Protein kinase A–induced myofilament desensitization to Ca^{2+} as a result of phosphorylation of cardiac myosin–binding protein C

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In skinned myocardium, cyclic AMP–dependent protein kinase A (PKA)-catalyzed phosphorylation of cardiac myosin–binding protein C (cMyBP-C) and cardiac troponin I (cTnI) is associated with a reduction in the Ca^{2+} responsiveness of myofilaments and an acceleration in the kinetics of cross-bridge cycling, although the respective contribution of these two proteins remains controversial. To further examine the relative roles that cTnI and cMyBP-C phosphorylation play in altering myocardial function, we determined the Ca^{2+} sensitivity of force (pCa_{50}) and the activation dependence of the rate of force redevelopment (k_{tr}) in control and PKA-treated mouse myocardium (isolated in the presence of 2,3-butanedione monoxime) expressing: (a) phosphorylatable cTnI and cMyBP-C (wild type [WT]), (b) phosphorylatable cTnI on a cMyBP-C–null background (cMyBP-$C^{-/-}$), (c) nonphosphorylatable cTnI with $serines^{23/24/43/45}$ and $threonine^{144}$ mutated to alanines ($cTnI_{Ala5}$), and (d) nonphosphorylatable cTnI on a cMyBP-C–null background ($cTnI_{Ala5}$/cMyBP-$C^{-/-}$). Here, PKA treatment decreased pCa_{50} in WT, $cTnI_{Ala5}$, and cMyBP-$C^{-/-}$ myocardium by 0.13, 0.08, and 0.09 pCa units, respectively, but had no effect in $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium. In WT and $cTnI_{Ala5}$ myocardium, PKA treatment also increased k_{tr} at submaximal levels of activation; however, PKA treatment did not have an effect on k_{tr} in cMyBP-$C^{-/-}$ or $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium. In addition, reconstitution of $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium with recombinant cMyBP-C restored the effects of PKA treatment on pCa_{50} and k_{tr} reported in $cTnI_{Ala5}$ myocardium. Collectively, these results indicate that the attenuation in myofilament force response to PKA occurs as a result of both cTnI and cMyBP-C phosphorylation, and that the reduction in pCa_{50} mediated by cMyBP-C phosphorylation most likely arises from an accelerated cross-bridge cycling kinetics partly as a result of an increased rate constant of cross-bridge detachment.

INTRODUCTION

Under conditions of increased circulatory demand, the activation of β-adrenergic receptors leads to an increase in systolic force generation as well as an acceleration in the rates of rise and fall of the myocardial twitch. These changes in cardiac contractility are largely mediated by the activation of cAMP-dependent PKA, which catalyzes the phosphorylation of several Ca^{2+}-handling proteins; e.g., sarcolemmal L-type Ca^{2+} channels, phospholamban, and the ryanodine-sensitive Ca^{2+} release channels of the SR, as well as several sarcomeric proteins involved in the regulation of acto-myosin interactions; e.g., cardiac troponin I (cTnI) and cardiac myosin–binding protein C (cMyBP-C) (for review see Bers, 2002; Lohse et al., 2003; Layland et al., 2005). Importantly, PKA-catalyzed phosphorylation of cTnI and cMyBP-C has been shown to decrease the Ca^{2+} sensitivity of force and to accelerate the kinetics of cross-bridge cycling in skinned myocardial preparations (Patel et al., 2001; Layland et al., 2005; Stelzer et al., 2007). The respective contribution of these proteins, however, remains controversial despite significant efforts to determine the effects of cTnI and cMyBP-C phosphorylation in isolation, given the importance of these proteins in regulating the contractile responses of myocardium to β-adrenergic stimulation (Layland et al., 2005; Sadayappan et al., 2005), the consequences of cTnI and cMyBP-C mutations in the pathogenesis of hypertrophic cardiomyopathy (Redwood et al., 1999), as well as the implication that there is altered β-adrenergic signaling in the pathophysiology of cardiac hypertrophy, apoptosis, and end-stage heart failure (Lohse et al., 2003).

Recent studies have used transgenic animals to investigate the relationship of cTnI and cMyBP-C phosphorylation and the changes in contractile function that occur in response to PKA treatment. In the first of these studies, cTnI was stoichiometrically replaced with slow skeletal TnI (ssTnI) to investigate the functional consequences of

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Abbreviations used in this paper: BDM, 2,3-butanedione monoxime; cMyBP-C, cardiac myosin–binding protein C; cTnI, cardiac troponin I; RLC, myosin regulatory light chain; ssTnI, slow skeletal TnI; WT, wild type.

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removing the 31–amino acid N-terminal domain of cTnI normally targeted by PKA (serines$^{23/24}$). Here, Fentzke et al. (1999) and Kentish et al. (2001) showed that expression of ssTnI induced a significant leftward shift in the force–pCa relationship. However, PKA treatment had no significant effect on either Ca^{2+} sensitivity of force or cross-bridge cycling kinetics, leading the authors to conclude that phosphorylation of endogenous cTnI most likely plays a predominant role in mediating the contractile effects of PKA treatment in skinned myocardial preparations. On the other hand, Stelzer et al. (2007) observed an accelerated rate of cross-bridge cycling in response to PKA treatment in transgenic myocardium expressing non-PKA phosphorylatable cTnI ($cTnI_{Ala2}$). Moreover, a small but statistically insignificant trend toward a reduction in Ca^{2+} sensitivity was also observed after PKA treatment, suggesting that cMyBP-C phosphorylation may also be involved in the regulation of myofilament Ca^{2+} sensitivity. Interestingly, several investigators have also reported a similar trend toward decreased Ca^{2+} sensitivity after PKA treatment in transgenic myocardium expressing either nonphosphorylatable cTnI (Pi et al., 2002) or phospho-mimetic cTnI (Yasuda et al., 2007). The variability in myofilament force response to PKA and the resulting lack of statistically significant differences in these measurements, however, have prompted these investigators to reach the same conclusion that cMyBP-C phosphorylation plays little role, if any, in the regulation of Ca^{2+} sensitivity.

Recently, Bardswell et al. (2010) have reexamined the function of cMyBP-C phosphorylation in $cTnI_{Ala2}$ myocardium previously treated with the β-adrenergic receptor antagonist propranolol, showing a significant desensitization of myofilament Ca^{2+} sensitivity in response to PKA treatment. Although the introduction of propranolol in that study markedly reduced the level of cMyBP-C phosphorylation in $cTnI_{Ala2}$ myocardium and allowed the authors to observe an unprecedented significant effect of PKA phosphorylation of cMyBP-C on Ca^{2+} sensitivity of force, it remains to be determined whether additional reductions in the overall background of myofilament protein phosphorylation affect the ability of cMyBP-C phosphorylation to alter Ca^{2+} sensitivity.

In the present study, we reduced the basal levels of myosin regulatory light chain (RLC) phosphorylation to uniformly low levels using 2,3-butanedione monoxime (BDM) as described previously (Olsson et al., 2004; Stelzer et al., 2006b) to allow for a more direct examination of the functional effects of cTnI and cMyBP-C phosphorylations on myofilament function. Because myocardial preparations used in mechanical studies are randomly chosen from ventricular wall homogenates, it is possible that inter-sample variations in the level of RLC phosphorylation preclude the reliable detection of decreased Ca^{2+} sensitivity mediated by PKA-dependent phosphorylation of cMyBP-C, given that RLC phosphorylation increases the Ca^{2+} responsiveness of myofilaments (Olsson et al., 2004; Stelzer et al., 2006b; Colson et al., 2010) in opposition to that of cMyBP-C phosphorylation. To reexamine the relative effects of PKA-mediated phosphorylation of cTnI and cMyBP-C on contractile properties in skinned myocardial preparations on a nominal phosphoprotein background, we used BDM-treated wild-type (WT) and transgenic mouse myocardium expressing either nonphosphorylatable cTnI ($cTnI_{Ala5}$) or lacking the expression of cMyBP-C ($cMyBP\text{-}C^{-/-}$) to fully uncover the effects of cMyBP-C and/or cTnI phosphorylation on the Ca^{2+} sensitivity of force (pCa_{50}) and the activation dependence of the rate constant of force redevelopment (k_{tr}). Our main results demonstrate that in the absence of basal RLC phosphorylation, both cMyBP-C and cTnI phosphorylation contribute to the reduction in Ca^{2+} sensitivity mediated by PKA treatment in skinned myocardial preparations, whereas only cMyBP-C phosphorylation plays a prominent role in accelerating the kinetics of cross-bridge cycling. These observations were confirmed in control experiments in which mice expressing nonphosphorylatable cTnI on a cMyBP-C–null background ($cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$) were used as an experimental platform after near stoichiometric reconstitution of full-length recombinant cMyBP-C.

