

# Loaded Shortening, Power Output, and Rate of Force Redevelopment Are Increased With Knockout of Cardiac Myosin Binding Protein-C

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**Abstract**—Myosin binding protein-C (MyBP-C) is localized to the thick filaments of striated muscle where it appears to have both structural and regulatory functions. Importantly, mutations in the cardiac MyBP-C gene are associated with familial hypertrophic cardiomyopathy. The purpose of this study was to examine the role that MyBP-C plays in regulating force, power output, and force development rates in cardiac myocytes. Skinned cardiac myocytes from wild-type (WT) and MyBP-C knockout (MyBP-C<sup>-/-</sup>) mice were attached between a force transducer and position motor. Force, loaded shortening velocities, and rates of force redevelopment were measured during both maximal and half-maximal Ca<sup>2+</sup> activations. Isometric force was not different between the two groups with force being 17.0±7.2 and 20.5±3.1 kN/m<sup>2</sup> in wild-type and MyBP-C<sup>-/-</sup> myocytes, respectively. Peak normalized power output was significantly increased by 26% in MyBP-C<sup>-/-</sup> myocytes (0.15±0.01 versus 0.19±0.03 P/P<sub>o</sub> · ML/sec) during maximal Ca<sup>2+</sup> activations. Interestingly, peak power output in MyBP-C<sup>-/-</sup> myocytes was increased to an even greater extent (46%, 0.09±0.03 versus 0.14±0.02 P/P<sub>o</sub> · ML/sec) during half-maximal Ca<sup>2+</sup> activations. There was also an effect on the rate constant of force redevelopment (k<sub>tr</sub>) during half-maximal Ca<sup>2+</sup> activations, with k<sub>tr</sub> being significantly greater in MyBP-C<sup>-/-</sup> myocytes (WT=5.8±0.9 s<sup>-1</sup> versus MyBP-C<sup>-/-</sup>=7.7±1.7 s<sup>-1</sup>). These results suggest that cMyBP-C is an important regulator of myocardial work capacity whereby MyBP-C acts to limit power output. (*Circ Res.* 2003;93:752-758.)

**Key Words:** cardiac myocytes ■ myosin binding protein-C ■ sarcomere proteins  
■ cardiac contractility ■ power output

Myosin binding protein-C (MyBP-C) is a thick filament associated protein<sup>1</sup> that is present in vertebrate striated muscle, and mutations in the cardiac MyBP-C gene have been implicated in the development of some familial hypertrophic cardiomyopathies (FHC). Although the exact role of MyBP-C in muscle development and function is unclear, it has been postulated that MyBP-C plays both structural and regulatory roles.<sup>2</sup> The role of MyBP-C as a structural element involves its carboxy terminus binding to the thick filament where it interacts with both the rod portion of myosin<sup>3</sup> and titin.<sup>4</sup> These C-terminal interactions are thought to stabilize the structure of the thick filament and produce a more ordered arrangement of myosin heads. Moolman-Smook et al<sup>5</sup> have provided evidence that the immunoglobulin-like domains C5 and C8 of MyBP-C preferentially interact with each other, which taken together with previous structural and biochemical data led them to propose that MyBP-C molecules form a collar around the backbone of the thick filament. They speculated that the collar packs the backbone more tightly and restricts actin-myosin interactions, whereas release of

the collar would result in a looser backbone and perhaps enhance crossbridge formation. Additionally, mutations in the MyBP-C gene that produce C-terminal truncated proteins have been found to result in acute changes in structure not only at the myofilament, but also in the structure of the heart that ultimately leads to impaired myocardial performance.<sup>6</sup> Cardiac MyBP-C (cMyBP-C) also contains a unique immunoglobulin-like domain at the N-terminal portion of the protein termed C0<sup>6</sup> and a distinct region between two Ig-like domains (C1 and C2), which is termed the MyBP-C motif.<sup>7</sup> This motif has been shown to bind to the S2 segment of myosin near the lever arm domain of the myosin head, and this interaction is modified in response to phosphorylation of the MyBP-C motif by cAMP-dependent protein kinase (PKA).<sup>7,8</sup> Mutations in the cardiac MyBP-C gene that lead to changes near the N-terminus have also been linked to familial hypertrophic cardiomyopathy.<sup>9</sup>

Recently, targeted ablation of the cMyBP-C gene was used to produce mice lacking MyBP-C in the heart. Mice lacking cMyBP-C (MyBP-C<sup>-/-</sup>) were viable and displayed well-

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developed sarcomeres, indicating MyBP-C is not essential for myofibrillogenesis. However, MyBP-C<sup>-/-</sup> mice exhibited profound concentric cardiac hypertrophy and impaired diastolic and systolic function in vivo. The impaired heart function was somewhat surprising in light of several studies showing enhanced myocyte contractility after manipulations of MyBP-C. For instance, disrupting the interactions between MyBP-C and S2 enhanced myocyte shortening<sup>10</sup> and increased Ca<sup>2+</sup> sensitivity of force in skinned myocardial preparations.<sup>11,12</sup> Consistent with these results, partial extraction of MyBP-C also increased Ca<sup>2+</sup> sensitivity of force in skinned myocytes<sup>13</sup> and sped shortening velocity in skinned skeletal muscle fibers.<sup>14</sup> These results are all consistent with a model in which MyBP-C affects myosin head flexibility and position perhaps by serving as collar or a tether. Removal of this collar/tether would increase myosin head flexibility and bring the head in closer proximity to actin, thereby enhancing the probability of crossbridge formation.

