Differential Roles of Cardiac Myosin-Binding Protein C and Cardiac Troponin I in the Myofibrillar Force Responses to Protein Kinase A Phosphorylation

Julian E. Stelzer, Jitandrakumar R. Patel, Jeffery W. Walker, Richard L. Moss

Abstract—The heart is remarkably adaptable in its ability to vary its function to meet the changing demands of the circulatory system. During times of physiological stress, cardiac output increases in response to increased sympathetic activity, which results in protein kinase A (PKA)-mediated phosphorylations of the myofilament proteins cardiac troponin (cTn)I and cardiac myosin-binding protein (cMyBP)-C. Despite the importance of this mechanism, little is known about the relative contributions of cTnI and cMyBP-C phosphorylation to increased cardiac contractility. Using engineered mouse lines either lacking cMyBP-C (cMyBP-C$^{-/-}$) or expressing a non-PKA phosphorylatable cTnI (cTnI$_{ala2}$), or both (cMyBP-C$^{-/-}$/cTnI$_{ala2}$), we investigated the roles of cTnI and cMyBP-C phosphorylation in the regulation of the stretch-activation response. PKA treatment of wild-type and cTnI$_{ala2}$ skinned ventricular myocardium accelerated stretch activation such that the response was indistinguishable from stretch activation of cMyBP-C$^{-/-}$ or cMyBP-C$^{-/-}$/cTnI$_{ala2}$ myocardium; however, PKA had no effect on stretch activation in cMyBP-C$^{-/-}$ or cMyBP-C$^{-/-}$/cTnI$_{ala2}$ myocardium. These results indicate that the acceleration of stretch activation in wild-type and cTnI$_{ala2}$ myocardium is caused by phosphorylation of cMyBP-C and not cTnI. We conclude that the primary effect of PKA phosphorylation of cTnI is reduced Ca^{2+} sensitivity of force, whereas phosphorylation of cMyBP-C accelerates the kinetics of force development. These results predict that PKA phosphorylation of myofibrillar proteins in living myocardium contributes to accelerated relaxation in diastole and increased rates of force development in systole. **(*Circ Res*. 2007;101:503-511.)**

Key Words: cross-bridge kinetics ■ β-adrenergic agonists ■ positive inotropy ■ contractile protein function

Enhanced cardiac contractile performance in response to increased circulatory demands is achieved in part through positive inotropy and lusitropy in response to increased sympathetic tone, resulting in increased stroke work during systole and earlier relaxation to optimize diastolic filling. Underscoring the importance of β-adrenergic stimulation in myocardial function, chronic hyperactivation of β-adrenergic pathways[1] or blunting of the β-adrenergic response[2] has been implicated in end-stage human heart failure.

At the level of the myofilament, the force at a given level of Ca^{2+} and the rate at which force is developed depend on properties that are intrinsic to the contractile proteins, such as protein isoforms, and on factors that affect protein function, such as phosphorylation status. β-Adrenergic stimulation effects on the heart are mediated via cAMP activation of protein kinase A (PKA), which in the myofilament, principally targets the thin filament protein cTnI and the thick filament protein cMyBP-C. In skinned myocardium, phosphorylation of cTnI and cMyBP-C is associated with increased rates of cross-bridge cycling and decreased Ca^{2+} sensitivity of force (reviewed elsewhere[3,4]), which together with altered Ca^{2+} handling (reviewed elsewhere[5]) would be expected to contribute to increased twitch force, decreased twitch duration, and increased rates of relaxation. Despite the physiological importance of the inotropic response in living myocardium, the respective contributions of cTnI and cMyBP-C to PKA-induced changes in myofibrillar contraction are not well understood.

The observation that PKA accelerates force development in skinned myocardium may have important implications for cardiac function in vivo. During both the isovolumic and ejection phases of systole, the left ventricle undergoes torsional deformation as the apex twists counterclockwise relative to the base.[6] Because the timing of electrical and mechanical activation varies across the ventricular wall,[7] the earliest activated regions of the wall (endocardium) contract and stretch regions that are activated later (epicardium).[8,9] It has also been observed that later in systole, late-activating epicardial fibers forcibly stretch the early-activating endocardial fibers.[9,10] The strain of endocardial fibers during systolic ejection[11] is thought to result from higher force production by the epicardial fibers, possibly as a result of greater levels of myosin regulatory light chain phosphorylation.[12,13] Stretch results in a delayed force response (stretch activation), which,

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when appropriately timed, could significantly increase force generation during late systole[14–16] and thereby contribute to late ejection.

Previous studies have shown that stretch activation is an intrinsic property of myocardium[17] and that the rate and amplitude of the delayed development of force following stretch varies with the level of Ca^{2+} activation,[16] suggesting that the response to stretch is regulated on a beat-to-beat basis[15] and contributes to myocardial power generation during ejection.[14,16,18] In this regard, we have recently shown that myocardial stretch activation in mice lacking cMyBP-C (cMyBP-$C^{-/-}$) differs dramatically from wild-type (WT) controls. The rates of force decay and delayed force development were accelerated, resulting in overall acceleration of the stretch-activation response.[19] We reasoned that the accelerated stretch-activation response in cMyBP-$C^{-/-}$ myocardium disrupts the timing of force generation such that the delayed increase in force attributable to stretch activation occurs prematurely, which truncates the period of ejection and reduces stroke volume, both of which have been observed in vivo.[20,21] Later, we observed that PKA treatment accelerated stretch activation in WT myocardium, such that it became indistinguishable from that of cMyBP-$C^{-/-}$ myocardium, but PKA treatment did not alter the stretch-activation response of cMyBP-$C^{-/-}$ myocardium.[22] These results suggest that the effect of PKA phosphorylation to accelerate stretch activation in WT myocardium involves cMyBP-C. However, recent studies[23–25] suggest that the cTnI phosphorylation state may affect the rate of cross-bridge cycling and thereby modulate the rate of force development in vivo. Thus, there is not a consensus regarding the roles of cMyBP-C and cTnI in the PKA-mediated contractile response of myocardium.

