Background: Myofilament length-dependent activation (LDA) is modulated by phosphorylation.

Results: Myosin binding protein C (MyBP-C) constitutes the dominant molecular mechanism underlying phosphorylation, with a minor and independent contribution of cardiac troponin-I (cTnI).

Conclusion: MyBP-C, and to a lesser extent cTnI, both contribute to enhance LDA.

Significance: Novel insights into the molecular basis LDA and its regulation by phosphorylation.

β-Adrenergic stimulation in heart leads to increased contractility and lusitropy via activation of protein kinase A (PKA). In the cardiac sarcomere, both cardiac myosin binding protein C (cMyBP-C) and troponin-I (cTnI) are prominent myofilament targets of PKA. Treatment of permeabilized myocardium with PKA induces enhanced myofilament length-dependent activation (LDA), the cellular basis of the Frank-Starling cardiac regulatory mechanism. It is not known, however, which of these targets mediates the altered LDA and to what extent. Here, we employed two genetic mouse models in which the three PKA sites in cMyBP-C were replaced with either phospho-mimic (DDD) or phospho-null (AAA) residues. AAA- or DDD-permeabilized myocytes (n = 12–17) were exchanged (~93%) for recombinant cTnI in which the two PKA sites were mutated to either phospho-mimic (DD) or phospho-null (AA) residues. Force-[Ca^{2+}] relationships were determined at two sarcomere lengths (SL = 1.9 μm and SL = 2.3 μm). Data were fit to a modified Hill equation for each individual cell preparation at each SL. LDA was indexed as EC_{50}, the difference in [Ca^{2+}] required to achieve 50% force activation at the two SLs. We found that PKA-mediated phosphorylation of cMyBP-C and cTnI each independently contribute to enhance myofilament length-dependent activation properties of the cardiac sarcomere, with relative contributions of ~67 and ~33% for cMyBP-C for cTnI, respectively. We conclude that β-adrenergic stimulation enhances the Frank-Starling regulatory mechanism predominantly via cMyBP-C PKA-mediated phosphorylation. We speculate that this molecular mechanism enhances cross-bridge formation at long SL while accelerating cross-bridge detachment and relaxation at short SLs.

The Frank-Starling Law of the Heart describes a cardiac regulatory control mechanism that operates on a beat-to-beat basis (1). In general, there is a unique relationship between ventricular end-systolic volume and end-systolic pressure, with a proportionality that is determined by cardiac contractility. Because of this relationship, for a given cardiac contractile state ventricular stroke volume is directly proportional to the extent of diastolic filling. In conjunction with heart rate and contractility, the Frank-Starling mechanism not only constitutes a major determinant of cardiac output, it also contributes to balance the output of the left and right ventricles. Although the Frank-Starling mechanism has been well established for over a century, the molecular mechanisms underlying this phenomenon are not well resolved. At the cellular level, a sustained increase in sarcomere length results in both an immediate and a secondary, slower, increase in twitch force that develops over several minutes (2). The latter phase, termed the slow force response, is caused by an alteration in cardiac myocyte Ca^{2+} homeostasis induced by a strain-dependent mechanism that, too, is incompletely understood (2). Existing data, mostly derived from permeabilized isolated myocardium, strongly supports the notion that the immediate increase in twitch force development upon stretch is directly due to an increase in the Ca^{2+} responsiveness of the cardiac contractile apparatus itself (3, 4), a phenomenon termed myofilament length-dependent activation (LDA)² (1).