The purpose of this study was to examine the effects of targeted deletion of cMyBP-C on contractile function in single skinned myocytes, working under the hypothesis that myocytes lacking MyBP-C would have faster loaded shortening velocities, greater power output, and increased force redevelopment rates all due to elevated crossbridge interaction kinetics resulting from removal of myosin head constraints normally imposed by MyBP-C.

## Materials and Methods

### Experimental Animals

Homozygous cardiac MyBP-C knockout mice (cMyBP-C<sup>-/-</sup>) were generated as previously described in detail.<sup>15</sup> Briefly, exons 3 through 10 of the murine cMyBP-C gene were deleted from mouse genomic DNA by homologous recombination. Properly targeted embryonic stem cells were injected into 357/B6 blastocysts and implanted into C57/B6 pseudopregnant mice (Taconic Farms, Germantown, NY). Appropriate breeding resulted in homozygous MyBP-C null mice. Animals were housed in groups of two to three and provided food and water ad libitum. All procedures involving animal use were performed according to the Animal Care and Use Committees of the University of Wisconsin and University of Missouri. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital and euthanasia followed by rapid excision of the heart.

### Cardiac Myocyte Preparation

Skinned cardiac myocyte preparations were obtained from mouse hearts using methods similar to those described previously for rats.<sup>16</sup> The compositions of relaxing and activating solutions were as follows (in mmol/L, obtained from Sigma at highest possible purity): free Mg<sup>2+</sup> 1, EGTA 7, MgATP 4, imidazole 20, and creatine phosphate 14.5 (pH 7.0); various [Ca<sup>2+</sup>] between 10<sup>-4.5</sup> (maximal Ca<sup>2+</sup> activating solution) and 10<sup>-9</sup> (relaxing solution); and sufficient KCl to adjust ionic strength to 180 mmol/L.<sup>17</sup> A portion of cardiac myocytes were aliquoted and stored in ATP-free relaxing solution containing phosphatase inhibitors (20 mmol/L NaF and 50 μg/mL microcystin LR) at -70°C for autoradiographic analysis. The experimental apparatus used for physiological measurements of single skinned myocytes has been described.<sup>16</sup> The dimensions of the myocyte preparations are included in Table 1.

### Force-Velocity and Power-Load Measurements

The protocol for obtaining force-velocity and power-load measurements has been described in detail<sup>18</sup> and all measurements

TABLE 1. Summary of Myocyte Dimensions

	Length, μm	Width, μm	SL at pCa 4.5, μm
Wild-type	132.8±28.4	20.1±2.3	2.31±0.097
MyBP-C <sup>-/-</sup>	123.8±31.4	24.3±4.3	2.28±0.089

Values are mean±SD. SL indicates sarcomere length.