The purpose of this study was to examine the respective effects of PKA-mediated phosphorylations of cMyBP-C and cTnI on the stretch-activation responses of murine skinned myocardium. In addition to the cMyBP-$C^{-/-}$ mouse, we used a knock-in mouse that expresses mutant cTnI that is not phosphorylated by PKA ($cTnI_{ala2}$),[26] and we have crossed cMyBP-$C^{-/-}$ with $cTnI_{ala2}$ mice to produce a line that has no functional PKA target sites on either protein (cMyBP-$C^{-/-}$/$cTnI_{ala2}$). Our results show that the primary effect of PKA phosphorylation of cTnI is to reduce the Ca^{2+} sensitivity of force, whereas cMyBP-C phosphorylation plays the dominant role in PKA modulation of the rate of cross-bridge cycling and force development.

Materials and Methods

An expanded Materials and Methods section is included in the online data supplement at http://circres.ahajournals.org.

Transgenic Animals

cMyBP-C–null (cMyBP-$C^{-/-}$) mice and $cTnI_{ala2}$ mice in which Ser23/24 were converted to alanines were generated previously.[20,26] cMyBP-$C^{-/-}$/$cTnI_{ala2}$ mice were generated by breeding homozygous male and female cMyBP-$C^{-/-}$ mice with homozygous male and female $cTnI_{ala2}$ mice. All procedures involving animal care and handling were reviewed and approved by the UW Medical School Animal Care and Use Committee.

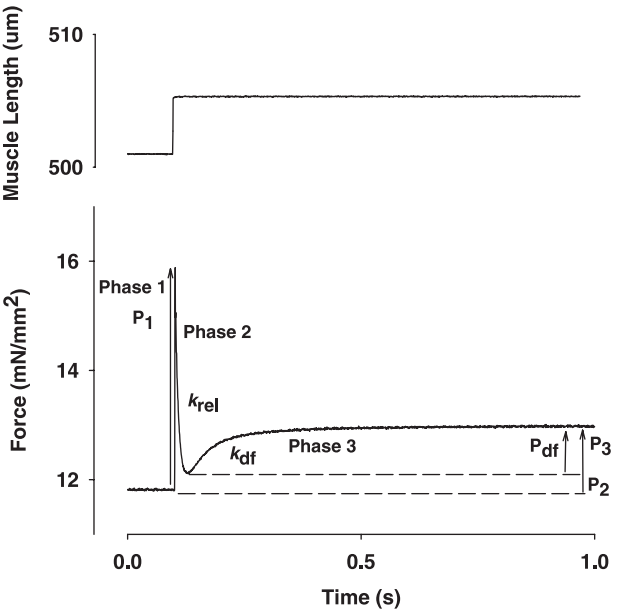

Figure 1. Stretch-activation response in murine myocardium. The force transient shown (bottom) is typical of the stretch-activation responses of WT myocardium following a stretch of 1% of muscle length (top). Once a steady-state isometric force of ≈50% of maximal was achieved in the presence of Ca^{2+}, the muscle was stretched and then held at the longer length, as described under Materials and Methods. The recorded variables are labeled on the force recording and described in the text.

Apparatus and Mechanical Experiments

Skinned ventricular myocardium was prepared and attached to a motor and force transducer as described previously.[13] Force–pCa relationships on skinned myocardium were constructed by varying the amount of activating Ca^{2+} in solutions, and stretch activation measurements were performed by imposing a rapid stretch of 1% of muscle length on maximally and submaximally activated fibers before and after PKA treatment.[13,22]

Determination of Protein Phosphorylation

Myofibrillar protein analysis was performed by SDS-PAGE using 10 or 12.5% Tris-HCl Criterion Precast gel (Bio-Rad) followed by SYPRO-Ruby (Molecular Probes) staining as previously described.[22] Detection of phosphorylated proteins was achieved with Pro-Q Diamond staining (Molecular Probes).[22] A UVP BioImaging System (UVP Inc) was used to quantify the relative abundance of total myofibrillar proteins and phosphoproteins.

In Vivo Cardiac Function

Noninvasive transthoracic echocardiography was performed as described previously.[20] Anesthetized mice were probed with a Visual Sonics 770 ultrasonograph with a 30-MHz transducer (RMV 707B) (Visual Sonics, Toronto, Canada) to acquire 2D M-mode and Doppler images of the left ventricle.[20] All echocardiography parameters were measured over at least 3 consecutive cardiac cycles.

Results

Responses of WT and Mutant Myocardium to Stretch

The recordings in Figure 1 exemplify stretch activation in WT Ca^{2+}-activated myocardium developing prestretch isometric force that was ≈50% maximal. The amplitudes corresponding to phases 2 and 3 were normalized to prestretch isometric force to allow comparisons of stretch-activation responses at different levels of activation. P_2 values

