

Cardiac MyBP-C Regulates the Rate and Force of Contraction in Mammalian Myocardium

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Abstract: Cardiac myosin-binding protein-C (cMyBP-C) is a thick filament-associated protein that seems to contribute to the regulation of cardiac contraction through interactions with either myosin or actin or both. Several studies over the past several years have suggested that the interactions of cardiac myosin-binding protein-C with its binding partners vary with its phosphorylation state, binding predominantly to myosin when dephosphorylated and to actin when it is phosphorylated by protein kinase A or other kinases. Here, we summarize evidence suggesting that phosphorylation of cardiac myosin binding protein-C is a key regulator of the kinetics and amplitude of cardiac contraction during β -adrenergic stimulation and increased stimulus frequency. We propose a model for these effects via a phosphorylation-dependent regulation of the kinetics and extent of cooperative recruitment of cross bridges to the thin filament: phosphorylation of cardiac myosin binding protein-C accelerates cross bridge binding to actin, thereby accelerating recruitment and increasing the amplitude of the cardiac twitch. In contrast, enhanced lusitropy as a result of phosphorylation seems to be caused by a direct effect of phosphorylation to accelerate cross-bridge detachment rate. Depression or elimination of one or both of these processes in a disease, such as end-stage heart failure, seems to contribute to the systolic and diastolic dysfunction that characterizes the disease. (*Circ Res.* 2015;116:183-192. DOI: 10.1161/CIRCRESAHA.116.300561.)

Key Words: cardiac myosin binding protein-C ■ phosphorylation ■ regulation

Cardiac myosin-binding protein-C (cMyBP-C) is one of a small family of homologous proteins that bind to myosin in mammalian-striated muscles (Figure 1). After its first description in skeletal muscle by Offer and colleagues,² MyBP-C (or C-protein as it was first named) was thought to play a principally structural role in the assembly and stabilization of the sarcomere, although early work by Moos suggested there were conditions under which C-protein modified the function of myosin in solution, in either the presence or absence of actin.³ Subsequent work by Hartzell⁴ showed in the amphibian heart that the phosphorylation state of C-protein varied with the infusion of a membrane receptor agonist (acetylcholine), an observation that led to a series of structural, biochemical, and functional studies of the protein.⁵⁻⁸ cMyBP-C was found to be phosphorylated at specific residues by protein kinase A (PKA) and calcium calmodulin kinase 2 delta (CaMK2 δ)⁶ and was also reversibly phosphorylated in vivo when β -adrenergic agonists were applied to the mammalian heart.⁹ The findings that biochemical extraction of cMyBP-C from the myofibril had no effect on the stability of the sarcomere⁷ and that genetic ablation of cMyBP-C in mice caused no abnormalities in sarcomere assembly¹⁰ led to the conclusion that cMyBP-C is not a structural protein, at least not in the strictest sense. In recent years, the picture that has emerged

is one in which cMyBP-C is a key determinant of the speed and force of cardiac contraction, a conclusion drawn from alterations in contractility that have been observed as a consequence of phosphorylation, ablation, or mutation of the protein.

cMyBP-C Regulates the Kinetics of Force Development in Myocardium

cMyBP-C as a Regulator of Contraction

The overarching conceptual framework for this review is that cMyBP-C binds to myosin or actin or both and thereby regulates the probability of cross-bridge interaction with actin, which in turn controls the rates of force development and relaxation in living muscle. In this regard, there is evidence (reviewed below) that dephosphorylated cMyBP-C preferentially binds to myosin and in so doing restricts spatial mobility of myosin and reduces the probability of myosin binding to actin. This MyBP-C-mediated depression of contractility is relieved by genetic ablation of *MYBPC3* (the gene encoding cMyBP-C)¹⁰ or phosphorylation of cMyBP-C by PKA, CaMK2 δ , and possibly other kinases.^{6,11-14} cMyBP-C also binds to actin, which seems to have activating effects on the thin filament (below), leading to the idea that phosphorylation increases contractility by shifting the balance of cMyBP-C from myosin to

Original received September 8, 2014; revision received October 31, 2014; accepted November 3, 2014. In November, 2014, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.96 days.

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DOI: 10.1161/CIRCRESAHA.116.300561

Nonstandard Abbreviations and Acronyms

CaMK2δ	calcium calmodulin kinase 2 delta
cMyBP-C	cardiac myosin binding protein C
PKA	protein kinase A
Ser	serine

actin. Such mechanisms would ensure that power generation and the efficiency of myocardial contraction are optimal in an individual at rest, but provides considerable contractile reserve for enhanced function when the heart is stressed by increases in circulatory load or neuro-humoral tone. As an example of the potential importance of these regulatory processes, failure of these mechanisms caused by depressed adrenergic signaling in heart failure most likely contributes to the reduced contractility of myocardium that is the hallmark of the disease. In contrast, hypertrophic cardiomyopathy mutations in cMyBP-C that are associated with hypercontractility even in an individual at rest presumably induce hypercontractility by disrupting the interactions of cMyBP-C with myosin or increasing its binding to actin or both.

Evidence for MyBP-C-Mediated Regulation of Cross-Bridge Cycling Kinetics

Early studies of possible roles of MyBP-C in muscle contraction found that biochemical extraction of MyBP-C from skinned skeletal or cardiac muscle increased the force

developed at Ca^{2+} concentrations that evoked forces less than maximal, that is, increased the Ca^{2+} sensitivity of force, and also increased the velocity of unloaded shortening at submaximal $[Ca]^{2+}$.⁷ Both results suggested that MyBP-C depressed contraction in muscle, a conclusion that was reinforced by reversal of these effects of extraction when the muscle preparations were reconstituted with MyBP-C.