MATERIALS AND METHODS

Transgenic mice

$cTnI_{Ala5}$ mice, in which five known phosphorylation sites on cTnI (serines$^{23/24/43/45}$ and threonine^{144}) were mutated to alanines, and $cMyBP\text{-}C^{-/-}$ mice were generated as described in detail by Pi et al. (2002) and Harris et al. (2002), respectively. $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ mice were generated by breeding homozygous male and female $cTnI_{Ala5}$ mice with homozygous male and female $cMyBP\text{-}C^{-/-}$ mice. All procedures involving animal care and handling were reviewed and approved by the University of Wisconsin School of Medicine and Public Health Animal Care and Use Committee.

Solutions

Solution compositions were calculated using the computer program of Fabiato (1988) and the stability constants (corrected to pH 7.0 and 22°C) listed by Godt and Lindley (1982). Unless otherwise stated, all solutions contained (in mmol/L): 100 BES, 15 creatine phosphate, 5 dithiothreitol, 1 free Mg^{2+}, and 4 MgATP. In addition, pCa 9.0 solution contained (in mmol/L) 7 EGTA and 0.02 $CaCl_2$, pCa 4.5 solution contained 7 EGTA and 7.01 $CaCl_2$, and preactivating solution contained 0.07 EGTA. Ionic strength of all solutions was adjusted to 180 mmol/L using potassium propionate. A range of activating solutions containing different $[Ca^{2+}]_{free}$ was prepared by mixing pCa 9.0 and pCa 4.5 solutions.

Skinned myocardial preparations

Skinned ventricular myocardium was prepared according to the protocol described previously (Stelzer et al., 2006b). In brief, beating hearts were excised in vivo from anesthetized WT, $cTnI_{Ala5}$, $cMyBP\text{-}C^{-/-}$, and $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ mice of either sex (~6 mo old) and dissected in Ringer's solution (in mmol/L: 120 NaCl, 19 $NaHCO_3$, 1.2 Na_2HPO_4, 1.2 $MgSO_4$, 5 KCl, 1 $CaCl_2$, and10 glucose, pH 7.4; 22°C) preequilibrated with 95% O_2/5% CO_2. Basal

levels of RLC phosphorylation were reduced to uniformly low levels as described previously (Olsson et al., 2004; Stelzer et al., 2006b) by perfusing hemisected hearts with Ringer's solution containing 30 mmol/L BDM (30 min) before rapid freezing in liquid nitrogen. To obtain multicellular preparations (600–900 μm × 100–250 μm), frozen ventricles were thawed and homogenized in ice-cold relaxing solution (in mmol/L: 100 KCl, 10 imidazole, 5 $MgCl_2$, 2 EGTA, and 4 ATP, pH 7.0) using a homogenizer (Polytron PT 10-35; Kinematica, Inc.). Cellular homogenates were then centrifuged and washed with relaxing solution, resuspended in relaxing solution containing 250 μg/ml saponin and 1% Triton X-100 (30 min), and washed with relaxing solution several times. The resulting skinned preparations were then used in mechanical experiments or solubilized in SDS sample buffer for subsequent protein analysis.

Apparatus and experimental protocol

Mechanical measurements were performed at 22°C as described previously (Stelzer et al., 2006b). Skinned preparations with well-defined edges were transferred into an experimental chamber containing relaxing solution and attached to the arms of a motor (model 350; Cambridge Technology) and force transducer (model 403; Cambridge Technology). The chamber assembly was then placed on the stage of an inverted microscope (Olympus) fitted with a 40× objective and a CCTV camera (model WV-BL600; Panasonic). Bitmap images were acquired using an AGP 4X/2X graphics card and associated software (ATI Technologies) to assess mean sarcomere length (SL) during the course of each experiment. Changes in force and motor position were sampled (16-bit resolution; model DAP5216a; Microstar Laboratories) at 2.0 kHz using SLControl software developed in the laboratory of Campbell and Moss (2003). At the start of each experiment, a skinned preparation was stretched to a mean SL of ∼2.2 μm and then transferred to preactivating solutions before being transferred into activating solutions of varying $[Ca^{2+}]_{free}$ (i.e., pCa 6.0–4.5) for simultaneous determination of Ca^{2+}-activated force and k_{tr}. Once forces reached steady state in Ca^{2+}-activating solutions, the length of the preparation was rapidly slackened by ∼20% to induce detachment of myosin cross-bridges from actin, held at the slack length for ∼15 ms to allow for brief periods of unloaded shortening, and then restretched back to the original length. Typical changes in forces recorded at pCa 4.5 and 9.0 during this protocol are illustrated in Fig. 1. After rapid decreases in muscle length, steady-state force abruptly fell to zero and remained at near zero until the preparation was restretched to its original length. Preparations were then transferred back into relaxing solution after forces recovered to near steady-state levels. Maximum levels of rundown in force and k_{tr} measurements were <15 and 35%, respectively. At the conclusion of each experiment, skinned preparations were solubilized in 10 μl of SDS sample buffer and stored at −80°C for subsequent protein analysis.

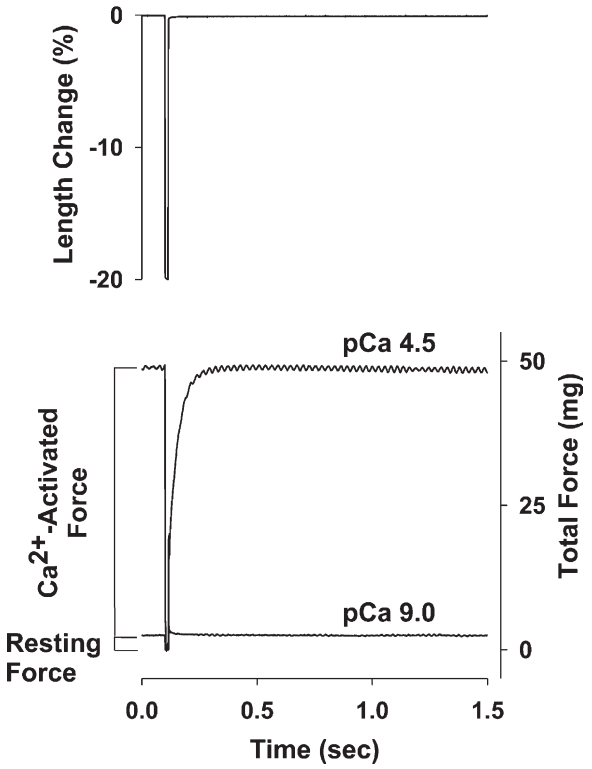

Figure 1. Experimental protocol for simultaneous determination of Ca^{2+}-activated force and the rate constant of force redevelopment (k_{tr}) in skinned myocardium. The bottom panel depicts the changes in force recorded before, during, and after a step change in length (top panel) of skinned myocardium. Once isometric force reached steady state in Ca^{2+}-activating solution (e.g., pCa 4.5), muscle length was rapidly slackened by 20%. After ∼15 ms of unloaded shortening, the preparation was restretched to its original length. Ca^{2+}-activated force at pCa 4.5 (P_0) was determined by subtracting the resting force measured at pCa 9.0 from the total force generated at pCa 4.5. k_{tr} was estimated by linear transformation of the half-time of force redevelopment after a rapid release–restretch maneuver.