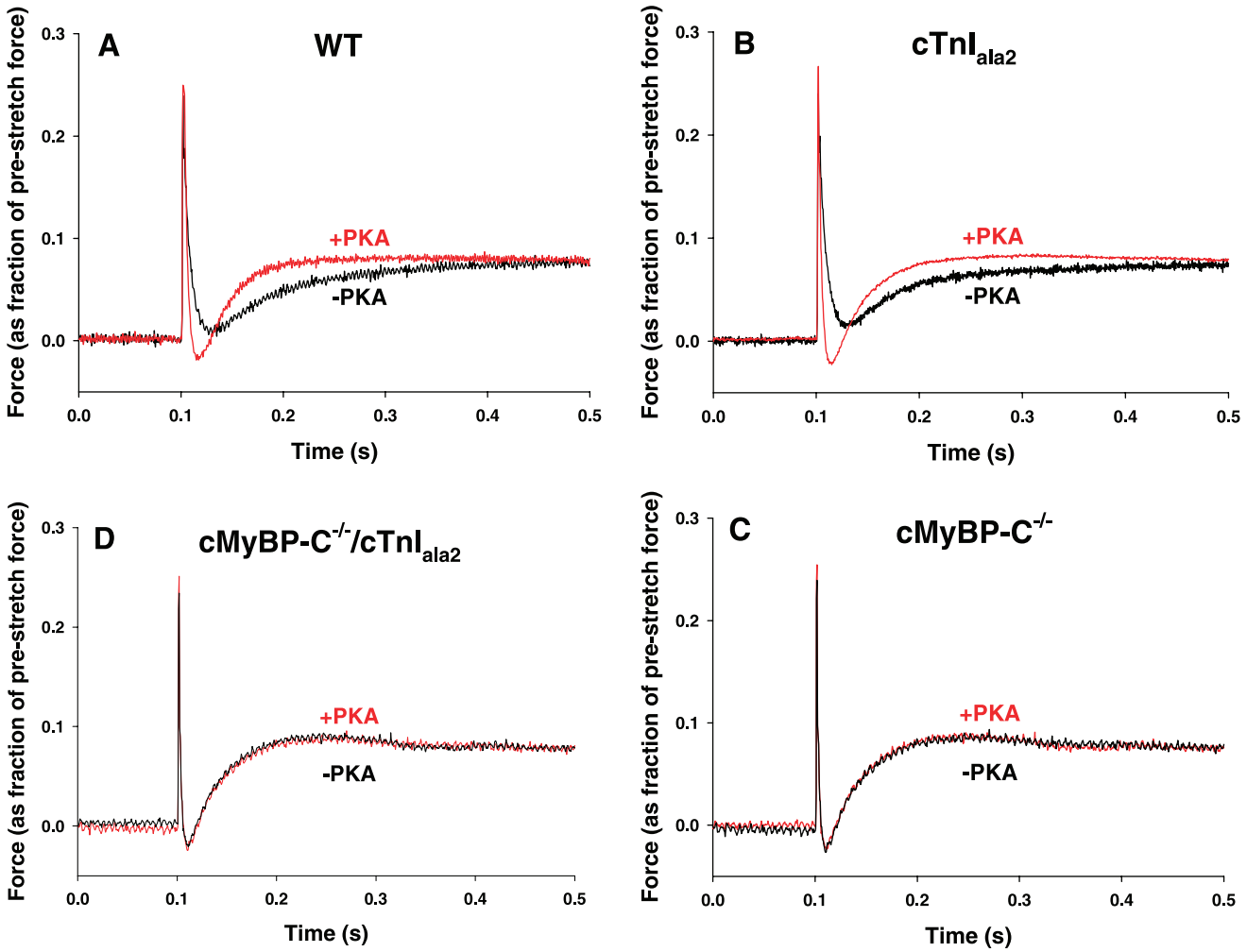

Figure 2. Effects of PKA treatment on the stretch-activation responses of WT and mutant myocardium. Force transients following a stretch of 1% of muscle length were recorded at [Ca^{2+}], yielding a prestretch isometric force of ≈50% maximal before (black traces) and following (red traces) PKA treatment in WT (A), cTnI$_{ala2}$ (B) , cMyBP-C$^{-/-}$ (C), and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ (D) myocardium. These representative transients are normalized to prestretch isometric force corresponding to the force baseline, which is arbitrarily set at 0.

in cMyBP-C$^{-/-}$ and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ myocardium differed significantly from WT and cTnI$_{ala2}$ myocardium (Figure 2A), in that the former were almost always less than the isometric force baseline at all levels of activation (Figure 3A). The apparent rate constant of force decay (k_{rel}) in phase 2 is determined by the rates of detachment and reattachment of strongly bound cross-bridges,[27,28] and at submaximal levels of activation, k_{rel} was significantly greater in cMyBP-C$^{-/-}$ and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ myocardium compared with WT and cTnI$_{ala2}$ myocardium (Figure 4A).

Cross-bridge recruitment (force, P_3) following stretch is related to prestretch isometric force.[16,29] Because the number of cross-bridges recruited by stretch progressively decreases as activation is increased (because fewer cross-bridges are in weakly bound or unbound states[15,16]), values of P_3 normalized to prestretch force also decreased as a function of increased activation. However, no differences in the normalized amplitude of P_3 were seen among the mouse lines studied here, suggesting that the numbers of cross-bridges recruited by stretch at a given activation level were similar. The trough-to-peak excursion of the phase 3 delayed force transient (P_{df}) was proportionally greater in cMyBP-C$^{-/-}$ and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ myocardium because of greater cross-bridge detachment (more negative P_2 values) during phase 2 compared with WT and cTnI$_{ala2}$ myocardium (Figure 3B).