A limitation of experiments involving biochemical extraction of cMyBP-C from permeabilized muscle fibers is the inability to determine the effects of stoichiometric depletion of the protein or the effects of depletion on myocardial function in vivo. The first cMyBP-C knockout mouse was developed to address the need for such data. Rather than causing disruption of sarcomere assembly, homozygous ablation of the *MYBPC3* gene resulted in a series of phenotypes, such as septal hypertrophy, chamber dilation, accelerated rate of rise of left-ventricular pressure, and slowed relaxation,¹⁰ all of which are consistent with changes reported in many cases of inherited hypertrophic cardiomyopathy.^{15–17}

Extension of these studies to permeabilized preparations of cMyBP-C null myocardium showed that ablation increased the rate of rise of force at submaximal $[Ca]^{2+}$,^{18,19} a phenomenon that accounts for the accelerated kinetics of pressure development in null hearts, but had no effect on the kinetics of force development at saturating $[Ca]^{2+}$. In contrast to the slowed myocardial relaxation (and resulting diastolic dysfunction) in null hearts in vivo,²⁰ relaxation of permeabilized null myocardium was accelerated compared with wild-type.¹⁸ This finding has been corroborated by data derived from a multicellular engineered cardiac tissue preparation incorporating neonatal cMyBP-C null cardiomyocytes, which exhibited an acceleration of the relaxation phase of the twitch.²¹ This suggests that slowed relaxation in vivo is related to alterations in Ca^{2+} handling as a result of functional remodeling of the heart as part of the compensatory response to ablation of cMyBP-C.¹² Consistent with this idea, the intracellular Ca^{2+} transient is substantively altered in intact single cardiomyocytes from cMyBP-C null myocardium, that is, the amplitude is reduced and the kinetics of rise and fall of the transient are slowed.²² Under these circumstances, the rate of relaxation of the twitch seems to be determined mainly by the rate of decay of the Ca^{2+} transient. This conclusion is consistent with the finding by David Yue and colleagues that most of the time-course of relaxation of force during the cardiac twitch follows the steady-state relationship between force and $[Ca]^{2+}$ determined in the same muscles.²³ Nevertheless, the kinetics of the calcium transient did not differ in null versus wild-type engineered cardiac preparation, which reinforces the idea that ablation per se of cMyBP-C accelerates relaxation.

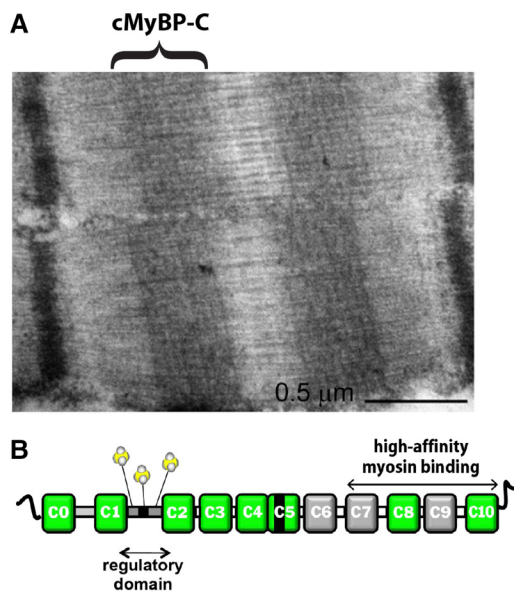


Figure 1. Salient features of cardiac myosin-binding protein-C. **A**, An electron micrograph of a cardiac sarcomere showing the transverse stripes corresponding to the alignment of cardiac myosin binding protein-C (cMyBP-C) in each half-sarcomere.¹ cMyBP-C was visualized by infusion of an N-terminal antibody before fixation. **B**, cMyBP-C showing its subunit structure and featuring the protein kinase A (PKA) phosphorylatable serines in the regulatory domain, that is, M-domain between subunits C1 and C2. cMyBP-C is a phospho-protein that is localized to 9 stripes (the C zone) in each half sarcomere. The stripes are 42 nm apart, corresponding to every third myosin crown.

Regulation of Cross-Bridge Cycling by PKA Phosphorylation of MyBP-C

Several earlier studies reported that cMyBP-C is reversibly phosphorylated by PKA or CaMK2 δ , indicating a regulatory role for cMyBP-C as an element of β -adrenergic stimulation of the heart.⁶ Consistent with this idea, infusion of PKA in permeabilized cardiac muscle from murine hearts accelerates the

rate of rise of force at submaximal $[Ca]^{2+}$ but has no effect on the kinetics of force development at saturating $[Ca]^{2+}$. These effects (and the lack of effect at saturating $[Ca]^{2+}$) are similar to the effects caused by ablation of cMyBP-C. In mouse models expressing non-PKA-phosphorylatable cMyBP-C, no acceleration in kinetics of force is measured during exposure to either PKA in skinned myocardium or dobutamine in living myocardium.¹² Thus, the mechanism underlying the effects of PKA mimics the contractile effects caused by ablation, perhaps reducing or eliminating regulatory interactions of cMyBP-C with myosin or with actin. PKA phosphorylates both cMyBP-C and cardiac troponin I in the cardiac myofibril, but the effects on kinetics are because of phosphorylation of cMyBP-C because the effects are observed in myocardium expressing nonphosphorylatable TnI but are not observed in myocardium expressing native TnI and nonphosphorylatable cMyBP-C.¹² Thus, phosphorylation of cMyBP-C accelerates force development in isolated muscle and would be expected to contribute to the acceleration of pressure development in the intact heart during adrenergic stimulation, which was also reported by Tong et al.¹²

Three highly conserved, physiologically relevant phosphorylation sites were identified by Gautel within the N-terminus region between C1 and C2, referred to as the MyBP-C motif or the M-domain.⁶ Three serine (Ser) residues (mouse: Ser-273, -282, and -302; human: Ser-275, -284, and -304) comprise the primary PKA substrates (Figure 1) and are thus implicated in mediating the cMyBP-C response to β_1 adrenergic stimulation. The initial studies by Gautel suggested nonequivalency of the phosphorylation sites based on mutagenesis data, indicating an essential function for Ser-282 phosphorylation for subsequent phosphorylations of Ser-273 and Ser-302.⁶ Although recent data suggest that PKA phosphorylation of Ser-282 may not be necessary for phosphorylation of the flanking sites,²⁴ functional data from transgenic models expressing an array of phosphomimetic and nonphosphorylatable combinations at these residues indicates that the individual sites do not exert equal effects on cross-bridge kinetics or force production.²⁵⁻²⁷ Thus, each site seems to exert unique, albeit subtly different, effects on contractile and morphological phenotypes.²⁶