In experiments assessing the effects of PKA (catalytic subunit of bovine PKA; Sigma-Aldrich) on mechanical properties, skinned preparations were first incubated for 1 h (∼22°C) in pCa 9.0 solution containing 1 U PKA/μl\e. Mechanical properties were then measured as described above. In reconstitution experiments using purified recombinant cMyBP-C, skinned preparations were incubated for 1 h (∼22°C) in pCa 9.0 solution containing 0.2 mg/ml cMyBP-C before the measurement of mechanical properties.

Analysis of myofibrillar protein expression, phosphorylation, and reconstitution

Phosphoproteins and myofibrillar proteins in untreated and PKA-treated WT, $cTnI_{Ala5}$, cMyBP-$C^{-/-}$, and $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium were examined according to the methods described previously (Stelzer et al., 2006c), with minor modifications. Myofibrillar proteins were quantified with the RC-DC protein assay (Bio-Rad Laboratories), serially loaded onto 12.5 or 10% Tris-HCl Precast Criterion gels (Bio-Rad Laboratories), separated by SDS-PAGE, and stained with Pro-Q Diamond (Invitrogen) and SYPRO Ruby (Invitrogen) according to the manufacturer's protocol. Phosphoproteins and myofibrillar proteins were then detected using UVP BioImaging System (UVP, LLC) and quantified with LaserPix software (Bio-Rad Laboratories). The product of the area and mean optical density of each protein or phosphoprotein band of interest was then plotted versus protein load (μg), and a first-order linear regression was fitted to the data points to determine the slope of the relationship between optical density and protein load.

To assess the incorporation of recombinant cMyBP-C in reconstitution experiments, silver staining of SDS-PAGE gels was performed as described previously (Stelzer et al., 2006b). In brief, gels were fixed in 50% methanol and 10% acetic acid and then (a) washed with distilled water, (b) incubated in 0.1% sodium thiosulfate solution for 90 s, (c) incubated in 0.09% silver nitrate solution for 20 min, and (d) imaged in developing solution containing 0.0004% sodium thiosulfate, 2% potassium carbonate, and 0.0068% formaldehyde until protein bands of interest became visible. The gels were then destained in 10% methanol and 10% acetic acid solution before imaging. Densitometric analysis was then performed using LaserPix software (Bio-Rad Laboratories),

and the ratio of the integrated optical density of the cMyBP-C band normalized to the integrated optical density of the corresponding α-actinin band was compared in reconstituted $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations and $cTnI_{Ala5}$ preparations containing endogenous cMyBP-C.

Expression and purification of recombinant cMyBP-C

Purified recombinant cMyBP-C was generated as described previously (Ge et al., 2009). In brief, DNA sequence encoding full-length cMyBP-C was PCR amplified from a clone containing full-length mouse cMyBP-C (provided by P.A. Powers, University of Wisconsin School of Public Health, Madison, WI) and cloned into pFastBac1 transfer plasmids (Invitrogen). The resulting vectors encoding native cMyBP-C and an 11–amino acid N-terminal FLAG-tag epitope were then used for site-specific transposition of expression cassettes into bacmids. Baculovirus strains were prepared according to the manufacturer's instructions. High titer viral stocks were then used to infect Sf9 cell monolayers (2.2×10^7 cells per 15-cm plate), and cells were collected 80–96 h after infection. Recombinant cMyBP-C was extracted and purified on anti-FLAG M2 agarose columns (Sigma-Aldrich).

Data analysis

Cross-sectional areas of skinned preparations were calculated by assuming the preparations were cylindrical and by equating the width (measured from video images of the mounted preparations) to diameter. The difference between steady-state force and the force baseline obtained after a 20% slack step was taken as the total force for that $[Ca^{2+}]_{free}$. Active force was then calculated by subtracting Ca^{2+}-independent force in pCa 9.0 solution from total force and normalizing to the cross-sectional area of the preparation. Each submaximal Ca^{2+}-activated force (P) was expressed as a fraction of the maximum Ca^{2+}-activated force (P_o) generated by the preparations at pCa 4.5, i.e., P/P_o. To determine the Ca^{2+} sensitivity of isometric force (pCa_{50}), the force–pCa data were fitted with the Hill equation: $P/P_o = [Ca^{2+}]^n/(k^n + [Ca^{2+}]^n)]$, where n is slope (Hill coefficient) and k is the Ca^{2+} concentration required for half-maximal activation (pCa_{50}). Apparent rate constants of force redevelopment (k_{tr}) were determined by linear transformation of the half-time of force recovery ($k_{tr} = -\ln 0.5 \times (t_{1/2})^{-1}$), as described previously (Patel et al., 2001). All data are presented as means ± SEM. Statistical analyses of the data were done using an unpaired t test. P values <0.05 were taken as indicating significant differences.