The rate at which stretch cooperatively recruits cross-bridges into force-generating states can be estimated from the apparent rate constant of phase 3 force development (k_{df}).[29] Increases in activation accelerate k_{df} (Figure 4C) and reduce the delayed force attributable to stretch activation (P_3) (as a fraction of prestretch force) because a high proportion of cross-bridges are initially bound to the thin filament, thereby reducing the number available for recruitment following stretch. Delayed force development following stretch in WT and cTnI$_{ala2}$ myocardium occurred as a biexponential process at submaximal activations yielding fast and slow rate constants (k_1 and k_2) and their corresponding amplitudes (a and b) (Table I in the online data supplement). In contrast, delayed force development in both cMyBP-C$^{-/-}$ and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ myocardium occurred as a single exponential process, corresponding to the fast rate of force development in WT and cTnI$_{ala2}$ myocardium (supplemental Table I). In the present

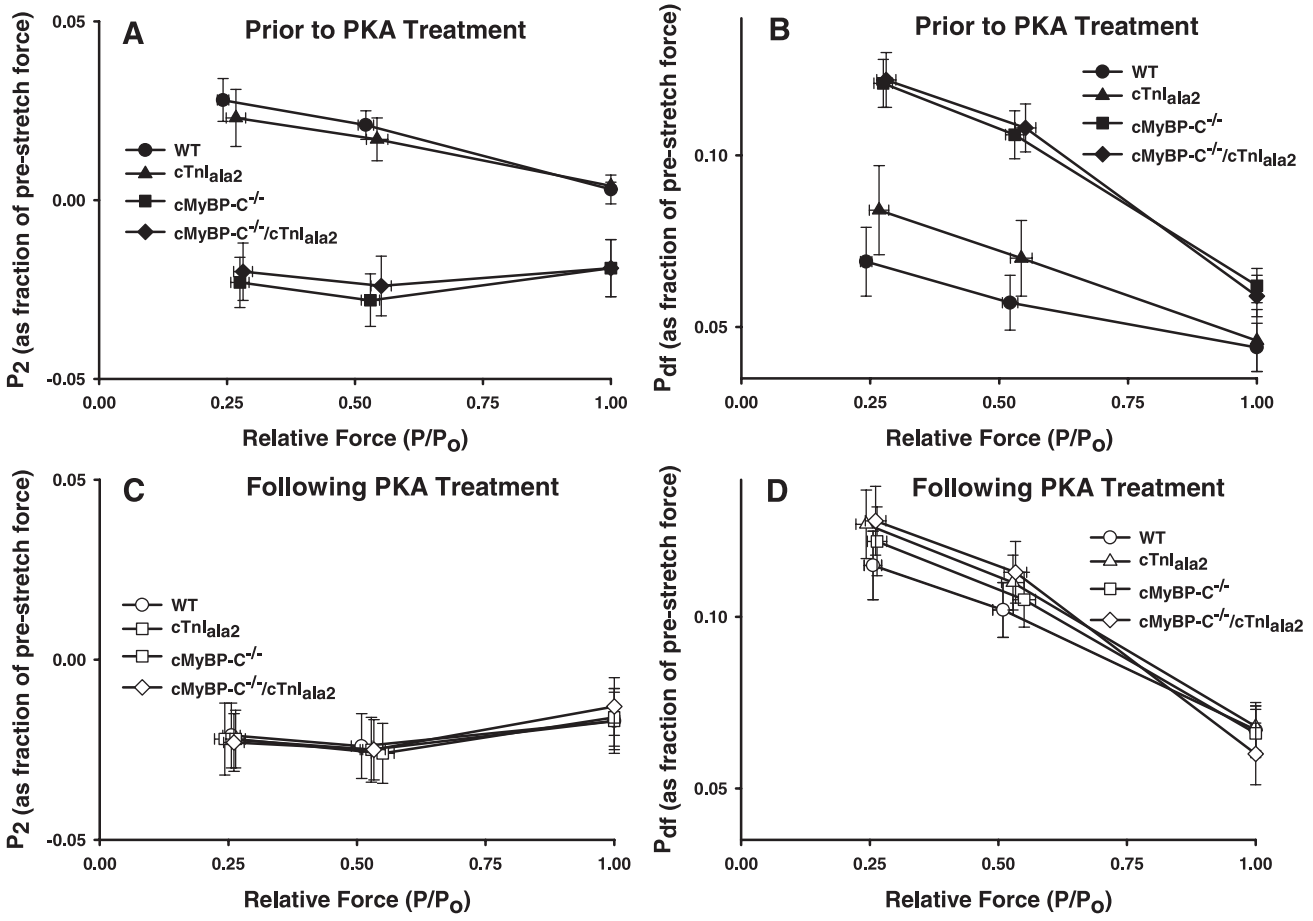

Figure 3. Effects of activation level and PKA treatment on stretch-activation parameters P_2 and P_{df}. P_2 and P_{df} values (normalized to prestretch isometric force) were measured from the force responses to stretches of 1% of muscle length as a function of activation. A, P_2 values before PKA treatment in WT (filled circles), $cTnI_{ala2}$ (filled triangles), cMyBP-$C^{-/-}$ (filled squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (filled diamonds) myocardium. B, P_{df} values before PKA treatment in WT (filled circles), $cTnI_{ala2}$ (filled triangles), cMyBP-$C^{-/-}$ (filled squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (filled diamonds) myocardium. C, P_2 values following PKA treatment in WT (open circles), $cTnI_{ala2}$ (open triangles), cMyBP-$C^{-/-}$ (open squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (open diamonds) myocardium. D, P_{df} values following PKA treatment in WT (open circles), $cTnI_{ala2}$ (open triangles), cMyBP-$C^{-/-}$ (open squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (open diamonds) myocardium. Data are means±SEM from 8 to 10 myocardial preparations.

study, there were no differences in k_{df} at maximal activation among the mouse lines studied, but k_{df} was significantly faster at submaximal levels of activation in cMyBP-$C^{-/-}$ and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ compared with WT and $cTnI_{ala2}$ myocardium (Figure 4C). Because the slow phase of force development is thought to manifest cooperative cross-bridge recruitment,[16] which acts to slow the overall rate of force development,[15] its absence in cMyBP-$C^{-/-}$ and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium suggests that ablation of cMyBP-C either accelerates or eliminates the cooperative recruitment of cross-bridges into force-generating states. As a result, the overall rate of stretch activation is faster in myocardium from these mouse lines.