Although PKA readily phosphorylates all 3 serine residues in the M-domain, additional kinases including CaMK2 δ , protein kinase C, protein kinase D, and perhaps ribosomal S6 kinase, have been shown to selectively phosphorylate a subset of these sites.²⁸ Work by Gautel⁶ suggested that residue Ser-282 in mouse cMyBP-C is readily phosphorylated by CaMK2 δ , whereas subsequent work has shown that CaMK2 δ phosphorylates Ser-282 only at high $[Ca]^{2+}$ but Ser-302 at low $[Ca]^{2+}$. Protein kinase C phosphorylation of cMyBP-C has been shown in human myocardium²⁹ and in mouse hearts to selectively target Ser-273 and Ser-302.³⁰ Phosphomimetic substitution at these protein kinase C sites in the absence of Ser-282 phosphorylation leads to severe cardiac hypertrophy, whereas a nonphosphorylatable Ser-273 and Ser-302 model with or without a concurrent phosphomimetic substitution at Ser-282 did not manifest any significant changes. There is need for additional work to elucidate the role(s) of protein kinase C and potentially other Gq protein-coupled receptor-activated

kinases, such as protein kinase D and ribosomal S6 kinase, in health and disease, but in any case, the work cited above suggests a role for cMyBP-C in mediating cardiac inotropic effects in response to α_1 -adrenergic stimulation.

In normal hearts, cMyBP-C may be highly phosphorylated at baseline, in some studies approaching 90% in mouse hearts, with a fairly even distribution among the mono-, di-, and tri-phosphorylated forms.²⁵ The possible functional implications of variable phosphorylation have been illuminated using an in vitro motility assay measuring the rate of movement of labeled thin filaments over bound myosin in the presence of C0-C3 fragments of cMyBP-C mutated at the 3 serine sites to either be phospho-mimetic or phospho-ablated.³¹ The relationship between sliding velocity (reflecting cross-bridge kinetics) and the extent of cMyBP-C phosphorylation is striking, implying a modulation capability based on the degree of phosphorylation, rather than a simple full-on/full-off response. Therefore, the mix of kinases, each associated with their unique physiological responses, as well as the identity of the serine(s) that confer functional effects when phosphorylated, are critical variables in the context of developing possible therapeutic interventions designed to improve function by targeting kinase activities or phospho-residues. The development by Sadyappan of highly specific phosphoserine antibodies to each of the 3 phosphoserines (Ser-272, Ser-283, and Ser-302) in mouse cMyBP-C provides a powerful set of tools with which to optimize the selection of therapeutic targets.³²

Model for the Regulation of the Kinetics of Force Development and Relaxation by cMyBP-C

Critical Constraint on Possible Mechanisms of Regulation by cMyBP-C

Consideration of the possible mechanisms by which phosphorylation of cMyBP-C influences contraction kinetics and myocardial force must take into account the observation that phosphorylation does not accelerate the rate of rise of force in skinned myocardium that is maximally activated with Ca^{2+} ,¹¹ as shown in Figure 2. The lack of effect of phosphorylation implies that the rate of cross-bridge cycling is indeed maximal at saturating Ca^{2+} , which places constraints on possible mechanisms by which phosphorylation accelerates kinetics at submaximal Ca^{2+} concentrations. Most notably, it seems unlikely that phosphorylation has its effects by increasing the proximity of cross bridges to actin, suggested previously,^{33,34} because such a mechanism should accelerate contraction kinetics even at maximal activation by increasing the probability of binding to actin. Thus, the previous observations of an apparent transfer of molecular mass from thick filaments to thin, inferred from greater equatorial $I_{1,1}/I_{1,0}$ intensity ratios from x-ray diffraction patterns recorded from phospho-mimetic cMyBP-C or cMyBP-C ablated myocardium,^{34,35} could represent a more subtle change in myosin structure because of these interventions. Because the changes in intensity ratios were observed in relaxed myocardium, the transfer of mass from one diffracting plane (thick filaments only in the 1,0 plane) to another (thick and thin filaments in the 1,1 plane) cannot be a straightforward result of increased cross-bridge binding, thus eliminating the possibility of a trivial explanation for these results. Instead, a cMyBP-C phosphorylation-induced structural change in

myosin inferred from the change in equatorial intensity ratios seems to be the cause rather than a result of increased cross-bridge binding to actin and the increased rate of force development under these conditions.

Plausible Mechanisms of Regulation of the Cross-Bridge Cycle via cMyBP-C

Although the detailed mechanism by which phosphorylation of cMyBP-C accelerates contraction is not yet understood, consideration of current models of the regulation of contraction by myofibrillar proteins provides useful clues and a framework for further mechanistic studies. Models of regulation are constrained to explain the activation dependence of the rate constant of force development (k_{fd}) in permeabilized myocardium after either photo-generation of Ca^{2+} from caged Ca^{2+} (yielding the rate constant k_{Ca}), photo-generation of adenosine diphosphate (ADP) from caged ADP (yielding k_{ADP}), or a release/restretch protocol during steady activation at varying Ca^{2+} concentrations (yielding k_{tr}). In myocardium, there is an ≈ 10 -fold acceleration of k_{fd} as $[\text{Ca}^{2+}]$ is increased from threshold levels for force generation to maximal activation.^{36,37}

