylated, changes in ATPase, force, calcium sensitivity) observed in the mutated myocardium.

As shown by van der Velden *et al.*, a largely reduced RLC phosphorylation was observed in end-stage heart failure patients [34–36]. The authors speculated that this could reflect an adaptive response of the myocardium to pathophysiological injury. Similar to what we think of phosphorylation-induced reduction in force and decreased Ca^{2+}-sensitivity in Tg-D166V muscle fibres, it was proposed that dephosphorylation of RLC in end-stage failing hearts was to compensate for the potentially detrimental effects of an abnormally increased Ca^{2+} responsiveness [34–36].

Molecular mechanisms of RLC phosphorylation in Tg-D166V hearts

The unique position of the D166V RLC mutation, located near the junction between the myosin head and filamentous rod, makes this region of myosin especially sensitive to charge-induced conformational changes that ultimately may affect the myosin–actin interaction and force generation. At the molecular level, it is postulated that phosphorylated myosin cross-bridges move away from the thick-filament backbone to be closer to the actin filaments increasing the probability of myosin–actin interactions [13]. This hypothesis was recently confirmed by low-angle X-ray diffraction studies showing a phosphorylation-induced 3.4% decrease in inter-thick filament spacing in permeabilized cardiac muscle fibres [37]. The authors also showed that following RLC phosphorylation, the myosin cross-bridges assume a new position, which is farther from the surface of the thick filament backbone and closer to the thin filament [37]. Based on our force data, we predict that phosphorylation of RLC in Tg-D166V muscle fibres would position the D166V myosin cross-bridges at a greater distance from actin than in Tg-WT fibres.

Studies in skinned muscle fibres further demonstrated that the ordered array of myosin heads, characteristic of relaxed thick filaments becomes reversibly disordered by RLC phosphorylation, thus increasing myosin head mobility and accessibility to actin [38]. Recent studies from the Cooke laboratory using epifluorescence of mant-nucleotides in the presence of blebbistatin suggest that the non-phosphorylated myosin heads are highly ordered in relaxed skinned skeletal [39] and cardiac [40] muscle fibres, and become vastly disordered upon phosphorylation. We hypothesize that the D166V mutation may stabilize a disordered conformation of myosin cross-bridges in Tg-D166V muscle fibres, and that their lack of ordered orientation could be further magnified by phosphorylation.

The importance of RLC phosphorylation was suggested by Davis *et al.* and a distinct balance of RLC phosphorylation was proposed to underlie the stretch activation response in the heart [24, 41]. Results from the Moss group supported this concept and showed that RLC phosphorylation affects cardiac muscle contractility by accelerating the stretch activation response [42]. These findings suggest that by decreasing RLC phosphorylation in the hearts of Tg-D166V mice, the D166V mutation may interfere with the proper ratio of phosphorylated to non-phosphorylated RLC that is required to maintain the stretch activation response and cardiac function.

Closing remarks

The results of this study indicate that RLC phosphorylation plays an important role not only in the physiological performance of the heart, but also helps to maintain normal cardiac function in the diseased myocardium. It seems to be especially important in the adaptive responses of the heart to pathophysiological injury. Our work leads us to the conclusion that phosphorylation of myosin RLC works as a regulator of the acto–myosin interaction in both normal and cardiomyopathic hearts. However, a phosphorylation-induced tuning of cardiac function in the diseased heart could be different from the healthy heart and may vary depending on the type and the level of cardiac insult.

Conflict of interest

No conflict of interest declared.

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