Effects of PKA Treatment on Stretch Activation in WT and Mutant Myocardium

To investigate the respective roles of cMyBP-C and cTnI in the PKA-induced acceleration of stretch activation, skinned preparations from WT, cMyBP-$C^{-/-}$, $cTnI_{ala2}$, and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium were treated with PKA. Because the amplitudes and rates of the phases of the stretch-activation response in mouse myocardium are activation dependent,[16] the pCa of the activating solutions was adjusted to ensure that prestretch isometric force in all myocardial preparations was similar both before and after treatment with PKA. For example, to achieve a force of ≈50% maximal, WT fibers were activated in solution of pCa 5.75 before treatment with PKA and in solution of pCa 5.65 following treatment with PKA. PKA treatment of WT and $cTnI_{ala2}$ myocardium significantly altered the stretch-activation response (Figure 2A and 2B) in that cross-bridge detachment during phase 2 was accelerated, causing P_2 to fall below prestretch isometric force (Figure 3C) and thereby increasing the overall amplitude of phase 3 (P_{df}) (Figure 3D). PKA also accelerated both k_{rel} (Figure 4B) and k_{df} (Figure 4D) such that the overall rate of delayed force development was accelerated. The acceleration of k_{df} following PKA treatment in WT and $cTnI_{ala2}$ myocardium was caused by the elimination of the slow rate process (k_2) rather than acceleration of the fast rate constant k_1, ie, delayed force developed as a single rate process similar to k_1 for cMyBP-$C^{-/-}$ and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium (supplemental Table I).

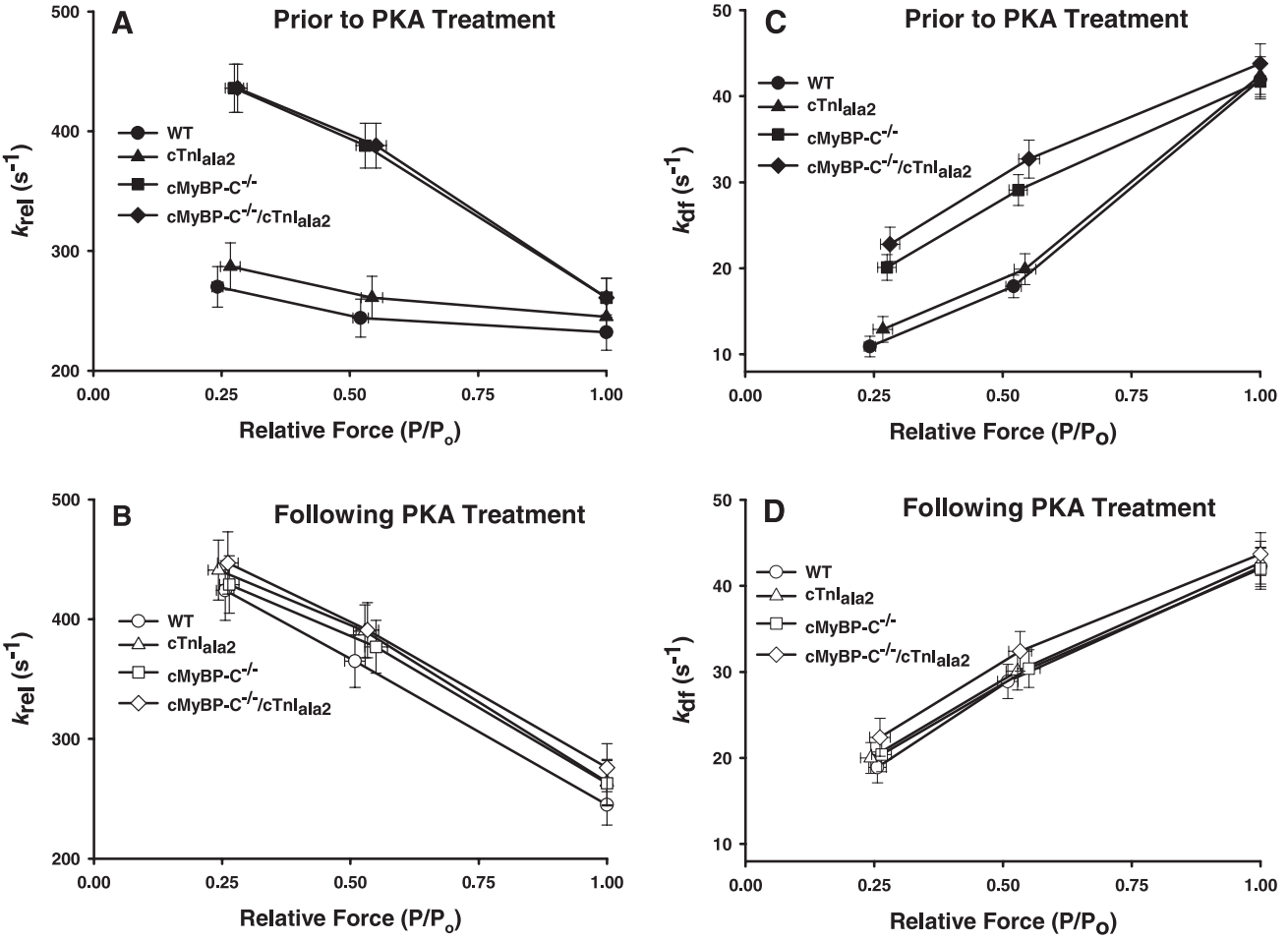

Figure 4. Effect of activation and PKA treatment on stretch k_{rel} and k_{df}. k_{rel} and k_{df} values were calculated from the force responses to stretches of 1% of muscle length at different levels of activation. A, k_{rel} values before PKA treatment in WT (filled circles), $cTnI_{ala2}$ (filled triangles), cMyBP-$C^{-/-}$ (filled squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (filled diamonds) myocardium. B, k_{rel} values following PKA treatment in WT (open circles), $cTnI_{ala2}$ (open triangles), cMyBP-$C^{-/-}$ (open squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (open diamonds) myocardium. C, k_{df} values before PKA treatment in WT (filled circles), $cTnI_{ala2}$ (filled triangles), cMyBP-$C^{-/-}$ (filled squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (filled diamonds) myocardium. D, k_{df} values following PKA treatment in WT (open circles), $cTnI_{ala2}$ (open triangles), cMyBP-$C^{-/-}$ (open squares), and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ (open diamonds) myocardium. Data are means±SEM from 8 to 10 myocardial preparations.