There are 3 general classes of regulatory mechanisms that could be invoked to explain the variation in k_{fd} as a function of $[\text{Ca}^{2+}]$, each of which could plausibly be modulated by the phosphorylation status of cMyBP-C and none of which has yet been excluded as a possibility. In one current model of regulation, this activation dependence was simulated by including $[\text{Ca}^{2+}]$ as a reactant in the force generating transition,³⁸ which would result in accelerated force development as $[\text{Ca}^{2+}]$ is increased. A second possibility is that kinetic transitions in the cross-bridge interaction cycle, notably the release of the products of ATP hydrolysis (P_i and ADP), are directly regulated as functions of thin filament activation caused by Ca^{2+} binding to the troponin complex. Such a mechanism would presumably involve allosteric modulation of these transitions as a consequence of changes in conformation of the thin filament regulatory strand as the amount of Ca^{2+} bound to troponin increases from threshold to saturating for force development. At saturating Ca^{2+} , the kinetics of cross-bridge turnover would presumably approach the intrinsic kinetics of the limiting step in the cross-bridge cycle, that is, the rate of ADP release. A third possibility is that the rate of force development is slowed at

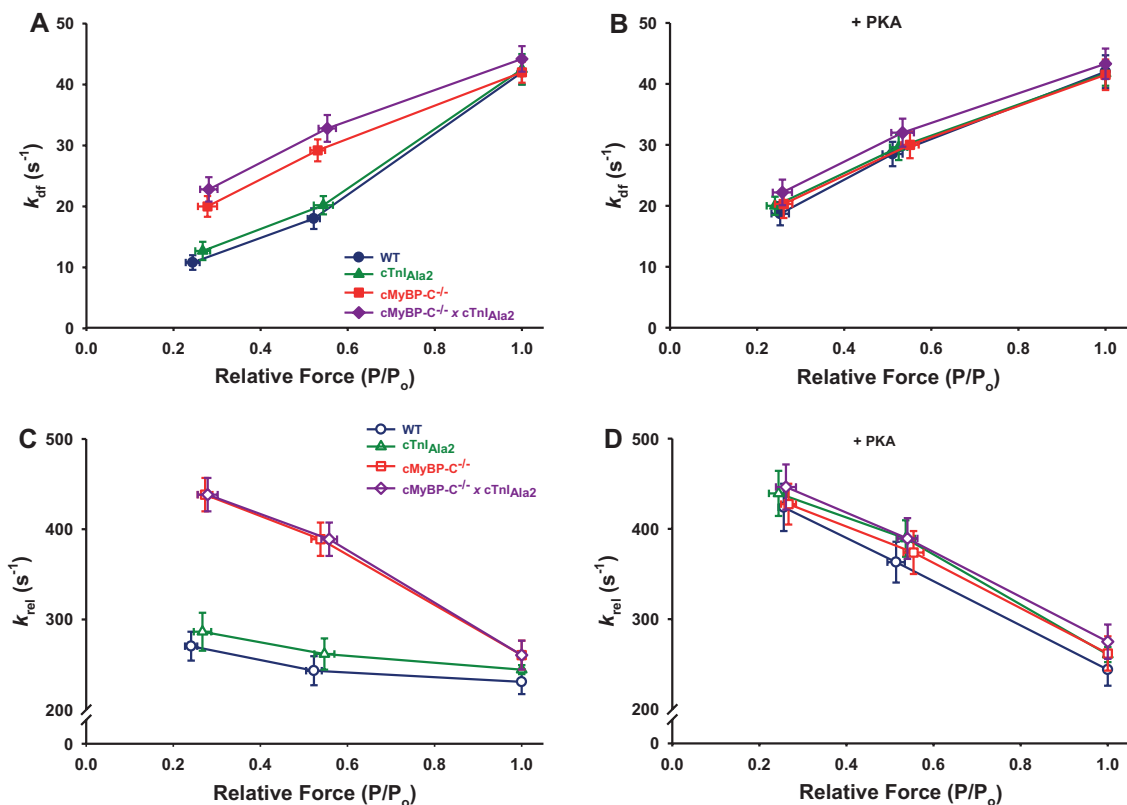


Figure 2. Effects of ablation or phosphorylation of cardiac myosin binding protein-C (cMyBP-C) on the kinetics of force development and relaxation. The rates of delayed force development (rate constant k_{dff}) and relaxation (k_{rel}) were measured during the force transient resulting from sudden stretch of skinned myocardium activated at different Ca^{2+} concentrations to develop forces of $\approx 25\%$, 50%, and 100% of maximum.¹¹ **A**, k_{dff} in wild-type increased nearly 4-fold when activation was increased from 25% to 100% of maximum; homozygous knockout of cMyBP-C accelerated the rate of force development during submaximal activations but not at maximal activation. **B**, Protein kinase A (PKA) phosphorylation of cMyBP-C in wild-type myocardium increased k_{dff} during submaximal activations, but there was no effect on k_{dff} at maximal activation. **C**, Knockout of cMyBP-C increased the rate of relaxation (k_{rel}) at submaximal but not at maximal activation. **D**, Similar to knock of cMyBP-C, PKA phosphorylation of cMyBP-C accelerated k_{rel} in wild-type myocardium at submaximal but not at maximal activation. Thus, phosphorylation of cMyBP-C had no effect on cross-bridge cycling kinetics at maximal activation but accelerated the rates of force development and relaxation during submaximal activation. These effects were as a result of phosphorylation of cMyBP-C and not cardiac troponin I (cTnI) because wild-type myocardium expressing nonphosphorylatable TnI (TnI_{ala2}) exhibited accelerated rates of force development and relaxation.

low Ca^{2+} concentrations because of the time taken for the cooperative recruitment of cross bridges to the thin filament.^{39,40} Such recruitment would be triggered by the initial binding of Ca^{2+} to troponin and is characterized by the progressive spread of cross-bridge binding away from the troponin with Ca^{2+} bound. This mechanism would be most pronounced at low $[\text{Ca}^{2+}]$ because relatively few troponins would have Ca^{2+} bound, leaving extended regions of thin filament available for cooperative activation; the mechanism would presumably not be operable at saturating Ca^{2+} because the thin filament would be fully activated by Ca^{2+} , leaving no regions of the thin filament available for cooperative recruitment of cross bridges. Thus, at high $[\text{Ca}^{2+}]$, the kinetics of force development would approach the fundamental rate of cross-bridge cycling.