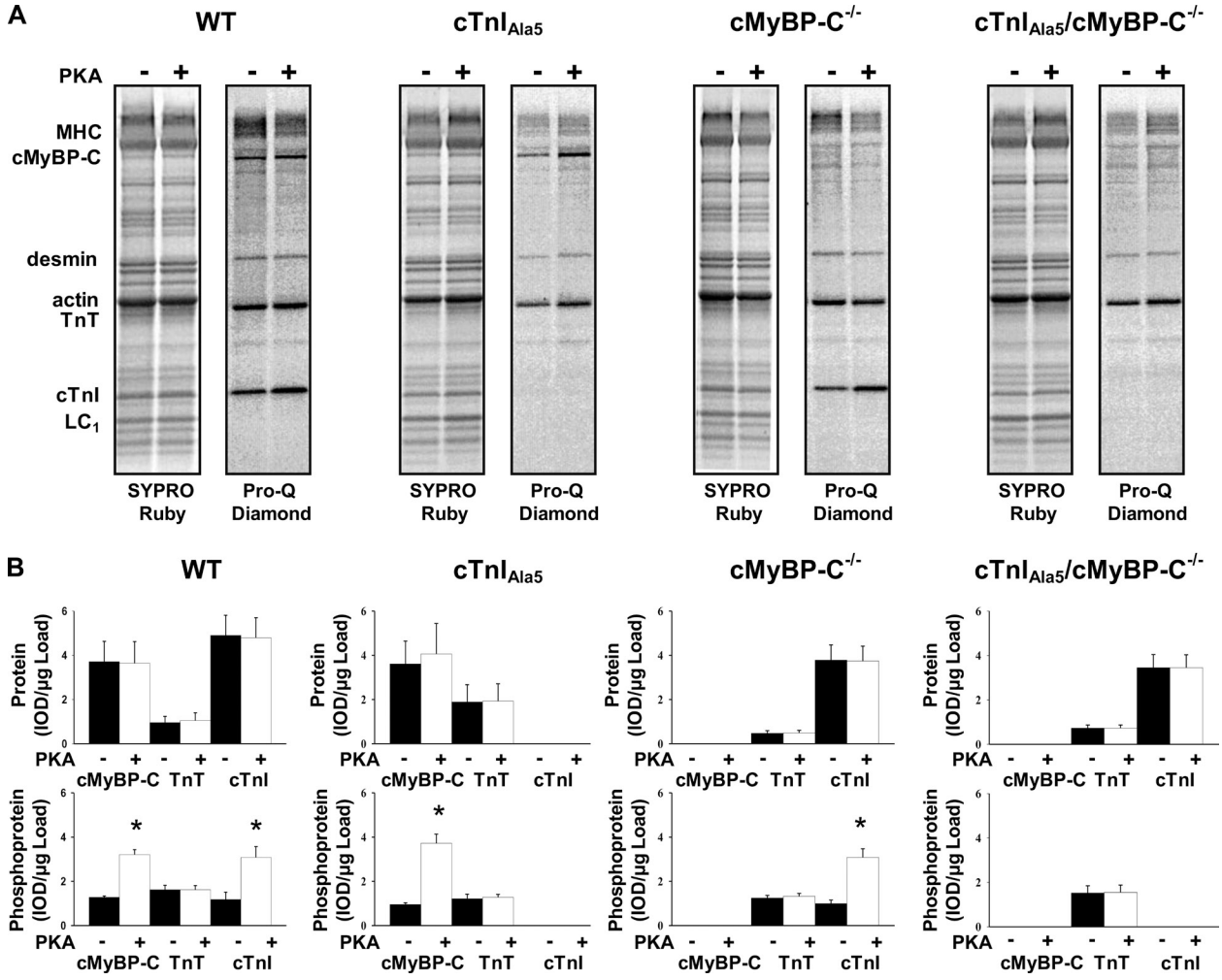

Figure 2. Myofibrillar protein phosphorylation in untreated and PKA-treated WT, $cTnI_{Ala5}$, cMyBP-$C^{-/-}$, and $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ skinned myocardium. (A) Representative 12.5% SDS-PAGE gel of myofibrillar proteins isolated from untreated (−) and PKA-treated (+) skinned myocardium and stained with SYPRO-Ruby to detect total protein and Pro-Q Diamond to detect phosphorylated proteins. (B) Slopes of proteins (top panel; $n = 8$) and phosphoproteins (bottom panel; $n = 8$) determined from regression analysis of plots of area × average optical density versus protein loaded (µg). Each bar represents the mean, and the error bar represents the SEM. *, $P < 0.05$.

Online supplemental material

Fig. S1 shows representative force redevelopment tracings following a mechanical slack–restretch maneuver in $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ preparations before and after cMyBP-C reconstitution and after PKA treatment of reconstituted preparations. It is available at http://www.jgp.org/cgi/content/full/jgp.201010448/DC1.

RESULTS

PKA-catalyzed phosphorylation of myofibrillar proteins in WT and transgenic skinned myocardium

Fig. 2 A shows a representative SDS-PAGE gel illustrating the expression profiles of phosphorylated and nonphosphorylated myofibrillar proteins isolated from WT, $cTnI_{Ala5}$, $cMyBP\text{-}C^{-/-}$, and $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ myocardium. Aside from the lack of cMyBP-C in $cMyBP\text{-}C^{-/-}$ and $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ myocardium, the expression of myofibrillar proteins in $cTnI_{Ala5}$, $cMyBP\text{-}C^{-/-}$, and $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ myocardium was similar to that observed in WT myocardium. Thus, neither ablation of cMyBP-C nor expression of nonphosphorylatable cTnI elicited any major changes in the expression pattern of myofibrillar proteins.

In control myocardial preparations, several myofibrillar proteins were found to exist in basally phosphorylated states in all four experimental groups. Specifically, the prominent phosphoproteins detected by Pro-Q Diamond staining included: cMyBP-C, desmin, TnT, and cTnI in WT myocardium; cMyBP-C, desmin, and TnT in $cTnI_{Ala5}$ myocardium; desmin, TnT, and cTnI in $cMyBP\text{-}C^{-/-}$ myocardium; and desmin and TnT in $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ myocardium. However, the basal levels of RLC phosphorylation were undetectable in either Pro-Q Diamond–stained SDS-PAGE gels or silver-stained two-dimensional gels (unpublished data). With the exception of desmin and TnT, PKA treatment elevated the phosphorylation status of both cMyBP-C and cTnI in WT myocardium, only cMyBP-C in $cTnI_{Ala5}$ myocardium, and only cTnI in $cMyBP\text{-}C^{-/-}$ myocardium (Fig. 2 B).

Ca^{2+} sensitivity of force in untreated and PKA-treated WT and transgenic skinned myocardium

Table I summarizes mechanical properties recorded from untreated and PKA-treated skinned preparations isolated from WT, $cTnI_{Ala5}$, $cMyBP\text{-}C^{-/-}$, and $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ myocardium. Under basal conditions, all four experimental groups generated similar amounts of maximum Ca^{2+}-activated force in pCa 4.5 solution. However, $cTnI_{Ala5}$ and $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ preparations generated less Ca^{2+}-activated force at submaximal levels of activation. As a result, the force–pCa relationships established in these preparations were shifted to the right (Fig. 3) of those established in WT controls and, most likely, reflected a functional consequence of the substitution of serines[43/45] and/or threonine[144] for alanines in cTnI because a similar effect was also observed in reconstituted thin filaments incorporating these same mutations (Noland et al., 1995).

In response to PKA treatment, phosphorylation of cTnI and cMyBP-C significantly reduced pCa_{50} in WT myocardium by 0.13 pCa units, whereas phosphorylation of cTnI alone produced a 0.09-pCa unit shift of the force–pCa curve to the right in $cMyBP\text{-}C^{-/-}$ myocardium (Fig. 3). In the absence of cTnI phosphorylation,

TABLE I

Effects of PKA treatment on mechanical properties in WT, $cTnI_{Ala5}$, $cMyBP\text{-}C^{-/-}$, and $cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ skinned myocardium

Treatment group	Resting force	Maximum Ca^{2+}-activated force	Hill coefficient (n_H)	Ca^{2+} sensitivity of force (pCa_{50})	Maximum rate of force redevelopment	Minimum rate of force redevelopment
	mN/mm^2	mN/mm^2			s^{-1}	s^{-1}
			WT (n = 16)			
(−) PKA ($n = 8$)	0.66 ± 0.07	14.43 ± 1.43	4.19 ± 0.15	5.82 ± 0.01	26.63 ± 0.88	2.22 ± 0.25
(+) PKA($n = 8$)	0.54 ± 0.08	12.62 ± 1.37	4.21 ± 0.15	5.69 ± 0.01[a]	27.98 ± 1.31	2.60 ± 0.12
			$cTnI_{Ala5}$ (n = 16)			
(−) PKA ($n = 8$)	0.89 ± 0.12	12.87 ± 1.03	5.03 ± 0.19[b]	5.48 ± 0.01[b]	22.00 ± 1.30[b]	2.51 ± 0.24
(+) PKA ($n = 8$)	0.99 ± 0.20	15.07 ± 2.65	4.09 ± 0.18[a]	5.40 ± 0.01[a]	23.23 ± 1.06	3.95 ± 0.26[a]
			$cMyBP\text{-}C^{-/-}$ (n = 16)			
(−) PKA ($n = 8$)	0.95 ± 0.12[b]	15.68 ± 1.67	3.95 ± 0.08	5.83 ± 0.01	34.48 ± 1.96[b]	3.66 ± 0.20[b]
(+) PKA ($n = 8$)	0.67 ± 0.12	14.30 ± 1.21	3.59 ± 0.09[a]	5.74 ± 0.01[a]	34.09 ± 1.87	4.00 ± 0.11
			$cTnI_{Ala5}/cMyBP\text{-}C^{-/-}$ (n = 16)			
(−) PKA ($n = 8$)	1.23 ± 0.17[b]	13.97 ± 1.40	4.25 ± 0.17	5.46 ± 0.01[b]	35.39 ± 1.66[b]	5.14 ± 0.43[b]
(+) PKA ($n = 8$)	1.23 ± 0.27	16.8 ± 2.47	4.23 ± 0.23	5.45 ± 0.01	34.34 ± 2.26	5.35 ± 0.70

Data are means ± SEM. Resting force was measured at pCa 9.0. Maximum force and the apparent rate constant of force redevelopment (k_{tr}) were measured at pCa 4.5. Minimum k_{tr} was measured at ∼10% of maximum force. pCa_{50} and n_H values were derived by fitting the force–pCa relationships to a Hill equation described in Materials and methods.

[a]Significantly different from values recorded in untreated (−PKA) skinned myocardial preparations.

[b]Significantly different from values recorded in untreated (−PKA) WT skinned myocardial preparations.

PKA treatment of $cTnI_{Ala5}$ myocardium reduced pCa_{50} to a similar degree as that observed in cMyBP-$C^{-/-}$ myocardium ($\Delta pCa_{50} = 0.08$). However, these effects of PKA treatment on Ca^{2+} sensitivity of force were completely abolished in $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium.