In contrast to the effects of PKA treatment on WT and $cTnI_{ala2}$ myocardium, PKA had no apparent effects on stretch activation in cMyBP-$C^{-/-}$ (Figure 2C) or cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium (Figure 2D), suggesting that the acceleration of stretch activation following treatment with PKA is attributable to the phosphorylation of cMyBP-C and not cTnI.

Effects of cMyBP-C and cTnI Phosphorylation on Force–pCa Relationships

Figure 5 presents an example of phosphoprotein analysis using SYPRO-Ruby and Pro-Q Diamond staining and shows that ablation of cMyBP-C did not change phosphorylation of cTnI in hearts from null mice[22,30] and that replacement of endogenous cTnI with mutant $cTnI_{ala2}$ did not change cMyBP-C phosphorylation.[31] Ablation of cMyBP-C, replacement of cTnI with $cTnI_{ala2}$, or both (cMyBP-$C^{-/-}$/$cTnI_{ala2}$) had little effect on the Ca^{2+} sensitivity of force, Ca^{2+}-independent force at pCa 9.0, or maximum force at pCa 4.5 when compared with WT controls (supplemental Table II). Figure 5 demonstrates that PKA phosphorylated both cTnI and cMyBP-C in WT myocardium, phosphorylated only cMyBP-C in $cTnI_{ala2}$ myocardium, and only cTnI in cMyBP-$C^{-/-}$ myocardium. No phosphorylation of cMyBP-C or cTnI was detected in PKA-treated cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium, confirming the absence of PKA target sites on cMyBP-C and cTnI in these mice (Figure 5).

PKA treatment significantly reduced the Ca^{2+} sensitivity of force in WT and cMyBP-$C^{-/-}$ myocardium but produced no significant changes in $cTnI_{ala2}$ or in cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium. Although the small reduction in the Ca^{2+} sensitivity of force in PKA-treated $cTnI_{ala2}$ myocardium did not reach statistical significance, a small decrease might be expected because of acceleration of the rates of cross-bridge cycling by cMyBP-C phosphorylation, in that increased rates would reduce the time cross-bridges spend in force-producing states. In any case, the contribution of cMyBP-C phosphorylation to decreased Ca^{2+} sensitivity appears to be minor, and, overall, our results support the idea that decreased Ca^{2+} sensitivity of force with PKA phosphorylation is mainly attributable to phosphorylation of cTnI, confirming earlier conclusions.[3,4,32]

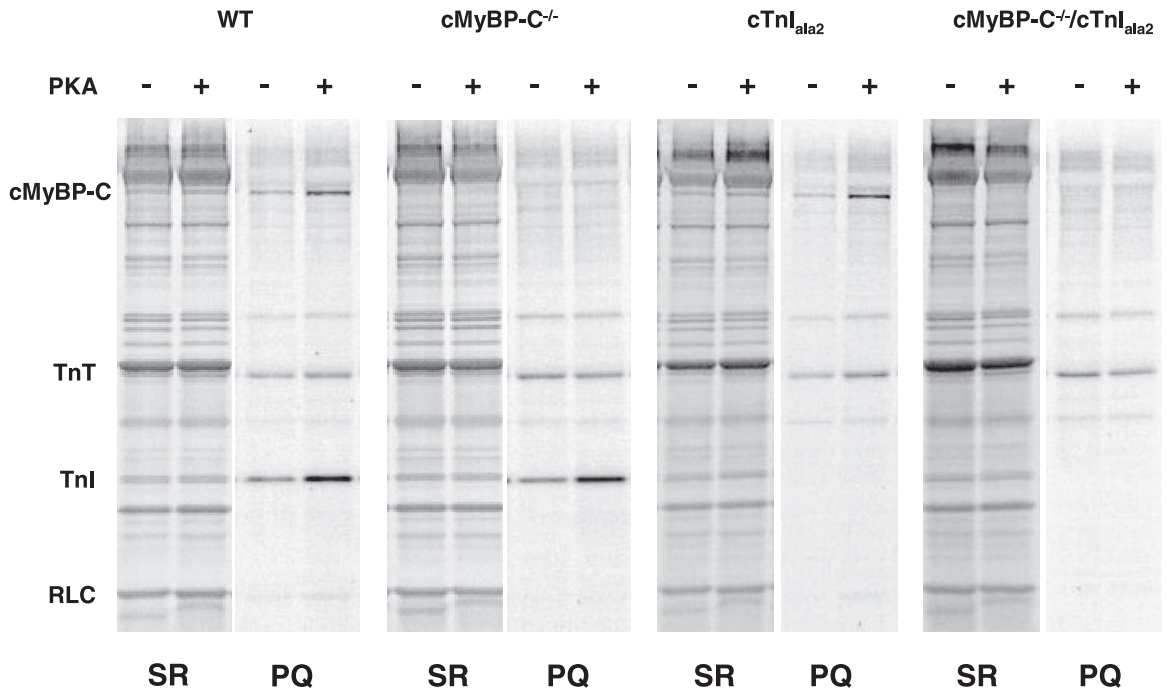

Figure 5. Determination of myofibrillar protein phosphorylation levels. Phosphorylation of myofibrillar proteins from WT, cMyBP-C$^{-/-}$, cTnI$_{ala2}$, and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ myocardium was assessed before and after PKA treatment, as shown in this representative SDS-PAGE. Preparation of myocardial preparations loaded on the gel and analysis of phosphorylation status of proteins were performed as described in the online data supplement. SR indicates SYPRO-Ruby–stained gel for total proteins; PQ, Pro-Q Diamond–stained gel specific for phosphorylated proteins; RLC, regulatory light chain.