Although all 3 of these mechanisms could conceivably contribute to the regulation of the kinetics of contraction, it is not presently known which of the 3 are involved, which of the 3 are predominant, or the conditions that may favor one versus another. Of course, these 3 mechanisms are not mutually exclusive—for example, any effect of variations in $[\text{Ca}^{2+}]$ to modulate cross-bridge cycling rates would be expected to modulate the rate of cooperative activation of the thin filament. Similarly, any effect of the phosphorylation status of cMyBP-C to modulate ≥ 1 of these mechanisms would be expected to influence any other mechanisms that contribute to the regulation of contraction.

Interactions of cMyBP-C With Myosin and Actin

The above discussion begs the identity of the molecular interactions that mediate the regulation of contraction by cMyBP-C. Of course, cMyBP-C has from the time of its discovery been thought to bind to myosin, an interaction that is weakened or eliminated by phosphorylation of serines within the cMyBP-C motif, or M-domain.⁶ More recent evidence obtained principally in simplified systems of proteins in solution but also using a yeast 2-hybrid approach suggest the possibility that cMyBP-C also binds actin.^{41,42} Such binding has been associated with the activation of regulated motility assays in the absence of added Ca^{2+} ,⁴³ the slowing of sliding velocity in motility assays as thin filaments slide into the region of the thick filament (C-zone) where cMyBP-C is bound,^{44,45} and the displacement of tropomyosin within the thin filament toward positions similar to Ca^{2+} activation.⁴⁶ Although the binding of cMyBP-C to actin is relatively weak,^{41,47} the affinity is in a range (a few micromolar) that is well suited to regulation in which phosphorylation modulates the interaction of cMyBP-C with its binding partners. Supporting this idea, it is evident that cMyBP-C alters the torsional dynamics of thin filaments in solution, an effect that varies with phosphorylation state of cMyBP-C.⁴⁸

The idea that cMyBP-C binds to both the thick and thin filaments, depending on the phosphorylation state of the protein, was first proposed by Moos³ and confirmed in several recent studies.^{42,49,50} As suggested by Gautel,⁵¹ phosphorylation of cMyBP-C would be expected to disrupt its binding to myosin, thereby increasing the rate of force development by increasing the probability of myosin binding to actin. At the same time, the now-phosphorylated cMyBP-C could bind to actin and further activate the thin filament. In this

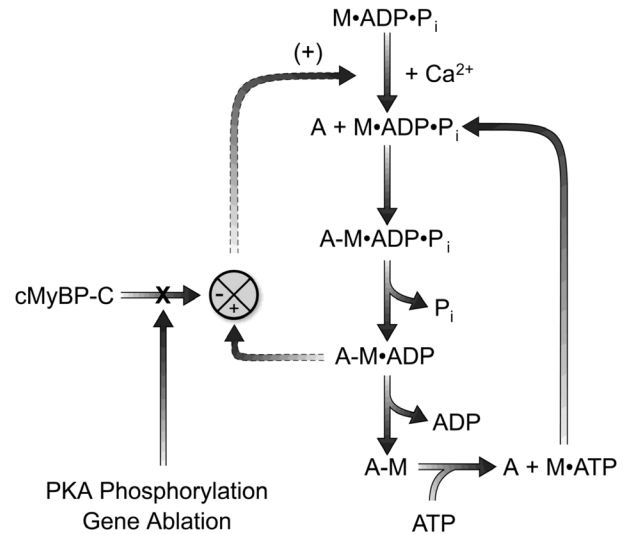


Figure 3. Modulation of cross-bridge cycling by phosphorylation of cardiac myosin binding protein-C (cMyBP-C). The diagram shows a plausible mechanism by which phosphorylation of cMyBP-C speeds the rate of force development (©Moss and Fitzsimons, 2010. Originally published in *Journal of General Physiology*. doi:10.1085/jgp.201010471). In the presence of Ca^{2+} nucleotide-bound myosin (M-ADP-P_i) binds actin (A), which is followed by P_i release and force development as cross-bridges populate the primary force generating state (A-M-ADP). On release of ADP, the resulting rigor complex rapidly binds ATP, which is followed by dissociation of M-ATP from A, hydrolysis of ATP, and re-entry of M-ADP-P_i into the cycle. According to Campbell's model of activation,³⁹ force-generating cross-bridges bound to the (A-M-ADP) cooperatively recruit near-neighbor cross-bridges to bind to actin, shown by the clockwise loop on the left.⁵² This cycle of recruitment may involve multiple iterations so that the time required to reach steady force, that is, a steady population of A-M-ADP cross-bridges, is determined principally by the time taken to complete the iterative recruitment process. In our modification of this model, cMyBP-C slows cooperative recruitment by placing a physical constraint on myosin, thereby reducing the probability of myosin binding to actin and slowing the cooperative spread of cross-bridge binding. Phosphorylation of cMyBP-C by protein kinase A (PKA), CaMK2 δ , or other kinases relieves this constraint either by disrupting the interaction of cMyBP-C with myosin, thereby increasing the probability of myosin binding to actin or binding to the thin filament, thereby increasing its activation state and accelerating cross-bridge binding. Both of these mechanisms, either singly or together, would be expected to increase the rate of force development.

regard, Craig⁴⁶ showed in 3D reconstructions of cardiac thin filaments that an N-terminal domain of cMyBP-C displaced tropomyosin to a position corresponding to an activated, Ca^{2+} -bound thin filament. Though not as pronounced as the effects of unphosphorylated cMyBP-C, infusion of the phosphorylated protein clearly displaces tropomyosin toward an activated position, which would presumably bias the filament toward the activated state. Thus, cMyBP-C would bind preferentially to myosin when unphosphorylated, which would depress activation caused by a structural constraint of cross bridges, and to the thin filament when phosphorylated, which would enhance activation caused by relief of the structural constraint of cross bridges and also increased activation of the thin filament as a consequence of cMyBP-C binding to actin.