Rate of force redevelopment in untreated and PKA-treated WT and transgenic skinned myocardium

Fig. 4 illustrates the activation dependence of k_{tr} in untreated and PKA-treated WT and transgenic myocardium. Compared with WT preparations, $cTnI_{Ala5}$ preparations redeveloped submaximal forces at nearly the same rates. However, maximum k_{tr} was significantly depressed in $cTnI_{Ala5}$ myocardium ($26.63 \pm 0.88\ s^{-1}$ vs. 22.00 ± 1.30), most likely as a result of a minor shift in the expression of α myosin heavy chain to the slower β myosin heavy chain isoform (unpublished data), which has been shown to reduce the maximum k_{tr} values in skinned myocardial preparations (Locher et al., 2009). In cMyBP-$C^{-/-}$ and $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium, however, k_{tr} was dramatically increased at all levels of activation when compared with WT preparations, similar to results published previously (Stelzer et al., 2007).

Compared with untreated control preparations, PKA-treated WT and $cTnI_{Ala5}$ preparations redeveloped submaximal forces at significantly faster rates. Hence, the curvilinear k_{tr}–force relationships in PKA-treated WT and $cTnI_{Ala5}$ preparations were shifted to the left of those established in untreated control myocardium. However, maximum k_{tr} was not significantly different in untreated and PKA-treated WT and $cTnI_{Ala5}$ preparations (Table I). Furthermore, in the absence of cMyBP-C expression, no effects were observed on the k_{tr}–force relationships in PKA-treated cMyBP-$C^{-/-}$ and $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium when compared with untreated controls. Thus, phosphorylation of cTnI by PKA treatment in these preparations had no effect on the rate of force redevelopment.

Reconstitution of $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ skinned myocardium with purified recombinant cMyBP-C

To further assess the functional effects of cMyBP-C phosphorylation on Ca^{2+} sensitivity of force and the kinetics of cross-bridge cycling, skinned $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations were reconstituted with purified recombinant cMyBP-C (Fig. 5) and subjected to PKA treatment. Here, the readdition of near stoichiometric levels of cMyBP-C into $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations (0.98 ± 0.13 in reconstituted $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations vs. 1.02 ± 0.09 in $cTnI_{Ala5}$ preparations) reduced k_{tr} at all levels of activation (Fig. 6 B) but had no effect on pCa_{50} (Fig. 6 A). In fact, reconstituted $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations were nearly indistinguishable from $cTnI_{Ala5}$ preparations, confirming the functionality of the recombinant protein. In response to PKA treatment, $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations reconstituted with cMyBP-C showed a significant reduction in Ca^{2+} sensitivity ($\Delta pCa_{50} = 0.07$) and a similar increase in k_{tr} values reported in $cTnI_{Ala5}$ preparations (Fig. 6, C and D). These mechanical properties are summarized in Table II.

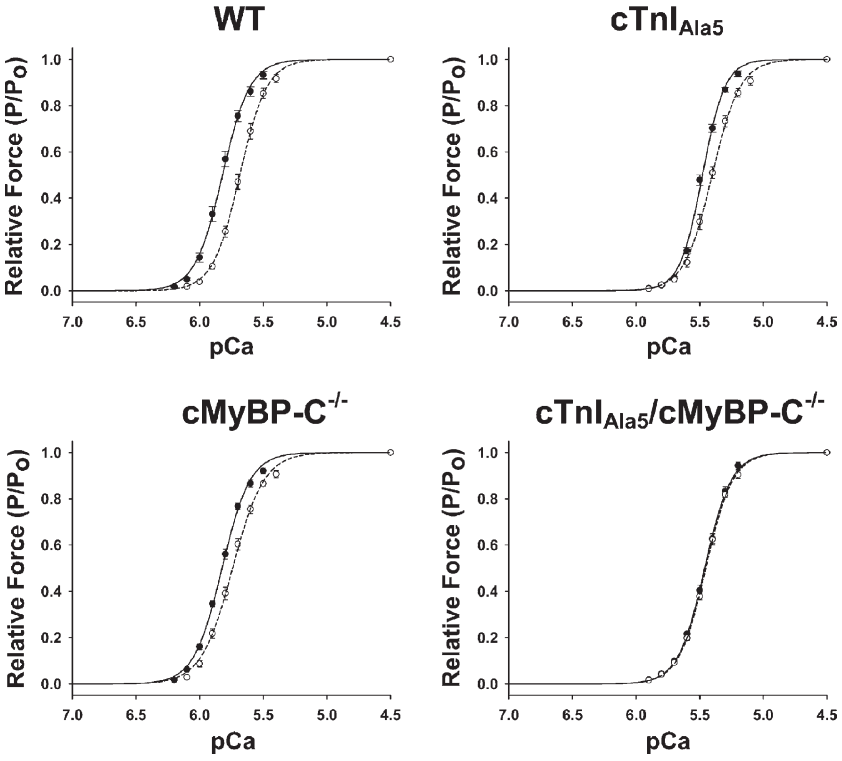

Figure 3. Effects of PKA treatment on Ca^{2+} sensitivity of force in WT, $cTnI_{Ala5}$, cMyBP-$C^{-/-}$, and $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ skinned myocardium. Relative isometric steady-state force (P/P_0) measured as a function of $-\log [Ca^{2+}]_{free}$ (pCa) in untreated (●; $n = 8$) and PKA-treated (○; $n = 8$) preparations. Smooth lines were generated by fitting the mean data to a Hill equation, as described in Materials and methods. All data are plotted as means ± SEM.

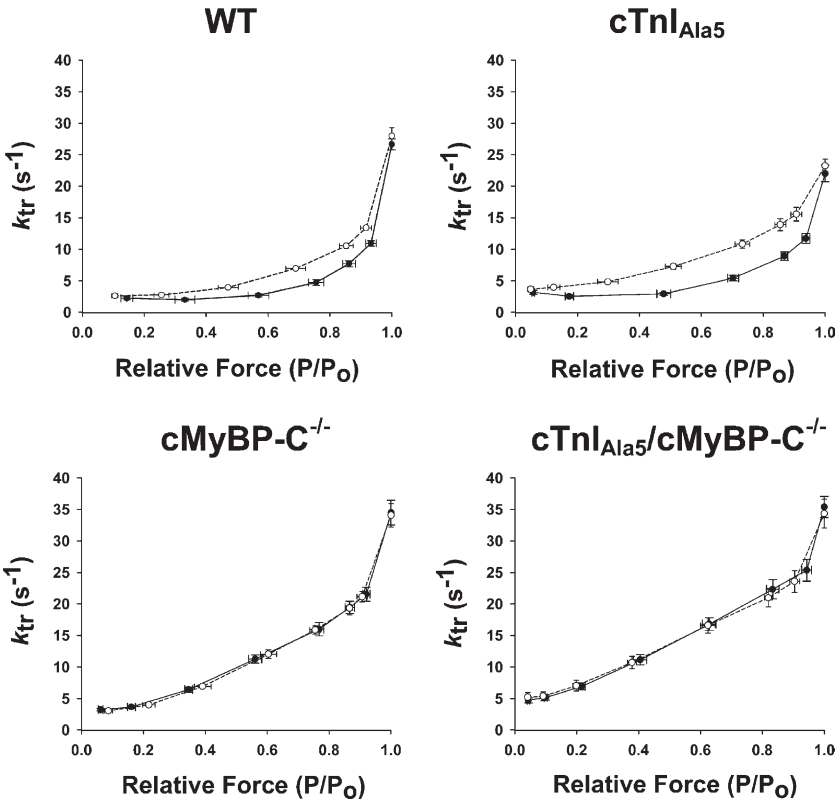

Figure 4. Effects of PKA treatment on the activation dependence of the rate of force redevelopment in WT, cTnI$_{Ala5}$, cMyBP-C$^{-/-}$, and cTnI$_{Ala5}$/cMyBP-C$^{-/-}$ skinned myocardium. The apparent rate constant of force redevelopment (k_{tr}) after a rapid release–restretch protocol measured as a function of relative isometric steady-state force (P/P_0) in untreated (●; $n = 8$) and PKA-treated (○; $n = 8$) preparations. All values are means ± SEM.