Echocardiography

Morphological data in supplemental Table III show that both cMyBP-C$^{-/-}$ and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ mice exhibited significant left ventricular hypertrophy. Echocardiographic indices show that ablation of cMyBP-C had profound effects on in vivo diastolic and systolic function, as indicated by reduced endocardial fraction shortening, ejection fraction, and prolonged isovolumic relaxation time in cMyBP-C$^{-/-}$ and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ mice (supplemental Table III). In contrast, cTnI$_{ala2}$ mice showed left ventricular morphology and in vivo cardiac function similar to WT mice (supplemental Table III).

Discussion

The goal of this work was to determine the respective roles of cMyBP-C and cTnI in PKA-mediated myocardial inotropy. We took advantage of existing (cMyBP-C$^{-/-}$ and cTnI$_{ala2}$) and novel (cMyBP-C$^{-/-}$/cTnI$_{ala2}$) transgenic mouse lines to investigate the effects of phosphorylation of cMyBP-C and cTnI, the main targets of PKA in the myofilaments, on force and the kinetics of force development in skinned myocardium. Our results show that the acceleration of stretch activation caused by treatment with PKA is primarily a consequence of cMyBP-C phosphorylation, whereas the decrease in Ca^{2+} sensitivity of force is mainly attributable to phosphorylation of cTnI. The acceleration of stretch activation observed here in skinned myocardium would predict increases in the rates and amplitude of systolic force and pressure generation resulting from β-adrenergic stimulation in vivo.

Stretch activation is an intrinsic property of cardiac muscle[17]; however, until recently, little was known about its functional role in the heart. Stretch activation has been postulated to be important for oscillatory power generation during systolic ejection.[12,14,18] Consistent with this idea, the kinetics of stretch activation appear to vary with heart rate in mammals.[17] We recently showed that the rate of delayed force development following stretch varies with the level of Ca^{2+} activation,[16] which determines the number of cross-bridges available for cooperative recruitment following stretch and consequently the ability of myocardium to perform oscillatory work. For optimal cardiac function, force development and pressure generation during systole must occur with precise timing. Thus, any disruption in the kinetics of the stretch-activation response could alter cardiac function because of premature or late stretch activation, resulting in reduced oscillatory power.[12,18,19] In this regard, mutations in sarcomeric proteins that slow[33] or accelerate[18,19] oscillatory work in myocardium also impair cardiac function.

We have recently shown that ablation of cMyBP-C dramatically accelerates cross-bridge cycling kinetics by eliminating or significantly accelerating cooperative cross-bridge recruitment[19] and ultimately results in cardiac dysfunction and hypertrophy. The mechanism by which ablation of cMyBP-C accelerates cross-bridge kinetics appears to be similar to that of cMyBP-C phosphorylation,[22] ie, an increase in the proximity of myosin heads to actin[34,35] caused by disruption of cMyBP-C binding to the S2 domain of myosin,[36–38] which increases the probability of cross-bridge binding to actin and accelerates the transition(s) to force-generating states. Because force development in myocardium is a highly cooperative process,[39] even a small change in the disposition of myosin heads resulting from ablation or phos-

phorylation of cMyBP-C could significantly alter the rates of cross-bridge attachment or detachment in response to stretch. In cMyBP-$C^{-/-}$ or cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium, chronic ablation of cMyBP-C accelerates stretch activation and leads to cardiac dysfunction, whereas reversible phosphorylation of cMyBP-C during β-adrenergic stimulation in the healthy heart provides a mechanism by which pump function can be transiently enhanced to match circulatory demands.

In submaximally activated WT and $cTnI_{ala2}$ myocardium, the acceleration of phase 3–delayed force development by treatment with PKA is attributable to the elimination of the slower rate constant of force development (k_2) related to cooperative cross-bridge recruitment, such that phase 3 proceeds with a single fast rate constant of force development similar to k_1 observed in cMyBP-$C^{-/-}$ and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium (supplemental Table I). On the other hand, in maximally activated WT and $cTnI_{ala2}$ myocardium, PKA treatment did not alter the rate of delayed force development (Figure 4C and 4D). During maximal Ca^{2+} activations a large fraction of cross-bridges is strongly bound to actin, leaving relatively few myosin heads available for recruitment by stretch, whereas at low levels of activation, fewer cross-bridges are bound to actin, leaving more cross-bridges for recruitment by stretch. Therefore, PKA phosphorylation of cardiac myofilaments either accelerates or eliminates cooperative recruitment of cross-bridges by stretch (supplemental Table I). Accelerated cross-bridge recruitment with PKA treatment would be expected to contribute to accelerated force development in the rising phase of the myocardial twitch. In cMyBP-$C^{-/-}$ and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ hearts, the chronic acceleration of the stretch-activation response effectively detunes stretch activation so that the timing of delayed force development occurs too early to sustain ejection in late systole, thus significantly diminishing ejection fraction (supplemental Table III).