The identity of binding partners involved in the regulation of contraction by post-translational modification of cMyBP-C has emerged as one of the most pressing issues today in myocardial biology. The importance of this information derives from our understanding that these interactions mediate cMyBP-C-phosphorylation-dependent effects on contraction, for example, adrenergic inotropy. Knowledge of the specific mechanism(s) of these effects would provide specific targets for development of therapeutics for treatment of myocardial dysfunction caused by reduced contractility, as in heart failure, or hypercontractility, as in hypertrophic cardiomyopathies.

Proposed Role of cMyBP-C in the Regulation of Cooperative Recruitment of Cross Bridges to the Thin Filament

As described earlier, the binding of cross bridges to the partially activated thin filament is a highly cooperative process, particularly in cardiac muscle, which operates at submaximal levels of activation due largely to the short duration of the intracellular Ca^{2+} transient that underlies the twitch. Cooperativity in the regulation of cardiac contraction kinetics has been described previously.^{52,53} For example, the rate constant of force development can be accelerated by an order of magnitude at low, steady Ca^{2+} concentrations by cooperatively activating the thin filament via infusion of N-ethylmaleimide-modified myosin subfragment 1,³⁶ a strong-binding, nonforce-generating derivative of myosin subfragment 1.

The activation dependence of cardiac contraction and the kinetics of force development has been modeled by Campbell as the effects of cooperative cross-bridge binding to actin to increase both the number of formed cross bridges and the amount of force that is generated.^{39,40} This model is shown diagrammatically in Figure 3, with a detailed explanation of the model in the figure legend. The key feature of the model is that Ca^{2+} binding to an isolated troponin activates the thin filament for initial binding of cross bridges to actin (Figure 4), but once a cross bridge binds, the activation state of the thin filament is greater than it would be because of the presence of bound Ca^{2+} alone.^{54–56} This enhancement of activation promotes the binding of cross bridges at a still greater distance from the Ca^{2+} -bound troponin. This cooperative spread does not extend beyond a certain distance, for example, ± 14 actin residues⁵⁷ because the activating effect of Ca^{2+} decays with distance from Ca^{2+} -bound troponin. In this model, the rate of force development is related not only to the rate of P_i release during the force-generating step but also the rate of propagation of cooperative cross-bridge recruitment away from the site of initial Ca^{2+} binding (Table 1).

We propose that phosphorylation of cMyBP-C accelerates the rate of force development by accelerating the rate of cooperative cross-bridge recruitment described by Campbell in his model.^{39,40} This mechanism is shown diagrammatically in Figure 4 as an effect of phosphorylation to increase the rate constants (k_1 and k_2) governing the rates of transition to progressively higher and broader activation profiles around sites of Ca^{2+} binding to troponin. The mechanism also accounts for the lack of effect of cMyBP-C phosphorylation on the kinetics of force development at saturating levels of Ca^{2+} because at these levels, adjacent regions of the thin filament are already

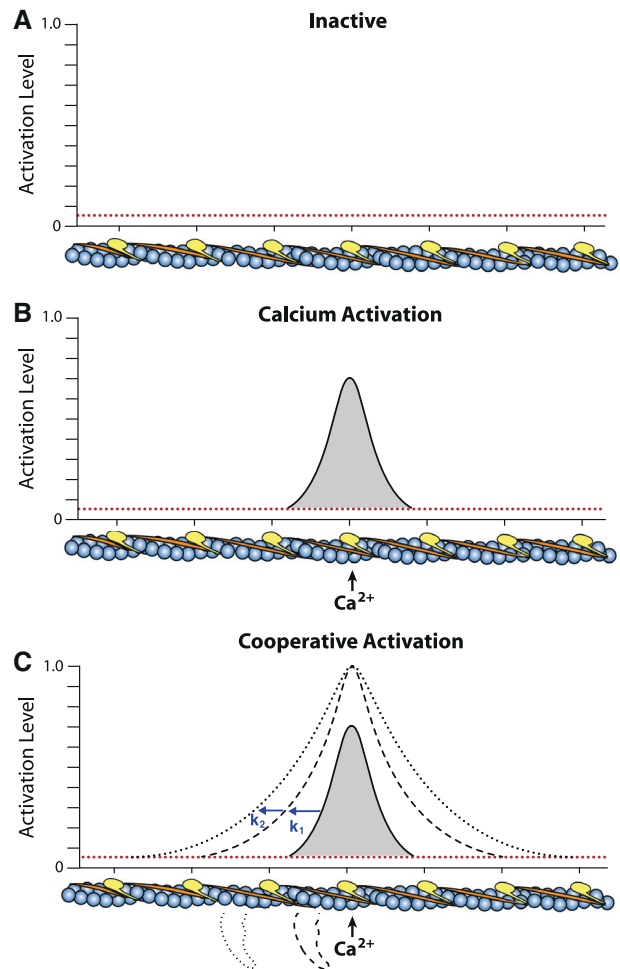


Figure 4. Maps of the cardiac thin filament showing the activating effects of Ca^{2+} and strongly bound cross-bridges.

The panels in this figure represent the state of activation of the thin filament (A) in the absence of Ca^{2+} (B) when Ca^{2+} binds to a single troponin (yellow structures), and (C) in response to the sequential cooperative binding of cross-bridges in response to Ca^{2+} binding to troponin. Binding of Ca^{2+} alone results in activation that is constrained with respect to amplitude and spread; subsequent binding of one and then another cross-bridge increases the amplitude and spread of activation. We propose that phosphorylation of cardiac myosin binding protein-C (cMyBP-C) increases the rates of transition from the Ca^{2+} activation envelope (shaded area) to the expanded envelope (dashed line) because of binding of 1 cross-bridge (rate constant k_1) and then the further expanded envelope (dotted line) because of binding of a second cross-bridge (k_2).

activated by Ca^{2+} , and cooperative cross-bridge recruitment would be absent or much diminished compared with a partially activated thin filament.