DISCUSSION

The primary focus of this study was to determine the contribution of cMyBP-C phosphorylation to the changes in contractile function that occur in response to PKA treatment. An important aspect of this study was the use of BDM treatments to reduce the basal levels of RLC phosphorylation to near zero to more accurately define the effects of cMyBP-C and/or cTnI phosphorylation on Ca^{2+} sensitivity of force and the kinetics of force redevelopment in skinned myocardium. Results here provide conclusive evidence to corroborate the role of cMyBP-C phosphorylation in the acceleration of cross-bridge cycling kinetics and the modulation of myofilament Ca^{2+} sensitivity. Importantly, these findings implicate and underscore the role of thick filament accessory proteins in the regulation of cardiac contractility during the positive inotropic and lusitropic effects of β-adrenergic stimulation in vivo.

Effect of PKA-dependent phosphorylation of cMyBP-C on Ca^{2+} sensitivity of force

In WT skinned myocardium, PKA-catalyzed phosphorylation of cTnI and cMyBP-C shifted the force–pCa

TABLE II

Summary of mechanical properties in cTnI$_{Ala5}$/MyBP-C$^{-/-}$ skinned myocardium before (- cMyBP-C), after reconstitution with purified cMyBP-C (+ cMyBP-C), and after reconstitution with purified cMyBP-C and PKA treatment (+ cMyBP-C/+ PKA)

Treatment group	Resting force	Maximum Ca^{2+}-activated force	Hill coefficient (n_H)	Ca^{2+} sensitivity of force (pCa_{50})	Maximum rate of force redevelopment	Minimum rate of force redevelopment
	mN/mm^2	*mN/mm^2*			*s^{-1}*	*s^{-1}*
			cTnI$_{Ala5}$/cMyBP-C$^{-/-}$			
(−) cMyBP-C ($n = 7$)	0.83 ± 0.15	14.70 ± 1.91	2.90 ± 0.08	5.49 ± 0.02	35.05 ± 2.52	2.28 ± 0.18
(+) cMyBP-C ($n = 6$)	1.26 ± 0.18	16.16 ± 1.08	3.28 ± 0.13[a]	5.51 ± 0.01	15.37 ± 2.00[a]	1.26 ± 0.22[a]
(+) cMyBP-C (+) PKA ($n = 6$)	1.03 ± 0.29	12.68 ± 0.62	2.73 ± 0.09[b]	5.44 ± 0.01[a,b]	22.64 ± 2.50[a,b]	3.48 ± 0.65[b]

Data are means ± SE. Resting force was measured at pCa 9.0. Maximum force and the rate constant of force redevelopment (k_{tr}) were measured at pCa 4.5. Minimum k_{tr} was measured at ~10% of maximum force. pCa_{50} and n_H values were derived by fitting the force–pCa relationships to a Hill equation.
[a]Significantly different from values recorded in cTnI$_{Ala5}$/cMyBP-C$^{-/-}$ skinned myocardial preparations (−cMyBP-C).
[b]Significantly different from values recorded in cTnI$_{Ala5}$/cMyBP-C$^{-/-}$ skinned myocardial preparations reconstituted with purified cMyBP-C (+cMyBP-C).

relationship to the right, decreasing the Ca^{2+} sensitivity of force by ~0.13 pCa units, in agreement with results published previously (Patel et al., 2001; Cazorla et al., 2006; Stelzer et al., 2007; Colson et al., 2008). Although a desensitizing shift in the myofilament force response to Ca^{2+} appears to be counterproductive in enhancing the inotropic state of β-adrenergic stimulation, this effect of PKA phosphorylation is thought to improve the relaxation of myocardium during increased heart rates to allow for adequate ventricular filling and, in turn, proper stroke volume and an appropriate level of cardiac output. The ability of myofilament proteins to enter a desensitized state in the presence of activator Ca^{2+}, however, has primarily been attributed to the phosphorylation of cTnI by PKA, which has been shown to weaken the interaction of the N-terminal domains of cTnI and cTnC (Ward et al., 2004) and to reduce the binding affinity of cTnC for Ca^{2+} (Robertson et al., 1982). In this way, PKA-catalyzed phosphorylation of cTnI is thought to enhance the relaxation of myocardium, primarily by limiting the activation of cardiac thin filaments and reducing the number of sites available on actin for strong-binding cross-bridges to form and generate force.

In cMyBP-$C^{-/-}$ myocardium, PKA treatment decreased the Ca^{2+} sensitivity of force by ~0.09 pCa units, indicating that cTnI phosphorylation had a lesser effect on myofilament Ca^{2+} sensitivity than simultaneous phosphorylation of cMyBP-C and cTnI in WT myocardium. This finding is consistent with the earlier results of Cazorla et al. (2006) and suggests that the absence of cMyBP-C either inhibits the ability of cTnI to decrease pCa_{50} or, more likely, removes the contribution of cMyBP-C phosphorylation to reduce pCa_{50} in cMyBP-$C^{-/-}$ myocardium. To investigate this issue further, we treated $cTnI_{Ala5}$ myocardium with PKA to isolate the effect of cMyBP-C phosphorylation on Ca^{2+} sensitivity and observed a similar rightward shift in the force–pCa relationship ($\Delta pCa_{50} = 0.08$). Further support for a role of cMyBP-C phosphorylation was provided by the lack of effect of PKA treatment in $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium, which was restored upon reconstitution of $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium with purified recombinant cMyBP-C. Collectively, these results strongly suggest that PKA-dependent phosphorylation of cMyBP-C and cTnI both contribute to the reduced Ca^{2+} sensitivity of force and the positive lusitropic effects of β-adrenergic stimulation.

Our finding that cMyBP-C phosphorylation contributes to the reduction in Ca^{2+} sensitivity mediated by PKA treatment was not predicted by earlier studies (Fentzke et al., 1999; Kentish et al., 2001; Konhilas et al., 2003) using ssTnI to elucidate the relative roles of cTnI and cMyBP-C phosphorylation. This implies that the cardiac isoform of TnI may be required for the phosphorylated species of cMyBP-C to influence the Ca^{2+} sensitivity of force in mouse myocardium. Indeed, previous studies using nonphosphorylatable cTnI (Pi et al., 2002) or phospho-mimetic cTnI (Yasuda et al., 2007) have reported a small but statistically insignificant decrease in Ca^{2+} sensitivity of force mediated by PKA-dependent phosphorylation of cMyBP-C in skinned myocardial preparations. In light of our findings, it

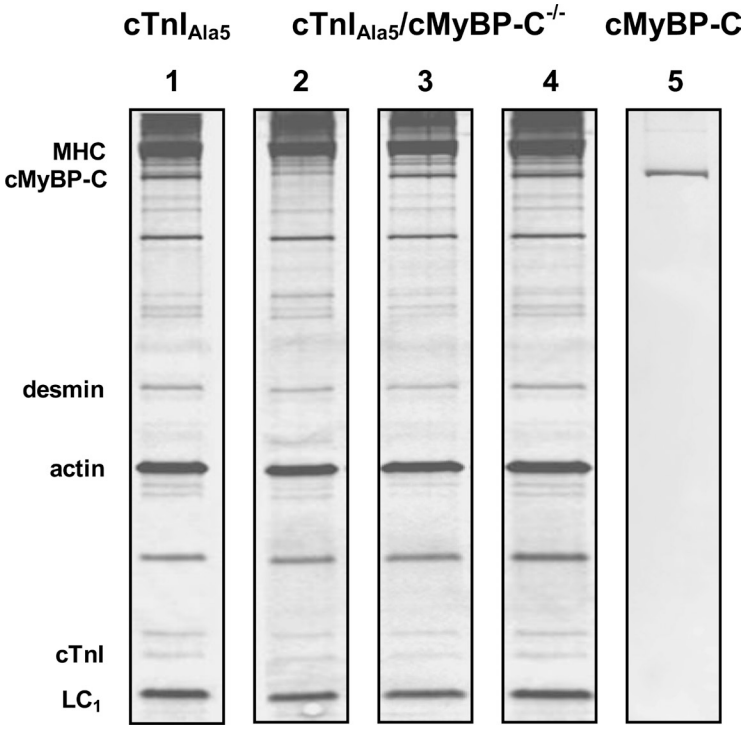

Figure 5. Reconstitution of $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ skinned myocardium with purified recombinant cMyBP-C. Representative silver-stained 10% SDS-PAGE gel of $cTnI_{Ala5}$ (lane 1) and $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations before cMyBP-C reconstitution (lane 2), after cMyBP-C reconstitution (lane 3), and after PKA treatment of reconstituted preparations (lane 4). Lane 5 shows the purified recombinant cMyBP-C protein expressed in baculovirus-infected Sf9 cells.

appears that the lack of statistical significance in these studies reflects an underlying variability in the level of RLC phosphorylation because near removal of RLC phosphorylation markedly reduced the variability in myofilament force response to PKA treatment, allowing us to observe a statistically significant effect of cMyBP-C phosphorylation on Ca^{2+} sensitivity.