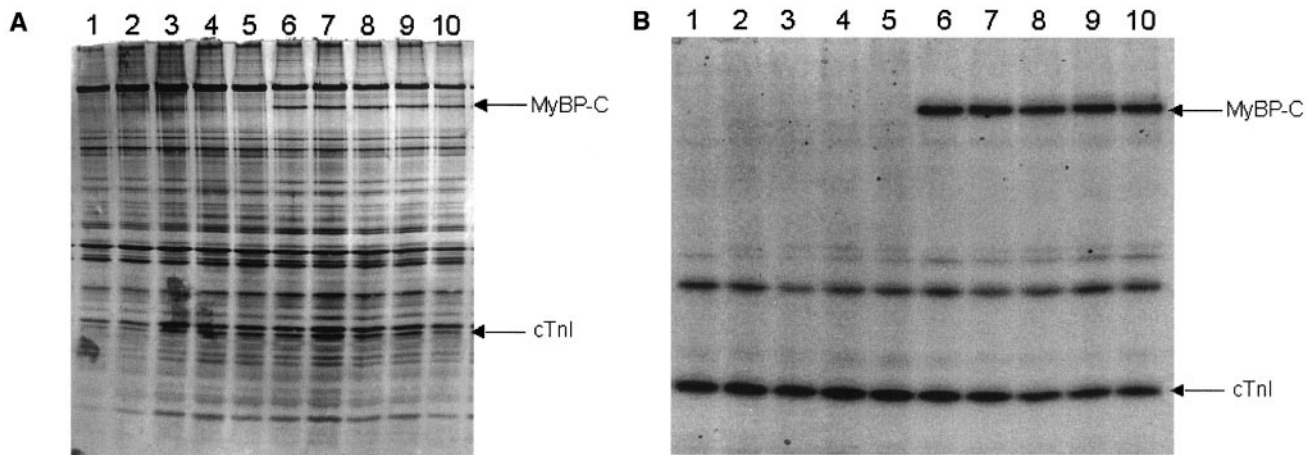
were done at 13±1°C. The attached myocyte was first transferred in maximal Ca<sup>2+</sup> activating solution and allowed to obtain a steady state force, after which a series of sub-steady state force clamps were applied to determine isotonic shortening velocities. The isotonic force was maintained using a servo system for 150 to 250 ms while the length change during this time is monitored. After the force clamp, the myocyte was slackened to near zero force to estimate the relative load sustained during the isotonic shortening, after which the myocyte was re-extended to its starting length. Due to the short lengths of single myocyte preparations, the rapid slackening after isotonic shortening did not always yield a baseline force value, the result of which is an underestimation of peak force and, thus, of the relative force during loaded contractions. In these cases, more accurate estimates of the relative force during isotonic shortening were obtained by interpolating between the peak forces in isometric contractions that were performed before and after every series of loaded contractions. The myocytes were kept in maximal Ca<sup>2+</sup> activating solution for 2 to 3 minutes during which 10 to 20 force clamps were performed without significant loss of force. If maximal force fell below 75% of the initial value while performing the force clamps, the myocyte was discarded and the data not included. Force-velocity measurements were also obtained during half-maximal Ca<sup>2+</sup> activations following the protocol above. Hyperbolic force-velocity curves were fitted to the relative force-velocity data using the Hill equation: (P+a)(V+b)=(P<sub>0</sub>+a)b, where P is force during shortening at velocity V, P<sub>0</sub> is the isometric force, and a and b are constants with dimensions of force and velocity, respectively. Power load curves were obtained by multiplying force by velocity at each load on the force-velocity curve.

### Rate of Force Redevelopment

The kinetics of force development were obtained using a procedure similar to that previously described.<sup>19</sup> While in activating solution (either maximal or half-maximal Ca<sup>2+</sup>), the myocytes were slackened by ≈15% of myocyte length to produce zero force and subsequently undergo a brief period of unloaded shortening. The myocyte was then rapidly restretched to its initial preslack length. The slack-restretch maneuver caused nearly complete dissociation of crossbridges, and the subsequent tension redevelopment is due to reattachment of crossbridges and their transition into force-generating states. Force redevelopment traces were fit by a single exponential function: F=F<sub>max</sub>[1-exp(-k<sub>tr</sub>t)]+F<sub>res</sub>, where F is tension at time t, F<sub>max</sub> is maximal tension, and k<sub>tr</sub> is the rate constant of tension redevelopment. F<sub>res</sub> represents any residual tension present immediately after the slack-restretch maneuver.

### SDS-PAGE and Autoradiography

To determine the effect that deletion of MyBP-C had on baseline phosphorylation of cardiac troponin I, myofibrillar samples were incubated for 30 minutes with the catalytic subunit of PKA (3 to 5 μg/mL, Sigma) in the presence of (γ-<sup>32</sup>P)-ATP (50 μCi, NEN). The reaction was terminated on the addition of electrophoresis sample buffer and heating at 95°C for 3 minutes. Equivalent protein loads were then separated by SDS-PAGE, silver stained, dried overnight, and exposed to a Kodak phosphorimaging screen for visualization on a phosphorimager. Individual radiolabeled protein bands were then excised from the gel and quantified using a scintillation counter (Packard 1900 TR). Stoichiometric cTnI phosphate incorporation was calculated using the following



**Figure 1.** Silver stain (A) and autoradiogram (B) of cardiac myofibrils from MyBP-C<sup>-/-</sup> (lanes 1 to 5) and WT (lanes 6 to 10) mice. Note the lack of phosphorylation of MyBP-C in MyBP-C<sup>-/-</sup> myofibrils. Also, the absence of MyBP-C did not appear to alter cTnI phosphorylation levels. PKA-induced phosphorylation of cTnI was similar between MyBP-C<sup>-/-</sup> ( $0.97 \pm 0.17$  mol P<sub>i</sub>/mol cTnI) and WT ( $0.96 \pm 0.14$  mol P<sub>i</sub>/mol cTnI) cardiac myofibrils.

equation:  $\text{cpm cTnI}/[\text{TnI}] \cdot [\text{P}_i]/\text{cpm total} = [\text{P}_i]/[\text{cTnI}]$ , where all concentrations are in moles and cpm is counts per minute. Figure 1 shows that MyBP-C and cTnI were phosphorylated by PKA in WT, whereas only cTnI was phosphorylated in myofibrils from MyBP-C<sup>-/-</sup> mice. Additionally, stoichiometric analysis indicated that PKA-induced phosphorylation of cTnI was similar between WT ( $0.96 \pm 0.14$  mol P<sub>i</sub>/mol cTnI) and MyBP-C<sup>-/-</sup> ( $0.97 \pm 0.17$  mol P<sub>i</sub>/mol cTnI) cardiac myofibrils.