The molecular mechanism by which phosphorylation of cMyBP-C would accelerate cooperative recruitment could involve an increased rate of cross-bridge binding to actin as a consequence of the disruption in binding of cMyBP-C to myosin. As discussed earlier, the mechanism may also involve an enhancement of thin filament activation caused by binding of phosphorylated cMyBP-C to actin, presumably as a result of a binding-induced displacement of tropomyosin.⁴⁶ Thus, inotropy involving phosphorylation

Table. Cooperative Activation of Cardiac Force Development

What is the role of Ca^{2+} in this model of regulation?

A given: Ca^{2+} binding to thin filament is necessary for cross-bridge binding to actin.

A condition: Ca^{2+} binding at one site on the thin filament increases the likelihood of cross-bridge binding ± 14 actins upstream and downstream from the site of binding, BUT the activating effect decays with distance from the site of binding.⁵⁷

Two related simplifying assumptions: Because Ca^{2+} binding to actin does not achieve equilibrium during the Ca^{2+} transient, binding occurs at discrete sites that are distributed randomly along the thin filament and throughout the sarcomere. For a Ca^{2+} transient of given amplitude, the amount of Ca^{2+} bound to troponin increases with the duration of the transient and vice versa, that is, the amount of bound Ca^{2+} is determined by the amplitude and duration of the Ca^{2+} transient, but in cardiac muscle the regulatory sites on troponin are never saturated under physiological conditions.

Initiation of cross-bridge binding: Ca^{2+} binding to troponin is necessary for opening actin sites for initial cross-bridge binding, that is, at low $[\text{Ca}^{2+}]_{\text{in}}$, such sites serve as nucleation sites for subsequent cross-bridge binding.

Activation dynamics: Activation of the thin filament during a Ca^{2+} transient is only loosely coupled to $[\text{Ca}^{2+}]_{\text{in}}$, that is, the number of nucleation sites will increase with increasing $[\text{Ca}^{2+}]_{\text{in}}$, but a corresponding increase in force depends on the rate of spread of cross-bridge binding along the thin filament, which in turn depends on cross-bridge binding kinetics and thus the level of cMyBP-C phosphorylation.

In contrast, during steady activation, force varies in a predictable way on $[\text{Ca}^{2+}]_{\text{in}}$, that is, the force–pCa relationship in skinned myocardium; in this model, Ca^{2+} activation of the thin filament is steady in time. However, at low $[\text{Ca}^{2+}]_{\text{in}}$, the filament is not fully activated by cooperative cross-bridge binding because Ca^{2+} binding to a nucleation site on actin has limited spatial influence on cross-bridge binding (± 14 actins).⁵⁷ Without this influence, cross-bridge binding is a rare event.

of cMyBP-C may be mediated by increases in the rate of cross-bridge binding and the extent of spread of cooperative binding during a given $[\text{Ca}^{2+}]_{\text{in}}$ transient. Graded effects on twitch amplitude and kinetics, for example, at low levels of β_1 -adrenergic stimulation, would in this model be caused by reduced phosphorylation of cMyBP-C. In such circumstances, the presence of partially phosphorylated or unphosphorylated cMyBP-C would slow the binding of nearby cross bridges and thereby slow the spread of cooperative cross-bridge recruitment. In this regard, cMyBP-C may be involved in signaling activation from the thin filament to the thick filament.⁵⁸

Can This Model Explain Physiological and Pathophysiological Phenomena?

The potential effect of the proposed model lies in its usefulness as a framework for explaining contractile phenomena. With respect to physiological phenomena, it is clear that cMyBP-C is phosphorylated during β -adrenergic stimulation⁹ and also as a result of increased frequency of stimulation.¹² In the case of adrenergic stimulation, PKA phosphorylation of cMyBP-C would be expected to contribute to accelerated dP/dt_{max} and increased twitch force as a consequence of acceleration of cooperative cross-bridge recruitment to the thin filament. Similarly, the increase in twitch force as a function of stimulus frequency, that is, the Bowditch effect or treppe, would increase force as a result of CaMK2 δ phosphorylation of cMyBP-C.¹²

With respect to pathophysiological phenomena, the reduced phosphorylation of cMyBP-C that has been observed in congestive heart failure^{29,59} would contribute to slowed twitch kinetics and reduced twitch force by slowing the rate of cooperative activation of the thin filament. In contrast, the hypercontractility observed in most cases of hypertrophic cardiomyopathy caused by mutations in cMyBP-C suggest an acceleration of the cooperative recruitment of force-generating cross bridges to the thin filament, possibly as a direct consequence of the hypertrophic cardiomyopathy mutation to alter the interaction of cMyBP-C with its binding partner(s) myosin and actin. Further, hypertrophic cardiomyopathy mutations in the *MYBPC3* gene resulting in the expression of truncated cMyBP-C often lead to reduced cMyBP-C content, which by itself would be expected to speed the rate of cooperative cross-bridge recruitment, thereby contributing to the cardiac hypercontractility observed in these patients.