Effect of PKA-dependent phosphorylation of cMyBP-C on the rate of force redevelopment

In skinned myocardium, there is compelling evidence to suggest that PKA-mediated phosphorylation of myofilament proteins also accelerates the kinetics of cross-bridge cycling, as indicated by an increase in unloaded shortening velocity (Strang et al.,1994), accelerated rates of force redevelopment (Patel et al., 2001; Stelzer et al., 2006c, 2007), and force relaxation (Zhang et al., 1995; Kentish et al., 2001) and faster rates of force decay and delayed force redevelopment after stretch activation (Stelzer et al., 2006c). This effect of PKA treatment, however, is somewhat controversial because several investigators have reported no effect on myofibrillar ATPase activity (de Tombe and Stienen, 1995), unloaded shortening velocity (Hofmann and Lange, 1994; Janssen and de Tombe, 1997), or rate of force relaxation (Johns et al., 1997). In this study, we found that PKA treatment accelerated the rate of force redevelopment at submaximal levels of activation in WT myocardium when compared with untreated WT controls. Moreover, a similar effect of PKA treatment was observed in $cTnI_{Ala5}$ myocardium expressing phosphorylatable cMyBP-C but was absent in cMyBP-$C^{-/-}$ myocardium. These results indicate that the acceleration in force redevelopment at submaximal $[Ca^{2+}]_{free}$ in skinned myocardial preparations after PKA treatment is most likely a result of the phosphorylation of cMyBP-C. In addition, reconstitution of $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations with recombinant cMyBP-C restored the values of k_{tr} to baseline and rendered the preparations sensitive to the effect of PKA treatment; i.e., phosphorylation of reconstituted cMyBP-C recapitulated the increase in k_{tr} values reported in $cTnI_{Ala5}$ myocardium that was otherwise absent in $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ myocardium. This acceleratory effect of cMyBP-C phosphorylation on the kinetics of force redevelopment at submaximal levels of activation has been proposed to involve several potential mechanisms (Stelzer et al., 2006c, 2007; Colson et al., 2008), including an increased probability of cross-bridge binding to actin, which would reduce the amount of time taken to cooperatively recruit cross-bridges into force-generating states, as well as an accelerated rate of cross-bridge cycling.

The possibility remains that cMyBP-C ablation may preclude the ability of cTnI phosphorylation to alter cross-bridge cycling kinetics in response to PKA treatment. Hence, future studies must examine the role of cTnI phosphorylation in the context of non-PKA phosphorylatable cMyBP-C to conclusively rule out any contributions of cTnI phosphorylation to the acceleration

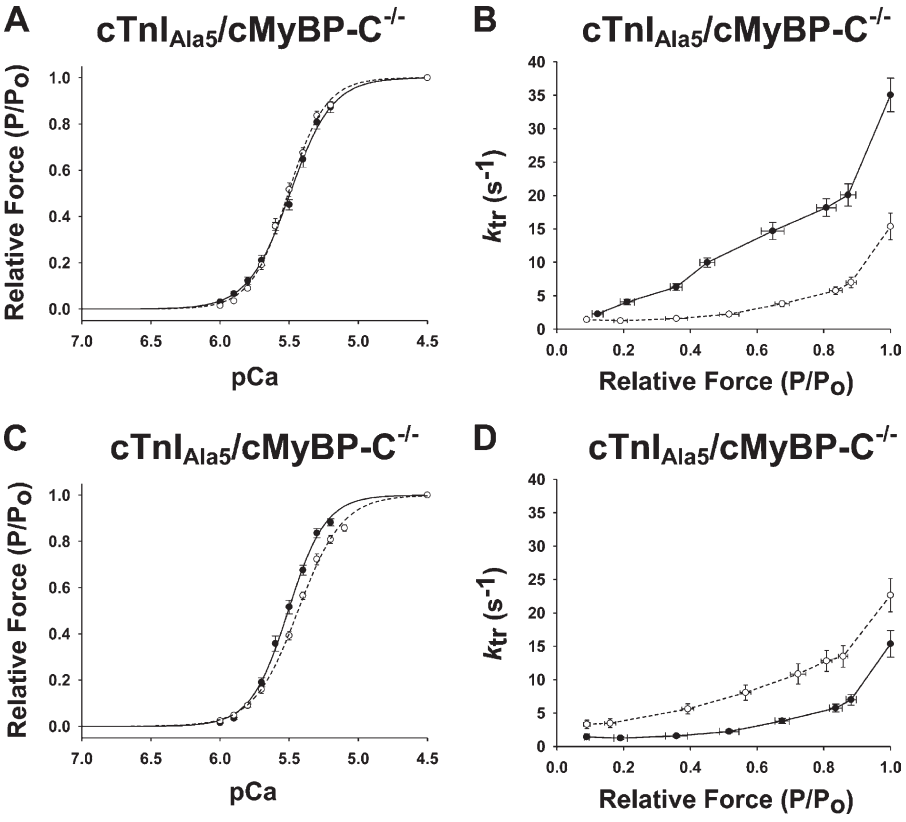

Figure 6. Effects of cMyBP-C reconstitution and PKA treatment in $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ skinned myocardium. Force–pCa (A) and k_{tr}–force (B) relationships in $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations before (●; n = 7) and after reconstitution (○; n = 6) with cMyBP-C. Force–pCa (C) and k_{tr}–force (D) relationships in cMyBP-C–reconstituted $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ preparations before (●; n = 6) and after (○; n = 6) PKA treatment. All data are plotted as means ± SEM.

in cross-bridge cycling, although previous studies using transgenic expression of nonphosphorylatable cMyBP-C at 74% on a cMyBP-C–null background have demonstrated no effect on the rate constants of force relaxation and delayed force development in stretch activation experiments after PKA treatment (Tong et al., 2008). Although several investigators have posited a role for cTnI phosphorylation in the acceleration of cross-bridge kinetics (Fentzke et al., 1999; Kentish et al., 2001; Turnbull et al., 2002), these studies do not demonstrate a direct effect of cTnI phosphorylation on cross-bridge cycling or an effect of cTnI phosphorylation in the absence of cMyBP-C phosphorylation. On the other hand, previous biochemical studies by Robertson et al. (1982) have demonstrated a faster rate of Ca^{2+} dissociation from cTnC in response to PKA-dependent phosphorylation of cTnI. Although this effect most likely contributes to the positive lusitropic effects of β-adrenergic stimulation, presumably by amplifying the effects of phospholamban phosphorylation on sarco/endoplasmic reticular Ca^{2+} ATPase reuptake of Ca^{2+} into the SR, it remains to be determined whether phosphorylation of cTnI has any direct effects on the dynamics of cross-bridge cycling.