### Data and Statistical Analysis

Skinned myocyte preparation length traces, force-velocity curves, power-load curves, and rate constants of force redevelopment were analyzed as previously described.<sup>19,20</sup> Student *t* tests were performed to determine significant differences between WT and MyBP-C<sup>-/-</sup> as well as between maximal Ca<sup>2+</sup> and half-maximal Ca<sup>2+</sup> measurements. A value of  $P < 0.05$  was chosen as indicating significance. All values are expressed as mean  $\pm$  SD.

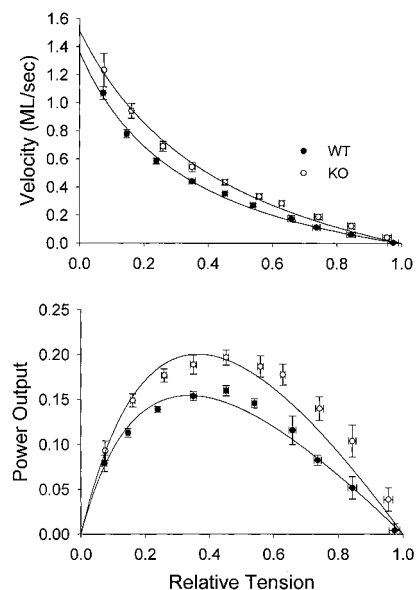
An expanded Materials and Methods section can be found in the online data supplement at <http://www.circresaha.org>.

### Results

Force-velocity and power-load curves were measured in MyBP-C<sup>-/-</sup> myocytes to assess the role of MyBP-C on work capacity of cardiac myocytes. Maximal Ca<sup>2+</sup>-activated force was unaffected by the absence of MyBP-C (WT,  $17.0 \pm 7.2$  kN m<sup>-2</sup> versus MyBP-C<sup>-/-</sup>,  $20.5 \pm 4.5$  kN m<sup>-2</sup>). On the other hand, force-velocity relationships were markedly altered in myocytes lacking MyBP-C. Deletion of MyBP-C shifted the force-velocity relationship upwards such that shortening velocity was faster at nearly all relative loads (Figure 2). The bottom panel of Figure 2 shows that power output (normalized to isometric force, ie, normalized power output) was also greater at nearly all relative loads in MyBP-C<sup>-/-</sup> myocytes, with peak normalized power output significantly increased by 26% (WT,  $0.15 \pm 0.01$  versus MyBP-C<sup>-/-</sup>,  $0.19 \pm 0.03$  P/P<sub>0</sub> · ML/sec;  $P < 0.05$ ) (Table 2). The mean shortening velocity at loads optimal for power output ( $V_{\text{opt}}$ ) was 25% faster in MyBP-C<sup>-/-</sup> myocytes (WT,  $0.45 \pm 0.03$  ML/s versus MyBP-C<sup>-/-</sup>,  $0.56 \pm 0.10$  ML/s;  $P < 0.05$ ) (Table 2). Overall, these results suggest that the absence of MyBP-C increased power output of single myocytes solely by increasing loaded

shortening rates (rather than an increase in force generating capacity), which likely resulted from faster crossbridge cycling rates at each given load.

Because Ca<sup>2+</sup> activation of cardiac myofilaments does not likely reach a maximum in vivo, we also examined the effects of MyBP-C ablation on force, velocity, and power output during half-maximal Ca<sup>2+</sup> activations. These effects are shown in Figure 3 and summarized in Table 3. The pCa solution that yielded half-maximal force was  $5.77 \pm 0.08$  in wild-type and  $5.67 \pm 0.08$  in MyBP-C<sup>-/-</sup>, indicating a slight reduction in Ca<sup>2+</sup> sensitivity to force in MyBP-C<sup>-/-</sup> myocytes, although this shift did not reach significance ( $P = 0.06$ ). Half-maximal Ca<sup>2+</sup> activation also yielded force-velocity and



**Figure 2.** Force-velocity (top) and power-load (bottom) curves from WT and MyBP-C<sup>-/-</sup> skinned cardiac myocytes during maximal Ca<sup>2+</sup> activations. Loaded shortening and power output were significantly increased at intermediate loads in MyBP-C<sup>-/-</sup> myocytes during maximal Ca<sup>2+</sup> activation. Data points are mean  $\pm$  SEM.