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2 The abbreviations used are: LDA, length-dependent activation; cMyBP-C, cardiac myosin binding protein C; NTG, non-transgenic; SL, sarcomere length; TnT, troponin T; cTnI, cardiac troponin I; cTnT, cardiac troponin T; F_{max} maximum, Ca^{2+}-saturated developed force; E_{cp} [Ca^{2+}] at which force development is 50% of F_{max}; AAA, MyBP-C in which the PKA sites are mutated to alanine (phospho-null); DDD, MyBP-C in which the PKA sites are mutated to aspartate (phospho-mimetic); AA, cTnI in which the PKA sites are mutated to alanine (phospho-null); DD, cTnI in which the PKA sites are mutated to aspartate (phospho-mimetic); BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; IP, inhibitory peptide.
PKA Modulation of Myofilament Length-dependent Activation

How the mechanical strain signal is transduced by the cardiac sarcomere is not known. We recently demonstrated that LDA develops within a few milliseconds after a change in sarcomere length (5), a finding suggestive of a molecular mechanism caused by strain-dependent geometric rearrangement of contractile proteins. Moreover, although LDA is a general property of striated muscle, it manifests itself to a much greater extent in cardiac muscle as compared with slow-twitch skeletal muscle (6). This is caused, in part, by the expression of the unique cardiac-specific isoform of troponin-I (cTnI) (7), the inhibitory subunit of the striated muscle regulatory troponin complex (8, 9). Cardiac LDA has been shown to be modulated by contractile protein phosphorylation by most (1, 8, 10, 11) but not all (12) reports. Moreover, LDA is affected by cardiac disease-associated mutations within various contractile proteins, including the troponins and cardiac myosin binding protein C (cMyBP-C) (13). Selective introduction of a PKA phosphomimic isoform of cTnI into the cardiac sarcomere increases LDA (10, 14). Finally, cardiac disease-causing mutations are associated with blunting of the myofilament Ca^{2+} desensitization normally associated with PKA-mediated phosphorylation (15, 16). Moreover, evidence has emerged that passive force originating from the giant elastic sarcomeric protein titin directly acts to modulate myofilament Ca^{2+} responsiveness (17–19).

cMyBP-C is a contractile protein that binds to myosin and is located in the C-zones of the sarcomere in 7–9 stripes on either side of the M-line (20, 21). Originally believed to be mainly a structural protein stabilizing the thick filament, more recent work strongly indicates a regulatory role for this protein (21, 22). Phosphorylation of cMyBP-C by protein kinase A (PKA), in addition to other kinases, has been shown to increase the mobility of the S1 globular domain of myosin, increase the velocity of muscle shortening, and enhance the rate of stretch activation (21). The functional effects of cMyBP-C phosphorylation are in many ways similar to the impact of complete removal of cMyBP-C either by protein extraction in isolated myocardium or by genetic knock-out of the protein in mouse models (21). Of importance to the current study, lack of cMyBP-C has been shown to blunt LDA (23, 24) and stretch activation (23); the impact of cMyBP-C phosphorylation on LDA, however, has not been studied. The molecular mechanisms underlying the impact of cMyBP-C on contractile regulation has generally been ascribed as a release from the inhibition of cross-bridge kinetics subsequent to detachment of the N\textsuperscript{+} domain of cMyBP-C from the S2 neck region of myosin upon PKA-mediated phosphorylation (21, 25). The PKA targets are within the M-domain of cMyBP-C, also located in the N\textsuperscript{+} domain (21, 25). These results, obtained in isolated muscle preparations with an intact sarcomere lattice, are consistent with results reported on motility assays using individual thin and thick filaments, or fragments thereof, indicating an inhibitory action of cMyBP-C or its N\textsuperscript{+} domains that is relieved upon phosphorylation of the protein (26, 27). More recently, however, evidence has emerged that cMyBP-C may also activate the thin filament by a direct interaction between its N\textsuperscript{+} domain and actin and/or tropomyosin (28, 29). These results suggest that the impact of cMyBP-C can be both inhibitory (via tethering of the myosin head away from actin toward the thick filament backbone) and activating (via binding to actin or displacing tropomyosin on the thin filament) (21). In this model, the N\textsuperscript{+} domain of the protein mediates both actions, and the balance between inhibition and activation is modulated by the cMyBP-C phosphorylation state. We recently showed in permeabilized isolated human myocardium a prominent impact on the myofilament function of exposure to the first three N\textsuperscript{+} domains of cMyBP-C at short sarcomere length (SL) that was absent at long SL (30). These results suggest that the impact of cMyBP-C on myofilament function may depend on SL in addition to its phosphorylation status.