Possible mechanisms of cMyBP-C phosphorylation–mediated attenuation of Ca^{2+} sensitivity of force

Because an important functional outcome of cMyBP-C phosphorylation (in this study and others; Stelzer et al., 2006c, 2007) is an acceleration of cross-bride cycling kinetics at submaximal levels of activation, it is reasonable to assume that such a mechanism could also account for a decrease in Ca^{2+} sensitivity of force. Indeed, an overall acceleration in the rate of cross-bridge cycling could reflect an underlying acceleration in the rate of cross-bridge detachment from actin, thereby reducing the amount of time cycling cross-bridges spend in the force-generating state. Interestingly, Colson et al. (2008) have shown that PKA-mediated phosphorylation of cMyBP-C radially displaces the heads of myosin cross-bridges closer to actin, suggesting that the faster rate of force redevelopment is most likely a result of a faster rate of cross-bridge attachment. Assuming that cMyBP-C phosphorylation only accelerates the attachment and transition of weakly bound cross-bridges into strongly bound force-generating states, such a mechanism would be expected to lead to an increase in Ca^{2+} sensitivity of force and oppose the reduction in Ca^{2+} sensitivity mediated by cTnI phosphorylation. Therefore, in light of our data, we propose that cMyBP-C phosphorylation speeds up both the kinetics of cross-bridge attachment (f_{app}) and detachment (g_{app}), such that the acceleration in g_{app} exceeds that of f_{app}, leading to a reduced fraction of cycling cross-bridges in the force-generating state (i.e., reduced $f_{app}/(f_{app} + g_{app})$) . Given that cMyBP-C phosphorylation has been shown to accelerate the kinetics of force decay after stretch activation (Stelzer et al., 2006c), which could possibly reflect an underlying acceleration in g_{app}, such a mechanism could potentially account for the attenuation in force generation at submaximal levels of $[Ca^{2+}]_{free}$ in WT and $cTnI_{Ala5}$ skinned myocardium after PKA treatment. This propounded acceleration in g_{app} after cMyBP-C phosphorylation, however, was not apparent in the minimum value of k_{tr} reported here in PKA-treated WT skinned myocardium (Table I), although a statistically significance acceleration in minimum k_{tr} was observed in PKA-treated $cTnI_{Ala5}$ skinned myocardium and PKA-treated $cTnI_{Ala5}$/cMyBP-$C^{-/-}$ skinned myocardium reconstituted with cMyBP-C (Table II). Thus, additional studies aimed at directly ascertaining the value of g_{app} in response to PKA treatment are needed to validate the mechanism of cMyBP-C phosphorylation–mediated desensitization of myofilament Ca^{2+} sensitivity. Lastly, although it is also possible that cMyBP-C phosphorylation may decrease Ca^{2+} sensitivity of force by reducing the number of cycling cross-bridges or the amount of force generated per cross-bridge, these alternatives appear less likely, given that maximum force generated at pCa 4.5 is unaltered by PKA treatment.

Presently, it is unclear how PKA-mediated phosphorylation of cMyBP-C enhances the rate of cross-bridge detachment. One possible mechanism may be that cMyBP-C phosphorylation increases the flexibility or compliance of myosin cross-bridges, thereby potentially reducing the stability of cross-bridges bound to actin during active force development (Stelzer et al., 2006c, 2007) and possibly enhancing the strain-dependent detachment of force-generating cross-bridges. Under normal conditions, the N-terminal cMyBP-C motif of cMyBP-C interacts with the subfragment 2 (S2) domain of myosin near the level arm domain of the myosin head (Gruen and Gautel, 1999). This interaction is thought to limit the availability of myosin cross-bridges for actin, possibly by tethering the heads of myosin cross-bridges closer to the thick filament backbone (Hofmann et al., 1991), although it is also probable that such a mechanism could potentially stabilize the structural integrity of the neck-hinge region of myosin, thereby affecting the transmission of force between cycling cross-bridges and actin. Recently, Nyland et al. (2009) have reported a reduction in the flexural rigidity of thick filaments in the absence of cMyBP-C, attributing the ability of cMyBP-C to stiffen the thick filament to its binding to myosin S2. Based on the putative interaction of cMyBP-C with myosin, we propose that the removal of the S2 interaction after PKA phosphorylation (Gruen et al., 1999) potentially destabilizes the hinge region of myosin in addition to moving the heads closer to actin (Colson et al., 2008), causing untethered cross-bridges to become more flexible and to attach at a faster rate but ultimately unable to remain strongly bound to actin in force-generating states. As a result, cross-bridges would detach at a faster rate, reducing the amount of force generated at

submaximal levels of $[Ca^{2+}]_{free}$ and thereby reducing the Ca^{2+} sensitivity of force. These mechanisms, however, have yet to be substantiated and require further investigation.

It is important to note here that although phosphorylation and ablation of endogenous cMyBP-C elicit similar effects on cross-bridge disposition (Colson et al., 2007, 2008) and cycling kinetics (Korte et al., 2005; Stelzer et al., 2006a,c, 2007) in skinned myocardium, our results indicate that these perturbations of cMyBP-C function are not absolutely equivalent, as indicated by the subtle differences in pCa–force and k_{tr}–force relationships reported in cMyBP-$C^{-/-}$ and PKA-treated $cTnI_{Ala5}$ myocardium. For example, in the absence of basal RLC phosphorylation, it appears that neither ablation nor subsequent reconstitution of cMyBP-C in cMyBP-$C^{-/-}$ myocardium has any effect on Ca^{2+} sensitivity of force in contrast to the reduction in Ca^{2+} sensitivity mediated by PKA-catalyzed phosphorylation of cMyBP-C in $cTnI_{Ala5}$ myocardium. In addition, it appears that the total removal of cMyBP-C in cMyBP-$C^{-/-}$ myocardium accelerates the rate of force development at all levels of activation and to a greater extent than cMyBP-C phosphorylation in PKA-treated $cTnI_{Ala5}$ myocardium. Although activation of potential compensatory mechanisms in cMyBP-$C^{-/-}$ myocardium may account for the differential effects of cMyBP-C ablation and phosphorylation on pCa_{50} and k_{tr}, it is also possible that the C-terminal interactions of cMyBP-C with titin (Freiburg and Gautel, 1996) and the light meromyosin portion of myosin (Moos et al., 1975) have functional roles in regulating the contractile properties of myofilament proteins.

Physiological significance of PKA-dependent phosphorylation of cMyBP-C

During β-adrenergic stimulation, the level of thin filament activation in myocardium is heightened such that peak force production is increased as a result of increased Ca^{2+} influx through the L-type Ca^{2+} channel. Although greater Ca^{2+} transients increase stroke work during systole and contribute to the positive inotropic effects of β-adrenergic stimulation, preservation of diastolic function during positive chronotropy follows from the attenuation of myofilament Ca^{2+} sensitivity, together with enhanced Ca^{2+} reuptake by sarco/endoplasmic reticular Ca^{2+} ATPase, to ensure that force decay occurs over an abbreviated time interval, though from an elevated systolic force. Because cMyBP-C and cTnI both constitute major physiological substrates of PKA phosphorylation at the myofilament level, it is plausible to consider these proteins as functional complements in the modulation of Ca^{2+} sensitivity. Based on the findings in this study, we propose that PKA-catalyzed phosphorylation of cMyBP-C contributes to the positive inotropic and lusitropic effects of β-adrenergic stimulation by accelerating the rate of force development and decreasing the Ca^{2+} sensitivity of force. Specifically, cMyBP-C phosphorylation accelerates the kinetics of cross-bridge cycling, most likely by inducing a disproportionate increase in the rate constant of cross-bridge detachment, which would not only accelerate the rate of force development but also allow the contractile apparatus to relax at a faster rate despite elevated levels of cytosolic $[Ca^{2+}]_{free}$. In conjunction with the PKA effects of cTnI phosphorylation on myofilament Ca^{2+} sensitivity and relaxation kinetics in vivo, these targeted modifications act to fine-tune the mechanical properties of the contractile apparatus in response to PKA-mediated changes in Ca^{2+} handling, and enable the contractile apparatus to expedite the rate of force development and relaxation during β-adrenergic stimulation.

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