INTRODUCTION
Activation of the β-adrenergic signaling pathway rapidly enhances ventricular contraction to increase cardiac output in response to enhanced systemic demand, via protein kinase A (PKA) activation, which phosphorylates key myofilament proteins involved in the regulation of myocardial force generation (1, 2). Previous studies have demonstrated that cardiac myosin binding protein-C (MyBP-C) phosphorylation regulates myofilament function by accelerating the rates of cross-bridge (XB) cycling and force generation in response to PKA treatment (3, 4). Animal models expressing phospho-ablated MyBP-C display an abolished PKA-mediated enhancement of the magnitude and rate of cooperative XB recruitment and force generation in skinned myocardium (5, 6), which limits the magnitude and the acceleration of left ventricular pressure generation in response to an acute β-agonist infusion (6–8). Thus, increased MyBP-C phosphorylation appears to be a primary mechanism by which the heart activates its contractile reserve under conditions of increased cardiac stress (7).

Despite growing evidence demonstrating the importance of MyBP-C phosphorylation in regulating in vivo cardiac contractile function, the respective roles of individual MyBP-C phosphorylation residues are not yet clear. In addition to PKA, which targets four M-domain MyBP-C residues (Ser273, Ser282, Ser302, and Ser307 mouse MyBP-C numbering) (9–13), individual MyBP-C residues are also selectively targeted by non-PKA kinases (9, 14). Previous studies have demonstrated a role for Ser282 phosphorylation in accelerating XB recruitment and force generation (15, 16), which contributes to the in vivo acceleration of systolic pressure development following β-adrenergic stimulation (16, 17). However, significant acceleration of in vivo pressure development was observed in Ser302 phospho-ablated mice following infusion of the β-agonist dobutamine, whereas a complete MyBP-C phosphorylation ablation (that is, Ser273, Ser282, and Ser307) abolished the acceleration in the rate of pressure development (6, 7, 16), demonstrating that phosphorylation of other PKA-targeted MyBP-C residues (Ser273 or Ser302) must be critical for modulation of the contractile response to increased β-adrenergic stimulation.

In addition to PKA, previous studies have shown that Ser302 can be phosphorylated in vitro by several kinases, which are known to modulate cardiac function, such as PKC-ε (18), PKD (19), and glycogen synthase kinase-3β (20). Furthermore, previous studies have suggested that increased Ca2+-dependent protein kinase II (CaMKII) activity due to increased cardiac pacing frequency enhances cardiac contractility, in part, due to increased Ser282 phosphorylation (21). We have previously demonstrated that Ser302 phosphorylation was lower than Ser273 or Ser282 phosphorylation at baseline and was increased by PKA treatment to a greater extent than Ser273 and Ser282 phosphorylation (7, 16). Thus, our hypothesis is that among MyBP-C phosphorylation residues, Ser302 phosphorylation may be the principal MyBP-C residue, which modulates the inotropic contractile response to enhanced β-adrenergic activation, and that preventing Ser302 phosphorylation impairs β-agonist–mediated enhancements in contractile response and reduces cardiac contractile reserve. However, to date, no study has examined the specific contribution of Ser302 phosphorylation to in vivo contractile function. Therefore, to elucidate the precise roles of Ser302 phosphorylation in modulating myofilament and in vivo contractile function, we generated a novel transgenic (TG) mouse model expressing MyBP-C with a nonphosphorylatable Ser302 (that is, Ser302 to Ala302; TG302A) and quantified the in vitro contractile response of skinned myocardium isolated from TG302A hearts to PKA treatment and in vivo contractile function of TG302A mice in response to β-agonist infusion.

### Cardiac myosin binding protein-C Ser302 phosphorylation regulates cardiac β-adrenergic reserve

**Ranganath Mamidi,**1* Kenneth S. Gresham,2* Jiayang Li,1 Julian E. Stelzer†

Phosphorylation of cardiac myosin binding protein-C (MyBP-C) modulates cardiac contractile function; however, the specific roles of individual serines (Ser) within the M-domain that are targets for β-adrenergic signaling are not known. Recently, we demonstrated that significant accelerations in in vivo pressure development following β-agonist infusion can occur in transgenic (TG) mouse hearts expressing phospho-ablated Ser282 (that is, TG3282A), but not in hearts expressing phospho-ablation of all three serines (that is, Ser273, Ser282, and Ser302 (TG332A)), suggesting an important modulatory role for other Ser residues. In this regard, there is evidence that Ser302 phosphorylation may be a key contributor to the β-agonist–induced positive inotropic responses in the myocardium, but its precise functional role has not been established. Thus, to determine the in vivo and in vitro functional roles of Ser302 phosphorylation, we generated TG mice expressing nonphosphorylatable Ser302 (that is, TG3302A). Left ventricular pressure-volume measurements revealed that TG3302A mice displayed no accelerations in the rate of systolic pressure rise and an inability to maintain systolic pressure following dobutamine infusion similar to TG3302A mice, implicating Ser302 phosphorylation as a critical regulator of enhanced systolic performance during β-adrenergic stress. Dynamic strain–induced cross-bridge (XB) measurements in skinned myocardium isolated from TG3302A hearts showed that the molecular basis for impaired β-adrenergic–mediated enhancements in systolic function is due to the absence of protein kinase A–mediated accelerations in the rate of cooperative XB recruitment. These results demonstrate that Ser302 phosphorylation regulates cardiac contractile reserve by enhancing contractile responses during β-adrenergic stress.
RESULTS