Here, to resolve whether cMyBP-C phosphorylation affects LDA and to determine whether the impact of cMyBP-C and cTnI phosphorylation are synergistic or merely additive, we employed two transgenic mouse models in which cMyBP-C is replaced by either PKA phospho-null (AAA) or phospho-mimic (DDD) versions of the protein (31–33). Isolated permeabilized single myocyte fragments were either treated with PKA or exchanged for troponin containing PKA phospho-null or phospho-mimic cTnI. Our results indicate that (i) both cMyBP-C and cTnI phosphorylation enhance LDA, (ii) the contribution of each protein is independent, and (iii) ¾ of the impact is due to cMyBP-C phosphorylation. Our results are consistent with a molecular model in which the N\textsuperscript{+} domain of cMyBP-C competes with both the inhibitory peptide domain of cTnI and myosin S1 for a common binding site on actin in a phosphorylation and SL-dependent manner. Such a mechanism would assist in the formation of cross-bridges at end-diastolic volumes to promote cardiac pressure development while promoting cross-bridge detachment at end-systolic volumes to promote cardiac relaxation. Moreover, β-adrenergic-stimulated PKA-mediated phosphorylation of cMyBP-C and cTnI would be expected to amplify both phenomena.

Experimental Procedures

cMyBP-C Non-transgenic (NTG), AAA, and DDD Mouse Models—The phospho-mimetic and phospho-null mouse models have been previously described (31–33). For the phospho-null mouse model, the PKA sites (Ser-273, Ser-282, and Ser-302) were mutated to alanine, whereas for the phospho-mimetic model these sites were mutated to aspartic residues. Molecular dynamics analysis indicates that the serine to aspartic substitution is in many ways equivalent to native phosphorylation of the serine residue, at least in the case of cardiac troponin-I (34). To ensure complete cMyBP-C replacement, both mouse models were crossed into a cMyBP-C truncation mutant mouse model (35). All experiments were conducted in accordance with institutional guidelines and approved by the Loyola University Chicago Stritch School of Medicine Animal Care and Use Committee. Mice (~8 weeks of age) were deeply anesthetized with pentobarbital (50 mg/kg), the hearts were removed from the chest, and left and right ventricles were snap-frozen in liquid nitrogen and stored at ~80 °C until use. For these studies, hearts from 10 NTG wild-type, 11 AAA, and 10 DDD mice were used. The AAA mouse model is associated with the development of hypertrophy, increased expression β-myosin, and potentially altered PKA activity (31–33). To
minimize the impact of this potentially confounding factor, relatively young mice were used for this study (~8 weeks of age). Furthermore, variable PKA activity was taken into account by the design of the study protocol, as the complete replacement of phospho-mimic cMyBP-C was achieved by crossbreeding with a cMyBP-C knock-out mouse line, essentially eliminating cMyBP-C PKA phosphorylation. Likewise, the impact of variable cTnI PKA phosphorylation was accounted for by the phospho-mimetic cTnI containing troponin exchange procedure, resulting in ~93% troponin replacement. Finally, the partial expression of β-myosin in the AAA group is unlikely to affect the results of the present study, as no impact on myofilament Ca^{2+} has been observed upon the complete replacement of α-myosin with β-myosin in rat myocardium (36, 37).