**TABLE 2. Maximal Calcium-Activated Force, Velocity, and Peak Power Output in Wild-Type and MyBP-C<sup>-/-</sup> Mouse Skinned Cardiac Myocytes**

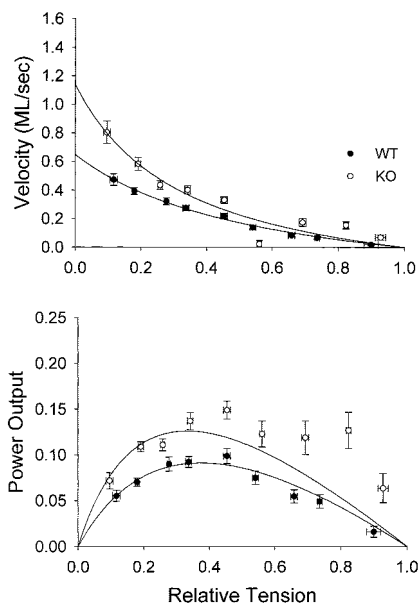
	Wild-Type	MyBP-C <sup>-/-</sup>
Maximum force, kN×m <sup>-2</sup>	17.03±7.22	20.48±4.45
V <sub>max</sub> , ML×s <sup>-1</sup>	1.29±0.19	1.60±0.36
F <sub>opt</sub>	0.348±0.028	0.351±0.026
V <sub>opt</sub> , ML×s <sup>-1</sup>	0.446±0.033	0.559±0.103*
Peak normalized power output, P/P <sub>0</sub> ×ML×s <sup>-1</sup>	0.154±0.011	0.194±0.032*

Values are mean±SD, n=6 for each. \*Significant difference from wild-type; P<0.05.

F<sub>opt</sub> indicates relative force at which power was optimal.

power-load curves that were shifted to higher velocities and loads. Interestingly, this upward shift was greater than that seen during maximal Ca<sup>2+</sup> activation. Deletion of MyBP-C increased loaded shortening and power output at all relative loads, and peak normalized power output was increased by 46% (WT, 0.09±0.03 versus MyBP-C<sup>-/-</sup>, 0.14±0.02 P/P<sub>0</sub> · ML/sec) as compared with 26% during maximal Ca<sup>2+</sup> activation. Likewise, V<sub>opt</sub> was 42% faster (WT, 0.25±0.05 versus MyBP-C<sup>-/-</sup>, 0.36±0.10) at half-maximal Ca<sup>2+</sup> activation, compared with 25% during maximal Ca<sup>2+</sup> activation. Overall, these results indicate that removal of MyBP-C results in faster loaded crossbridge cycling and that this effect is relatively greater at half-maximal Ca<sup>2+</sup> activation than maximal Ca<sup>2+</sup> activation.

To further examine the effects of MyBP-C ablation on crossbridge turnover kinetics, the rate of force redevelopment was measured during maximal and half-maximal Ca<sup>2+</sup> activations. Results are summarized in Figure 4. Deletion of MyBP-C did not alter the rate constant of force redevelopment (k<sub>tr</sub>) during

**Figure 3.** Force-velocity (top) and power-load (bottom) curves from WT and MyBP-C<sup>-/-</sup> skinned cardiac myocytes during half-maximal Ca<sup>2+</sup> activations. Loaded shortening and power output were significantly increased in MyBP-C<sup>-/-</sup> myocytes at all loads. Data points are mean±SEM.**TABLE 3. Half-Maximal Calcium-Activated Force, Velocity, and Peak Power Output in Wild-Type and MyBP-C<sup>-/-</sup> Mouse Skinned Cardiac Myocytes**