Possible Contributions of cMyBP-C Phosphorylation to Relaxation Kinetics in Myocardium

Relaxation of the myocardial twitch is triggered by the reduction of myoplasmic Ca^{2+} caused by extrusion of Ca^{2+} via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and sequestration of Ca^{2+} by the sarcoplasmic reticulum.⁶⁰ The initial relaxation of force is delayed with respect to the initial decline in the Ca^{2+} transient from its peak (Figure 5). This is most likely caused by ≥ 2 processes: (1) Ca^{2+} continues to bind to troponin when $[\text{Ca}^{2+}]_{\text{in}}$ is declining but still near its peak, and Ca^{2+} remains bound to the thin filament even as $[\text{Ca}^{2+}]_{\text{in}}$ declines; (2) once a cross bridge binds to the thin filament under the influence of Ca^{2+} and neighboring bound cross bridges, the cross bridge completes its cycle of interaction even if $[\text{Ca}^{2+}]_{\text{in}}$ has returned to resting levels, and troponin no longer has Ca^{2+} bound to the regulatory site. In support of the first mechanism, force during much of the relaxation phase of the twitch has been observed to follow the force– $[\text{Ca}^{2+}]_{\text{in}}$ relationship.²³ With regard to the second mechanism, the relaxation of twitch force is delayed relative to the decay of the intracellular Ca^{2+} transient (Figure 5).¹²

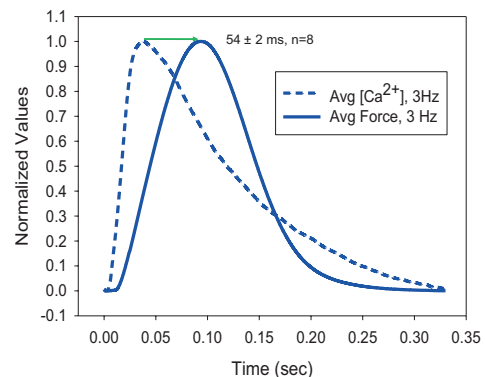


Figure 5. Time-courses of force and intracellular $[\text{Ca}^{2+}]_{\text{i}}$ during the cardiac twitch. Force and intracellular $[\text{Ca}^{2+}]_{\text{i}}$ were recorded during twitches (3 Hz stimulus frequency) from mouse myocardium at room temperature.¹² The traces presented here are the averaged traces from multiple muscle preparations and exhibit the time delay between the peak of the Ca^{2+} transient and the peak of the twitch.

Based on our proposal that cMyBP-C phosphorylation increases cooperative recruitment of cross bridges, it is difficult to envision how phosphorylation (eg, caused by an adrenergic agonist) would speed twitch relaxation of the twitch because increased cooperativity would be expected to prolong thin filament activation.⁵² However, earlier studies of stretch activation responses of skinned myocardium at a fixed $[Ca]^{2+}$ showed that the rate of relaxation of force is accelerated by phosphorylation of cMyBP-C (Figure 2).¹¹ The acceleration is most likely caused by an increased rate of cross-bridge detachment, although the underlying mechanism is not yet known. In the context of a twitch, an increase in detachment rate would be expected to increase the rate of relaxation: the decay of Ca^{2+} back to resting levels would slow or eliminate cooperative recruitment of cross bridges, so that relaxation of force would be determined by cross-bridge detachment rate.

Modulation of relaxation rates via cMyBP-C phosphorylation could be important during physiological interventions, such as increased adrenergic tone or increased stimulus frequency. Under these conditions, the decay phase of the Ca^{2+} transient is accelerated because of phosphorylation of Ca^{2+} handling proteins by the same kinases that phosphorylate cMyBP-C. In these instances, the coordinated phosphorylations of cMyBP-C and Ca^{2+} -handling proteins tune the kinetics of cross-bridge detachment to the kinetics of Ca^{2+} decay. Without this synchronization of kinetics and particularly the acceleration of cross-bridge detachment, twitch duration would be prolonged, which could depress diastolic filling under conditions of increased stimulation frequency. As an illustration of the importance of synchronization, even though cross-bridge detachment kinetics are accelerated in cMyBP-C null myocardium, cardiac relaxation is slowed in vivo because of a slowing of the Ca^{2+} transient.¹²

Summary

In the past decade, our understanding of the roles played by cMyBP-C has evolved from an emphasis on structural stabilization of the thick filament to a consensus view that the protein regulates cardiac contractility through phosphorylation-dependent interactions with ≥ 1 proteins in the cardiac sarcomere. This review has focused on putative interactions with myosin or actin as possible mediators of cMyBP-C regulation of cardiac contraction kinetics, which has narrowed the focus of the review but has not taken into account the observations that cMyBP-C may also bind other sarcomeric proteins, such as myosin regulatory light chain and titin, and may exert regulatory effects that are not controlled by phosphorylation.^{61,62} Even with this narrower focus, the range of questions that need to be answered to understand the regulatory roles of cMyBP-C is daunting and important. Does phosphorylation of cMyBP-C directly regulate the kinetics of cross-bridge state transitions or does this involve indirect effects, such as the modulation of the rate of cooperative recruitment of cross-bridges discussed here? Are the mechanisms of phosphorylation-dependent modulation of contractility and relaxation similar or different? Does the phosphorylation state of cMyBP-C influence its binding to myosin, to actin, or to both? What are the contributions of kinases other than PKA to the

regulation of cMyBP-C phosphorylation and function? Are the binding phenomena involving myosin and actin that have been observed in simplified biochemical systems functional within the intact filament lattice of myocardium? Which are the key residues in these interactions, and do these residues provide a targeting framework for development of therapeutic interventions designed to modify contractility? How do mutations in cMyBP-C contribute to the altered contractility of inherited or acquired cardiomyopathies?

The complexity implied by this list of questions, which is undoubtedly incomplete, points to the need to use a range of approaches from biochemical and biophysical to intact myocardium and living animals and ultimately human systems, to more fully understand the functions of this intriguing protein. Remarkably, cMyBP-C phosphorylation has only recently emerged as an important player in the regulation of myofibrillar function in heart muscle after several decades of consensus beliefs that such regulation principally involved interactions between and modulation of Ca^{2+} delivery systems and myofibrillar Ca^{2+} binding proteins within the myocyte. The complexity introduced into this system by regulation via cMyBP-C, and the possibility of other as yet unrecognized regulatory processes, presumably improves the precision and redundancy of control of contraction and relaxation, but also increases the possibility that genetic or environmental derangements might give rise to cardiac disease.

Acknowledgments

We gratefully acknowledge Nancy Bintz for technical assistance with this article.

Sources of Funding

Research conducted in the Moss Laboratory was supported by grants from the National Institutes of Health (R37 HL082900 and P01 HL094291 to R.L. Moss).

Disclosures

None.

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