Expression, Purification, and Reconstitution of Recombinant Troponin—The methods used to clone, express, and purify the troponins and reconstitution of the recombinant troponin complex have been previously described (38). Briefly, murine DNA clones of cardiac troponin C, cardiac myc-tagged troponin T, and phospho-null or phospho-mimetic cTnI (S22A, S23A; S22D, S23D respectively) were transformed into Rosetta BL21 (DE3) Escherichia coli bacteria (Novagen) and grown at 37 °C overnight. High protein yield was achieved by isopropyl 1-thio-β-D-galactopyranoside induction. Proteins were isolated by lysis and centrifugation followed by column chromatography purification (15). Water dialyzed and lyophilized proteins were stored at ~20 °C. The troponin complexes were formed by mixing equimolar amounts of cTnI, cardiac troponin C, and myc-TnT in 8 M urea followed by serial dialysis to a final ionic strength of 180 mM in relaxing solution. Next, the troponin complexes were purified by FPLC (ResourceQ) and stored at ~80 °C until use.

Exchange for Recombinant Troponin in Myocytes—Myocytes were harvested from frozen left ventricular tissue by mechanical homogenization (39). Briefly, a portion of the left ventricular (30–40 mg) was homogenized at 10,000 rpm for 1 s in relaxing solution. The relaxing solution contained 97.92 mM KOH, 6.24 mM ATP, 10 mM EGTA, 10 mM Na2CrP, 47.58 mM potassium propionate, 100 mM BES, 6.54 mM MgCl2, 1 mM DTT, 0.01 mM leupeptin, 0.1 mM PMSF, and 0.001 mM pepstatin. Next, the homogenate slurry was filtered through a 70-μm cell strainer followed by centrifugation at 120 g for 1 min at 4 °C. The cells were skinned by resuspending the pellet in relaxing solution with 1% Triton X-100 added for 10–15 min on a slowly rotating shaker. Thereafter, Triton X-100 was removed by resuspending the single cells two times in relaxing solution after centrifugation at 120 × g for 1 min at 4 °C. The cells were skinned by resuspending the pellet in relaxing solution with 1% Triton X-100 added for 10–15 min on a slowly rotating shaker. Thereafter, Triton X-100 was removed by resuspending the single cells two times in relaxing solution after centrifugation at 120 × g for 1 min at 4 °C. Skinned myocytes were exchanged for exogenous recombinant mouse cardiac troponin as described previously (15). Briefly, after permeabilization and Triton X-100 removal, the myocytes were resuspended in 4 °C relaxing solution containing 2 mg/ml recombinant phospho-null or phospho-mimetic troponin and incubated overnight on a slowly rotating orbital shaker at 4 °C. The next day, exchanged myocytes were pelleted 3 times by centrifugation at 300 × g for 5 min at 4 °C and each time resuspended in 300 μl of 4 °C relaxing solution. After a final centrifugation, resultant myocytes were suspended in 1 ml of 4 °C relaxing solution and kept on ice until use. Thereafter, myocytes were used for isometric force measurement as described below within 8 h. A portion of the cell suspension was also collected to determine the extent of troponin exchange by Western blot analysis.

Western Blot Analysis of Recombinant Troponin Exchange—The Western blot method to measure the extent of recombinant troponin exchange has been previously reported (40). Briefly, isolated skinned myocytes were exchanged overnight with recombinant troponin and after twice resuspending in relaxing solution myocytes were solubilized in 8 M Tris-urea denaturing buffer, and Laemmli buffer was added. Total pro-
PKA Modulation of Myofilament Length-dependent Activation

| TABLE 1 |
|----------|
| Impact of PKA treatment on mechanical properties of cMyBP-C NTG, AAA, and DDD myocytes |

Force-Ca\textsuperscript{2+} relationships were recorded in Triton X-100-permeabilized myocytes prepared from wild-type non-transgenic (NTG) and phospho-null (AAA) and phospho-mimetic (DDD) mice both before and after PKA treatment. Each individual relationship was fit to a modified Hill equation yielding maximum Ca\textsuperscript{2+} saturated force (F\textsubscript{max}), myofilament Ca\textsuperscript{2+} sensitivity (EC\textsubscript{50}), the [Ca\textsuperscript{2+}] at half-maximal force development, and the Hill coefficient (n\textsubscript{H}), a measure of cooperative activation. Data were collected at both SL and PKA treatment status in all cells, allowing for the calculation of SL\Delta EC\textsubscript{50} and the difference in EC\textsubscript{50} between the short and long SL as well as \Delta F\textsubscript{PKA}, the difference in each Hill parameter between the pre- and post-PKA condition. Passive force (SL\Delta F\textsubscript{pass}) was indexed as the difference in passive myocyte force in relaxing solution between the short and the long SL, mN, millinewtons.