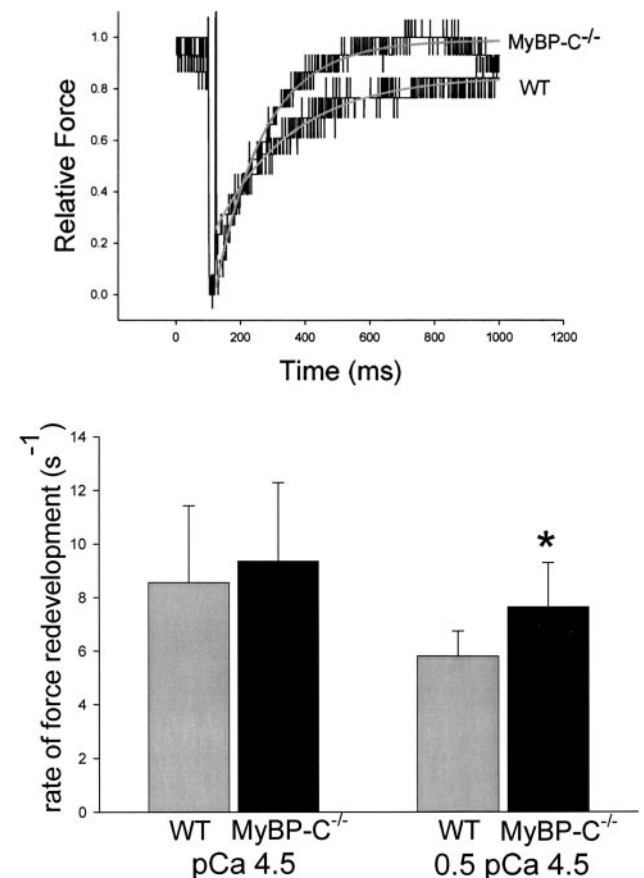
	Wild-Type	MyBP-C <sup>-/-</sup>
Maximum force, kN×m <sup>-2</sup>	10.82±6.32	10.51±1.53
V <sub>max</sub> , ML×s <sup>-1</sup>	0.687±0.224	0.833±0.252
F <sub>opt</sub>	0.377±0.059	0.388±0.057
V <sub>opt</sub> , ML×s <sup>-1</sup>	0.251±0.049	0.356±0.098*
Peak normalized power output, P/P <sub>0</sub> ×ML×s <sup>-1</sup>	0.093±0.017	0.135±0.031*

Values are mean±SD n=6 for each. \*Significant difference from wild-type; P<0.05.

maximal Ca<sup>2+</sup> activations. However, k<sub>tr</sub> at half-maximal Ca<sup>2+</sup> activation was significantly greater in MyBP-C<sup>-/-</sup> myocytes compared with WT (Figure 4, top).

## Discussion

We examined the effects of targeted MyBP-C ablation on the functional properties of mouse skinned cardiac myocytes. Deletion of MyBP-C resulted in significantly in-

**Figure 4.** Summary of rates of force redevelopment in WT and MyBP-C<sup>-/-</sup> skinned cardiac myocytes during maximal (left) and half-maximal (right) Ca<sup>2+</sup> activations. Top, Force traces after a slack-restretch maneuver in WT and MyBP-C<sup>-/-</sup> myocytes. Rate of force redevelopment was significantly increased in MyBP-C<sup>-/-</sup> myocytes at half-maximal Ca<sup>2+</sup> activations compared with WT. \*P<0.05 for MyBP-C<sup>-/-</sup> half-maximal Ca<sup>2+</sup> activation vs WT half-maximal Ca<sup>2+</sup> activation.



creased loaded shortening and power output during both maximal and half-maximal  $\text{Ca}^{2+}$  activation, as well as yielded faster rates of force redevelopment at half-maximal  $\text{Ca}^{2+}$  activation. These increases in contractile function imply faster crossbridge cycling rates under load in myocytes lacking MyBP-C.

### Function of MyBP-C on Myocyte Contraction

Various functions have been suggested for MyBP-C, including a role in the assembly and structural support of the thick filament, and serving as a regulator of crossbridge movement. Studies of the binding of MyBP-C to myosin have revealed MyBP-C binding sites on both light meromyosin (LMM) and the S2 subfragment of myosin, but not on the S1 subfragment.<sup>21–23</sup> The strong affinity of MyBP-C for LMM suggests a structural role for MyBP-C in the assembly and stabilization of the thick filament; however, MyBP-C does not appear to be necessary *in vivo* for assembly of highly organized myofibrils, which were observed in MyBP-C<sup>−/−</sup> mice.<sup>15</sup> MyBP-C also binds actin and regulated thin filaments in solution, and the latter is  $\text{Ca}^{2+}$  dependent.<sup>22,24</sup> Because the length of the MyBP-C molecule is sufficient to simultaneously contact both the thick and thin filaments in the intact filament lattice, an interaction between MyBP-C and actin in myofibrils is possible. In fact, MyBP-C has been observed to alter actin-activated myosin ATPase in solution,<sup>25</sup> an effect that varies depending on the ionic strength and molar ratio of actin and myosin, suggesting MyBP-C may facilitate positioning myosin and actin for interaction.