Although the acceleration of cross-bridge recruitment and delayed force development with β-adrenergic stimulation would be expected to accelerate force development during systolic ejection in vivo, optimization of stroke volume also requires that ventricular filling is maintained during diastole by accelerating the rates of cross-bridge detachment and force relaxation. In this study, PKA phosphorylation of WT and $cTnI_{ala2}$ myocardium produced a dramatic acceleration in the rate of cross-bridge detachment (k_{rel}) following stretch (Figure 4). Genetic ablation or acute biochemical extraction of cMyBP-C has been shown to increase shortening velocity,[30,36] presumably because of increased rates of cross-bridge detachment. Thus, it appears that ablation or phosphorylation of cMyBP-C result in similar accelerations in the rates of cross-bridge detachment, perhaps because of a decrease in a putative internal viscous load normally provided by cMyBP-C, which acts to slow the speed of shortening.[40] PKA phosphorylation of WT and $cTnI_{ala2}$ myocardium also increased the number of cross-bridges that detach following stretch, which is evident in more negative values of P_2 (Figure 3). Because more negative values of P_2 have been interpreted as indicating greater reversal of force-producing steps in response to stretch,[41] cMyBP-C phosphorylation appears to accelerate cross-bridge reverse transitions from strongly bound to weakly bound states so that force relaxation is accelerated. Davis et al[41] have proposed that reversal of force-producing steps following stretch may improve contractile efficiency in myocardium because cross-bridges could conceivably detach from actin and quickly reattach without consuming ATP.[41]

The mechanism by which PKA phosphorylation enhances cross-bridge detachment is not known for certain but might be attributable to increased myosin head flexibility and range of movement[42] caused by relief of spatial constraints imposed on the S2 domain of the myosin molecule. The effect of cMyBP-C to slow the rates of cross-bridge cycling may appear to be deleterious to contractile function; however, slowed rates of cross-bridge detachment may be beneficial in vivo by prolonging systolic ejection and perhaps increasing contractile efficiency by minimizing ATP utilization. Conversely, ablation of cMyBP-C accelerates cross-bridge detachment and significantly truncates the period of systolic ejection[21] such that less blood is pumped with each beat. Myocardial force generation is proportional to the number of attached cross-bridges interacting with the thin filament and the amount of time those cross-bridges remain attached.[43] Therefore, cMyBP-C–induced phosphorylation increases the number of detached cross-bridges following stretch (as indicated by decreased P_2) and accelerates the apparent rate of cross-bridge detachment (as indicated by accelerated k_{rel}) following stretch, such that the number of strongly bound cross-bridges is diminished and the time cross-bridges remain in strongly attached states is reduced. Such a mechanism would be beneficial during β-adrenergic stimulation in vivo in accelerating force relaxation following ejection and thereby enhancing diastolic filling.

cTnI plays an important role in the regulation of contraction by interacting with actin to inhibit actomyosin ATPase activity at low cytoplasmic Ca^{2+}, whereas PKA phosphorylation of cTnI at Ser23/24 alters its interaction with troponin C and reduces Ca^{2+}-binding affinity.[3,4] In the present study, the substitution of Ser23/24 residues for nonphosphorylatable Ala23/24 in cTnI ($cTnI_{ala2}$ and cMyBP-$C^{-/-}$/$cTnI_{ala2}$ myocardium) effectively eliminated the decrease in Ca^{2+} sensitivity of force attributable to PKA (supplemental Table II). However, the stretch-activation response of $cTnI_{ala2}$ myocardium did not differ from WT myocardium before PKA treatment and was greatly accelerated following PKA treatment (Figure 2B). Recent studies[23,44] show that mutation of PKA phosphorylation sites on cTnI (Ser23/24) to Asp23/24 to mimic PKA phosphorylation had little or no effect on basal systolic function (dP/dt_{max}) but significantly enhanced basal diastolic function (dP/dt_{min}) in whole hearts. However, treatment of the cTnI mutant hearts with the β-agonist dobutamine had only small effects on diastolic function but significantly accelerated systolic function. Taken together, these results support the idea that PKA-mediated phosphorylation of cTnI is the main mediator of the decrease in Ca^{2+} sensitivity of force and contributes to the rate of relaxation during diastole, whereas cMyBP-C phosphorylation accelerates the rate and extent of force development during systole.

There is growing evidence that cMyBP-C and its phosphorylation by PKA are important regulators of cardiac

contractility and cardiac output. The lack of cMyBP-C[19–22] and decreased levels of cMyBP-C phosphorylation[45,46] in myocardium have been implicated in cardiac dysfunction. Here we show that ablation of cMyBP-C causes severe hypertrophy and impaired in vivo systolic and diastolic function in both cMyBP-C$^{-/-}$ and cMyBP-C$^{-/-}$/cTnI$_{ala2}$ mice. Furthermore, ablation of cMyBP-C has been shown to significantly attenuate the cardiac responsiveness to β-adrenergic agonist stimulation, suggesting that cMyBP-C plays an important role in the contractile response to β-adrenergic stimulation in vivo.[47] Myocardial mechanical data demonstrate that the acceleration of cross-bridge cycling in myocardium lacking cMyBP-C accelerates stretch activation during submaximal contractions, which would reduce stretch-induced oscillatory power in late systole and prematurely terminate ejection. Thus, we propose that in healthy myocardium, cMyBP-C normally acts to slow the rates of cross-bridge attachment and transition to force-generating states, so that the peak of force generation is delayed, and also slows cross-bridge detachment and force relaxation by stabilizing force-generating states. The delayed force development in early systole will also delay the stretch-activation of force in late systole, thereby prolonging the period of ejection. However, during adrenergic stimulation, phosphorylation of cMyBP-C accelerates the rates of cross-bridge cycling and force generation such that the timing of the stretch-activation response matches the increased heart rate and contributes to the accelerated rate of twitch force generation. In this scheme, the main effect of PKA phosphorylation of cTnI is to decrease the Ca^{2+}-binding affinity of troponin, which causes an earlier onset of relaxation, whereas the main effect of cMyBP-C phosphorylation is to accelerate stretch activation early in systole because of closer juxtaposition of myosin heads to actin. Together, PKA-mediated phosphorylations of cTnI and cMyBP-C tune myofibrillar contraction to accelerate systolic ejection and optimize the durations of systolic ejection and diastolic filling.

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Disclosures

None.

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