![FIGURE 2. PKA impact on LDA. Myofilament LDA was indexed as \( \Delta EC_{50} \), the difference in myofilament Ca\textsuperscript{2+} sensitivity between long and short sarcomere length. *p < 0.05 (−) PKA vs. (+) PKA.](https://example.com/figure2.png)

PKA Treatment—In some experiments (cf. Fig. 1 and Table 1), the attached skinned myocyte was incubated with relaxing solution containing 100 units of the catalytic subunit of PKA (Leica DMRB). Myocytes to be studied were selected based on a visual scan at 10\times magnification, identifying uniformity of the cell and a regular striation pattern. Next, myocytes were attached to two metal micro-needles connected to a Piezo translator (Thorlabs, Newton, NJ) and a force transducer (Aurora Scientific Inc., Aurora, ON, Canada) and attached to XYZ stage manipulators. The cell attachment was accomplished using UV-sensitive glue (Norland, Cranbury, NJ). All measurements were made under bright field conditions at 40\times magnification using a long working distance objective lens. The attached skinned myocyte was perfused with skinned fiber solutions emanating from a closely placed perfusion pipette attached to a solenoid valve controlled perfusion system (VC-8 M 8-channel mini-valve perfusion system, Warner Instrumetns). A maximum Ca\textsuperscript{2+} saturating activation solution was applied at the beginning of the experiment to test the strength of the cell attachment. Subsequently, cells were continuously perfused with relaxing solution interspersed with random Ca\textsuperscript{2+} activating solutions with varying calcium concentrations (pCa 10.0 to pCa 4.5) to elicit an isometric force response at either the short sarcomere length (SL = 1.9 \( \mu \text{m} \)) or the long sarcomere length (SL = 2.3 \( \mu \text{m} \)). Sarcomere length was continuously monitored using a custom-designed video microscopy based Fourier transform method as previously described (41). Isometric force was recorded at each activating cycle; zero baseline force level was subtracted from all force recordings. All force measurements were corrected for rundown (42) and normalized to cross-sectional area. Cell cross-sectional area was measured at the end of the experiment by slackening the attached myocyte until buckling; the dimensions of the “ellipse” of the two sides of the buckled cell were measured with a calibrated screen monitor. Cells were discarded if their total rundown was greater than 20% for either SL. Three to six cells per heart were used for the experiments. Data were acquired using custom-designed Labview software (National Instruments) and were analyzed offline using Origin Pro 8.0.

PKA Treatment—In some experiments (cf. Fig. 1 and Table 1), the attached skinned myocyte was incubated with relaxing solution containing 100 units of the catalytic subunit of PKA.
PKA Modulation of Myofilament Length-dependent Activation

FIGURE 3. Phosphorylation status of cMyBP-C and cTnI. Panel A, Western blot using phospho-specific and pan nonspecific antibodies was employed to determine the phosphorylation status of cMyBP-C (residue Ser-302) and cTnI (residues Ser-22, Ser-23). Actin was probed with an actin antibody. The intensities of both phospho-specific and pan nonspecific blot intensities were determined using ImageJ. Panel B, average cTnI phosphorylation, normalized to cTnI-P post-PKA.