The binding of MyBP-C to S-2 and actin suggests that MyBP-C is involved in regulating the contractile process and hypotheses regarding its possible regulatory function have been investigated using both cardiac myocytes and skeletal muscle fibers. Along these lines, the incorporation of soluble S2 myosin into intact cardiac myocytes,<sup>10</sup> the addition of the N-terminal MyBP-C motif into skinned skeletal muscle fibers,<sup>11</sup> and knock-in expression of a shortened N-terminal domain of cMyBP-C<sup>12</sup> have all been shown to increase  $\text{Ca}^{2+}$  sensitivity of force. These results are consistent with the idea that the N-terminal domain of endogenous cMyBP-C constrains the flexibility and position of the myosin head via its interaction with S2. Also consistent with this idea is the finding that extraction of 60% to 70% of endogenous MyBP-C in skinned cardiac myocytes and skeletal muscle fibers resulted in reversible increases in isometric tension at submaximal  $\text{Ca}^{2+}$  concentrations and a decrease in the apparent cooperativity of  $\text{Ca}^{2+}$ -dependent force development.<sup>13</sup> Based on these results, a model was proposed in which MyBP-C modulates the range of movement of myosin crossbridges, perhaps by tethering the crossbridges to the thick filament backbone, so that following MyBP-C extraction crossbridges are less constrained and the probability of myosin binding to actin is increased.<sup>13</sup> This model is consistent with a more recently proposed model in which three MyBP-C molecules interact to form a collar around the backbone of the thick filament, with projections from this collar able to interact with S2.<sup>5</sup>

MyBP-C was also shown to modify the apparent kinetics of crossbridge interaction during muscle shortening,<sup>14</sup> in that partial extraction of MyBP-C sped maximum shortening velocity ( $V_o$ ) of fast skeletal muscle fibers at low levels of  $\text{Ca}^{2+}$  activation. In control fibers at low levels of activation, the time course of unloaded shortening is normally biphasic, ie, an initial phase of high-velocity shortening is followed by low-velocity shortening. The sudden slowing of  $V_o$  as shortening proceeds is thought to result from an activation-dependent internal load that arises once there is a given amount of active shortening.<sup>26</sup> Partial extraction of MyBP-C reversibly increased  $V_o$  in the low velocity phase of unloaded shortening but had no effect on the high-velocity phase. One interpretation of this result is that at low levels of activation, MyBP-C gives rise to an internal load by simultaneously binding actin and myosin. An alternative hypothesis is consistent with the model mentioned earlier (as proposed by Hofmann et al<sup>13,14</sup>), whereby MyBP-C tethers the myosin head to the thick filament backbone, such that at low levels of  $\text{Ca}^{2+}$  when the rate of crossbridge detachment is slow, crossbridges give rise to an internal load once their useful working stroke is completed. A similar hypothesis could arise from the trimeric collar model proposed by Moolman-Smook et al<sup>5</sup> in which the MyBP-C C-terminal collar restricts crossbridge formation while the N-terminal end is free to interact with S-2 and/or actin. A prediction from these models is that both loaded shortening velocity and force redevelopment rates will be increased if extraction of MyBP-C is complete, and thus the tether point or collar is removed. This idea was addressed in this study using cardiac myocytes from MyBP-C-null mice and, indeed, both loaded shortening and force development rates were increased in MyBP-C<sup>−/−</sup> myocytes and these effects were greater during half-maximal  $\text{Ca}^{2+}$  activation. These results are consistent with the idea that MyBP-C constrains interactions of myosin heads with actin, as well as contributing to an internal load that tends to slow shortening velocity. These results also agree with the idea that phosphorylation of cMyBP-C relieves constraint of the myosin head<sup>27</sup> because phosphorylation of cMyBP-C by cAMP-dependent protein kinase A has been correlated with both faster force redevelopment in mouse skinned myocardium<sup>28</sup> and faster loaded shortening in rat skinned cardiac myocytes.<sup>29</sup> Interestingly, we saw significantly increased power output in single myocytes lacking MyBP-C despite the increased presence of a small amount of  $\beta$ -MyHC in MyBP-C<sup>−/−</sup> myocytes.<sup>15</sup>  $\beta$ -MyHC is known to result in decreased power output in rat skinned cardiac myocytes<sup>16</sup> even if the amount of  $\beta$ -MyHC is small.<sup>30</sup> Because the upregulation of  $\beta$ -MyHC would likely depress the work capacity of singly myocytes, the observed increase in power output may actually be an underestimation of what would be observed if MyBP-C ablation could be studied on a 100%  $\alpha$ -MyHC background.

There are, however, two predictions based on the above models that have not been borne out experimentally. First,  $\text{Ca}^{2+}$  sensitivity of force was predicted to increase because both competitive inhibition of S2 binding by MyBP-C<sup>11</sup>