Results

Impact of PKA Treatment—Fig. 1 summarizes force-[Ca$^{2+}$] relationships recorded on wild-type (NTG) and cMyBP-C AAA or DDD phosphorylation mutant myocytes. The average fit parameters were reported in Table 1. At baseline and long SL, no significant differences in myofilament Ca$^{2+}$ sensitivity between the groups were recorded, consistent with previous reports (43). At short SL, however, cMyBP-C DDD was associated with a significantly lower Ca$^{2+}$ sensitivity. Treatment with PKA resulted in decreased Ca$^{2+}$ sensitivity in all groups that was most pronounced in NTG myocytes. Increasing SL enhanced Ca$^{2+}$ sensitivity, particularly in NTG and DDD myocytes. Fig. 2 summarizes average ΔEC$_{50}$, a convenient parameter to index LDA, and the impact of PKA treatment on this parameter. LDA increased upon PKA treatment in NTG myocytes, consistent with previous reports (1, 8, 10, 11, 13) but was without impact in either the AAA or DDD myocytes. In addition, after PKA treatment, LDA in NTG myocytes approached LDA recorded in DDD myocytes regardless of PKA treatment, whereas it was ~50% lower in AAA myocytes. In all myocytes, PKA treatment resulted in comparable reduction in passive force.

Phosphorylation Status of cMyBP-C and cTnI—The data in Fig. 2 suggest that increased LDA after PKA treatment is predominantly due to phosphorylation of cMyBP-C and only marginally due to phosphorylation of cTnI. In contrast to the present results, introduction of PKA phospho-mimetic cTnI into the cardiac sarcomere has been reported to increase LDA (10, 14). This discrepancy may be explained by baseline cTnI and cMyBP-C phosphorylation present before PKA treatment (Fig. 4A). This discrepancy may be explained by baseline cTnI and cMyBP-C phosphorylation present before PKA treatment (Fig. 4A). In addition, others have shown that ~50% cTnI phosphorylation is sufficient to induce a maximal response on myofilament calcium sensitivity (44). Fig. 3A shows representative Western blots of myofilament proteins pre- and post-PKA treatment. PKA treatment induced robust increases in both cMyBP-C and cTnI phosphorylation in NTG myocytes, whereas cMyBP-C phosphorylation was virtually absent in the AAA and DDD myocytes. Fig. 3B shows average cTnI PKA phosphorylation levels at baseline, normalized to post-PKA treatment levels. Importantly, a significant and variable phosphorylation baseline level was detected in all groups for cTnI and for cMyBP-C in the NTG myocytes. Thus, rendering conclusions regarding the relative impact of cTnI and cMyBP-C phosphorylation on LDA in this series of experiments is difficult.

LDA after Recombinant Troponin Exchange—To overcome the confounding impacts of baseline cTnI phosphorylation, we next exchanged endogenous troponin for recombinant troponin containing either PKA phospho-null (AA) or phospho-mimetic (DD) cTnI into either AAA or DDD myocytes. The recombinant troponin also contained myc-tagged TnT, allowing for quantification of troponin exchange by Western blot analysis (cf. Fig. 4A and B, inserts; 93 ± 3% average exchange; C = non-exchanged controls). The force-[Ca$^{2+}$] relationships recorded in exchanged AAA cells revealed a modest level of LDA in the absence of cTnI and cMyBP-C PKA pseudophosphorylation, increasing slightly in the presence of phospho-mimetic cTnI (Fig. 4A). In contrast, phospho-mimetic cMyBP-C (DDD) already displayed robust LDA in the presence of phospho-mimetic cTnI (Fig. 4B).
PKA Modulation of Myofilament Length-dependent Activation

![Diagram](image)

**FIGURE 4.** LDA after recombinant troponin exchange. Force-[Ca^{2+}] relationships recorded on myocytes from AAA (panel A) and DDD hearts (panel B) at two SLs (cf. Fig. 1) after troponin exchange containing either phospho-null (open symbols, solid lines) or phospho-mimetic (closed symbols, dashed lines) cTnI. Insets: Western blot analysis of troponin exchange. Average fit parameters are summarized in Table 2. C = non-exchanged controls. Panel C, average LDA and estimate of the relative contribution of cMyBP-C and cTnI phosphorylation. *p < 0.05 AAA versus DDD; and #, p < 0.05 AA versus DD using 2-way analysis of variance; this analysis furthermore revealed that the average impact on ΔEC_{50} of cMyBP-C-DDD versus cMyBP-C-AAA was ~0.48 μM, and that of cTnI-DD versus cTnI-AA was ~0.24 μM.

Phosphorylation on LDA was ~2× greater than that of cTnl phosphorylation, and the impacts were, moreover, proportional for each PKA target with no apparent evidence for synergism. Passive force was similar among the groups, indicating comparable levels of titin phosphorylation (Table 2).

**Discussion**

Myofilament LDA is a fundamental sarcomeric property that underlies the Frank-Starling of the heart. The main new findings of our study are 1) PKA phosphorylation of cMyBP-C enhances LDA, 2) both cMyBP-C and cTnI phosphorylation each independently contribute to enhance LDA, and 3) cMyBP-C phosphorylation is the major contributor to the enhanced LDA.

CMyBP-C has emerged in recent reports as a contractile protein possessing prominent regulatory properties within the cardiac sarcomere, as opposed to merely being a structural protein (21, 28, 29). The underlying molecular mechanisms include both inhibition and activation of the contractile apparatus, with the relative balance between these two modes modulated by cMyBP-C phosphorylation status (21, 28, 29). Of note, the binding affinity between actin and cMyBP-C has been shown to be either reduced (45) or not affected by PKA-mediated phosphorylation (46). However, because the binding affinity of cMyBP-C toward myosin is an order stronger than that for actin (21, 45, 46), the net impact of cMyBP-C phosphorylation is expected to induce a redistribution of the average position of cMyBP-C away from the thick filament and toward the thin filament.

A recent study in skeletal muscle has shown that the relative overlap between MyBP-C and the thin filament in the so-called C-zone of the sarcomere affects myosin structure that was suggested to contribute to LDA (47). However, considering the range of sarcomere lengths studied here (SL = 1.9–2.3 μm), the lengths of the cardiac thick and thin filaments (48, 49) and the structure of the cardiac C-zones (48), full overlap is predicted between cMyBP-C and the cardiac thin filament at both the short and long SL. Hence, in the heart a mere change in position between cMyBP-C and the cardiac thin filament is unlikely to underlie LDA or its modulation by PKA.

The mechanism underlying cTnI PKA phosphorylation may be related to a molecular conformational change upon introduction of the negatively charged phosphates into the intrinsically disordered N’ domain of the molecule (50). This rearrangement could involve new intramolecular interactions between the N’ and the inhibitory peptide (IP) domain of cTnI (34). This in turn may strengthen the interaction between cardiac troponin C and the switch peptide domain of cTnI, thereby inducing a reduction in myofilament Ca^{2+} sensitivity and at the same time weaken the interaction between the IP domain and actin. PKA treatment resulted in a reduction of passive force, as has been described previously (17, 51). The passive force is believed to originate from titin strain that is reduced upon PKA (or protein kinase G)-mediated phosphorylation. Titin strain has also been suggested to modulate LDA (17–19). Hence, the possibility exists that a reduction in passive force would actually reduce LDA, potentially offsetting the impact of PKA-mediated phosphorylation reported here. Although not the focus of this
TABLE 2
Summary of mechanical properties of AAA and DDD myocytes after exchange with recombinant mutant troponin

| Group          | n   | Fmax | EC50 | nH | SL = 1.9 μm | Fmax | EC50 | nH | SL = 2.3 μm | Fmax | EC50 | nH | SLΔEC50 | SLΔFmax | SLΔFmax |
|----------------|-----|------|------|----|-------------|------|------|----|-------------|------|------|----|----------|----------|----------|
| cMyBP-C AAA    | 17  | 52.0 ± 2.0 | 3.44 ± 0.17 | 2.2 ± 0.1 | 10.3 ± 1.3 | 14.6 ± 0.7 |
| rTnI AA        | 17  | 61.6 ± 2.4 | 3.53 ± 0.24 | 2.1 ± 0.1 | 16.7 ± 2.1 | 13.9 ± 0.7 |
| rTnI DD        | 12  | 0.03  | 0.05  | 0.05  | 0.05  | 0.05  | 0.05  |