and partial MyBP-C extraction<sup>13</sup> increased  $\text{Ca}^{2+}$  sensitivity. It is reasonable to predict that if removal of MyBP-C reduces S1 constraints, more crossbridges would be able to interact with the thin filaments, which in turn would cooperatively activate additional regulatory units and result in more force at a given  $[\text{Ca}^{2+}]$ . However, ablation of MyBP-C was found to decrease  $\text{Ca}^{2+}$  sensitivity<sup>15</sup> and our current results are in agreement with this finding because MyBP-C<sup>-/-</sup> myocytes required slightly higher  $\text{Ca}^{2+}$  concentration to reach a force value that was half-maximal, although this difference was not considered significant (see Results). This unexpected finding may be accounted for by the fact that previous experiments in which MyBP-C was chemically extracted resulted in only partial relief of the constraint of myosin heads by MyBP-C. As mentioned, previous studies that used N-terminal truncations,<sup>12</sup> C-terminal truncations,<sup>31,32</sup> MyBP-C extraction,<sup>13,14</sup> and addition of phosphorylatable MyBP-C motif to skeletal muscle,<sup>11</sup> all maintained at least some intact portions of MyBP-C, which may be involved in mediating mechanical interactions between myosin and actin. For example, Witt et al<sup>12</sup> produced an N-terminally shortened MyBP-C, which led to increased  $\text{Ca}^{2+}$  sensitivity. In that instance, the carboxy terminal end was unaffected and thus able to normally interact with the rod portion of myosin. Also, the increased  $\text{Ca}^{2+}$  sensitivity seen after MyBP-C extraction<sup>13</sup> occurred with many MyBP-C molecules still bound to S2, the myosin rod, and titin. Interestingly, Yang et al<sup>31,32</sup> developed two different mouse models with a C-terminal truncation that also exhibited increased  $\text{Ca}^{2+}$  sensitivity of force. Thus, it appears that both the N-terminal and C-terminal ends of MyBP-C exert separate regulatory effects and that complete deletion of MyBP-C yields an effect opposite to C-terminal and N-terminal deletions at least with regard to  $\text{Ca}^{2+}$  sensitivity of force. A second possibility to explain the discrepancy between the expected and actual results is that some studies involved acute modifications of MyBP-C content whereas others, including this one, involved chronic changes. For instance, our knockout model completely lacks MyBP-C from the onset of myocardial development. Thus, some type of chronic compensatory changes in the myofilaments may account for the unexpected decrease in  $\text{Ca}^{2+}$  sensitivity. However, the decreased  $\text{Ca}^{2+}$  sensitivity does not appear to be due to increased baseline phosphorylation of cardiac troponin I because stoichiometric analysis indicated no significant difference in PKA-induced phosphate incorporation between MyBP-C<sup>-/-</sup> and wild-type myofibrils (Figure 1). Another possible explanation for reduced  $\text{Ca}^{2+}$  sensitivity is that  $\text{Ca}^{2+}$  affinity of TnC is reduced in MyBP-C<sup>-/-</sup> myocardium due to changes in the normal feedback by which strongly bound crossbridges increase the binding affinity of TnC for  $\text{Ca}^{2+}$ .<sup>33</sup> For instance, the loss of the normal constraint of S1 by MyBP-C<sup>5</sup> would perhaps yield more flexible and compliant myosin crossbridges, which could result in less developed force per crossbridge. Reducing the force per crossbridge may adversely affect the normal allosteric changes induced by

crossbridges on the thin filament that tend to enhance  $\text{Ca}^{2+}$  and/or crossbridge binding.

Another apparent contradiction in the present results is the finding that loaded shortening, power output, and force development rates were increased in MyBP-C<sup>-/-</sup> myocytes, whereas MyBP-C<sup>-/-</sup> hearts demonstrate decreased contractile function in vivo. This does not appear to be due to presence of intercellular connections because control experiments yielded qualitatively similar differences between multicellular preparation from WT and MyBP-C<sup>-/-</sup> mice (data not shown). It is possible that temperature differences between in vitro and in vivo measurements or some type of compensatory changes in myofibrillar proteins may contribute to these differences. An alternative mechanism is compensated hemodynamic loads in MyBP-C<sup>-/-</sup> mice. For instance, when Calaghan et al<sup>10</sup> introduced exogenous S2 into rat ventricular myocytes to compete with endogenous MyBP-C, intact myocytes exhibited a 30% increase in contractility, but also an increase in the time to half-relaxation. This is consistent with the finding that isovolumic relaxation is prolonged in MyBP-C<sup>-/-</sup> mouse hearts.<sup>15</sup> Slowed relaxation rates would lead to reduced filling time and decrease the end diastolic volume, which would depress systolic performance in accordance with the Frank-Starling relationship. Prolonged relaxation is consistent with a model in which ablation of MyBP-C reduces structural constraint on myosin heads, allowing myosin heads to move closer on average to actin binding sites, thereby increasing the probability of crossbridge binding, delaying inactivation of thin filaments, and prolonging relaxation. Another possible explanation for prolonged relaxation in MyBP-C<sup>-/-</sup> hearts in vivo is that there are compensatory alterations in  $[\text{Ca}^{2+}]_i$  handling, but this remains to be investigated.

Overall, we found that complete removal of cMyBP-C led to faster loaded shortening and force development rates in mouse skinned cardiac myocytes. Our results are consistent with a model whereby MyBP-C acts to constrain actin-myosin interaction, which limits loaded shortening velocity and ultimately power output.

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