Cardiac TnI phosphorylation

FIGURE 5. Proposed model for cMyBP-C and cTnI PKA phosphorylation impacts on LDA. Four states of phosphorylation are shown: cMyBP-C and cTnI both dephosphorylated (top left), cTnI phosphorylated (top right; horizontal), cMyBP-C phosphorylated (bottom left; vertical), and both proteins phosphorylated (bottom right). Myosin is in blue, with the S1 head domain; cMyBP-C is in red, with the N terminus (N) containing the phospho sites (AAA or DDD); cTnI is in yellow, containing the inhibitory peptide (IP) and switch peptide (SW) domains, and the N-terminal phospho sites (AA or DD). See "Discussion" for details.

study, we did perform limited experiments employing PKA treatment in the recombinant troponin-exchanged myocytes. In particular, cMyBP-C AAA phospho-null myocytes in which endogenous troponin was exchanged for DD phospho-mimetic cTnI (n = 4) displayed a 10.5 ± 1.0% reduction in passive force (14.0 ± 0.5 versus 12.5 ± 0.3 millinewtons/mm²) upon PKA treatment that was associated with a significant reduction in ΔEC50 (0.67 ± 0.08 versus 0.49 ± 0.03 μM), supporting the notion that a PKA-mediated reduction in titin strain by itself reduces LDA. In addition, maximum Ca²⁺-activated myofilament force was reduced upon treatment with PKA in all muscle groups (NTG, AAA, and DDD), consistent with previous reports by others and us (1, 8, 10, 11). In contrast, exchange of either AAA or DDD myocytes with the cTnI phospho-mimetic containing troponin resulted in an increase in maximum force (Table 1). These data suggest that the decrease in force development after PKA treatment may be due to the reduction in titin-based passive force caused by PKA-mediated phosphorylation, consistent with the reduced LDA observed in other experimental models where the titin strain is reduced (17–19).
PKA Modulation of Myofilament Length-dependent Activation

Opposing this reduction in force development is the interaction of the N′-cMyBP-C domain with actin induced by PKA-mediated phosphorylation (see below). Such a mechanism would explain the elevated maximum force that was recorded in DDD cMyBP-C phospho-mimetic as compared with AAA cMyBP-C phospho-null myocytes regardless of cTnI phosphorylation status (Table 2). Presumably, here, titin phosphorylation levels were comparable as passive force was not different between the cMyBP-C and cTnI phospho-mimetic groups.

Our results are consistent with a molecular model (Fig. 5) in which the cMyBP-C N′ domain competes with both the cTnI IP domain and myosin S1 for a common binding site on actin in a phosphorylation and SL-dependent manner. In this proposed model, cTnI phosphorylation (horizontal) induces reduced affinity of its IP domain for actin, thereby allowing for enhanced cooperative cross-bridge attachment upon Ca⁺² activation. cMyBP-C phosphorylation (vertical) induces its release from myosin, resulting in enhanced actin activation by cMyBP-C, which also promotes cooperative cross-bridge attachment. Moreover, although both pathways are separate and independent, they are additive because of a downstream target formed by the common thin filament myosin-binding site. Such a mechanism would assist in the formation of cross-bridges at end-diastolic volumes to promote cardiac pressure development while accelerating cross-bridge detachment at end-systolic volumes to enhance cardiac relaxation. Moreover, β-adrenergic-activated PKA-mediated phosphorylation of cMyBP-C and cTnI are expected to amplify both phenomena through enhanced myofilament LDA and, consequently, amplification of the Frank-Starling mechanism.

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