Sarcomeric protein expression and phosphorylation

To examine the role of MyBP-C Ser302 phosphorylation in regulating β-adrenergic enhancement of ventricular contractile function and relaxation, we generated a novel TG mouse line in which Ser302 was mutated to an Ala residue to abolish phosphorylation (Fig. 1A). Expression of TG MyBP-C in TG302A myocardial samples was confirmed by Western blot analysis (Fig. 1B) and was detected as a full-length MyBP-C protein band at a molecular mass of ~150 kDa. Ser273 and Ser282 phosphorylation was detected in TG302A samples and was similar to non-TG (NTG) phosphorylation (TG302A Ser273 phosphorylation was 106 ± 6% and Ser282 phosphorylation was 104 ± 14% of NTG phosphorylation; n = 5), but Ser302 phosphorylation was absent, confirming Ser302 phospho-ablation in TG302A mice (Fig. 1B). TG MyBP-C expression in TG302A mice was determined to be 79 ± 15% of NTG MyBP-C expression levels, similar to expression levels of MyBP-C TG mice in previous studies (6, 16). MyBP-C phosphorylation at Ser273, Ser282, and Ser302 was not detected in TG3SA mice, as previously reported (Fig. 1B) (6, 7). The percentage of β-myosin heavy chain (MHC) expression in myofibrillar isolated from TG302A (3.6 ± 1.5%) was found to be similar to NTG myocardium (2.2 ± 1.2%), whereas β-MHC levels were slightly elevated in TG3SA myocardium (8.3 ± 1.7%; P < 0.05) (Fig. 1C). The expression and phosphorylation of other sarcomeric proteins, including troponin I (TnI), TnT, and regulatory light chain (RLC), were similar among all three groups (Fig. 1, D and E).

Following PKA treatment, Ser273 and Ser282 phosphorylation levels were similar between TG302A and NTG samples (TG302A Ser273 phosphorylation was 97 ± 10% and Ser282 phosphorylation was 95 ± 15% of PKA-treated NTG phosphorylation; n = 3 to 5), demonstrating normal PKA-mediated phosphorylation of these sites in the absence of Ser302 phosphorylation (Fig. 1B). Ser302 phosphorylation was not detected in TG302A samples following PKA treatment, confirming Ser302 phospho-ablation. TnI and TnT phosphorylation levels were similar between TG302A and NTG samples following PKA treatment. Phosphorylation of RLC was unaffected by PKA treatment and was similar between TG302A and NTG samples under all conditions. Ser273, Ser282, and Ser302 phosphorylation was not detected in TG3SA samples following PKA treatment. These results demonstrate that TG expression of S302A MyBP-C does not disrupt phosphorylation of other MyBP-C Ser residues or phosphorylation of other sarcomeric proteins.

Assessment of cardiac morphology

Representative formalin-fixed hearts and cross sections for NTG, TG3SA, and TG302A hearts are presented in Fig. 2A. TG302A hearts exhibited similar overall size and morphology with no noticeable

Fig. 1. Determination of expression, PKA-mediated phosphorylation of MyBP-C, and other sarcomeric proteins. (A) MyBP-C is composed of eight immunoglobulin (ovals) and three fibronectin III (rectangles) domains labeled C0 to C10 (N to C terminus). The conserved M-domain in the linker between domains C1 and C2 contains three serines (S273, S282, S302; mouse numbering) in the NTG sequence that are targets for PKA phosphorylation. The substitution used to prevent S302 phosphorylation in TGS302A is shown in red. (B) Representative Western blot showing S273, S282, and S302 phosphorylation, before and after PKA incubation. No Ser302 expression was detected in TGS302A samples, whereas fully phosphorylatable Ser273 and Ser282 were observed. (C) Representative 5% tris-HCl gel, stained with silver stain, showing MHC isoform expression in the myocardial samples. (D) Representative gels shown are stained by Pro-Q (left) for protein phosphorylation, and the same gel is shown for total protein (right) stained with Coomassie Blue. cMyBP-C, cardiac myosin binding protein-C; pMyBP-C, phosphorylated cardiac myosin binding protein-C. (E) Relative protein phosphorylation (phosphorylated signal/total protein signal) was calculated for each protein and is expressed as % of PKA-treated NTG values for that protein. Values are expressed as means ± SEM, from three to six hearts in each group. *P < 0.05, different from non-PKA-treated samples from the same line; †P < 0.05, different from the corresponding NTG group.
alterations in chamber geometry or increased cardiac fibrosis (Fig. 2B) when compared to control NTG hearts. In contrast to the cardiac hypertrophy produced by complete MyBP-C phospho-ablation (Fig. 2 and Table 1), left ventricle (LV) mass and wall thickness were similar between TGS302A and NTG mice as measured by echocardiography. These results demonstrate that Ser302 phospho-ablation does not result in pathological cardiac hypertrophy.

**Basal cardiac function**

Cardiac contractile and hemodynamic function was assessed by echocardiography and pressure-volume (P-V) loop analysis to determine the functional effects of Ser302 phospho-ablation. No changes in systolic function or diastolic function as evidenced by measurements of ejection fraction (EF) or isovolumic relaxation time (IVRT) were detected in TGS302A mice compared to NTG controls (Table 1). Similarly, no differences were detected in the rate of pressure development (dP/dt\(_{\text{max}}\)) or relaxation (\(t_{\text{a base}}\)) at baseline between TG\(^{302A}\) mice and NTG controls (Table 2). In contrast, complete MyBP-C phospho-ablation in TG\(^{35A}\) hearts resulted in slowed IVRT and reduced EF at baseline (Table 1), consistent with previous studies (6, 7, 22).

**β-Adrenergic reserve**

The contribution of Ser\(^{302}\) phosphorylation to the contractile response to β-adrenergic stimulation was assessed in TG\(^{302A}\) mice following dobutamine infusion. The postdobutamine dP/dt\(_{\text{max}}\) in TG\(^{302A}\) mice was slower than in dobutamine-treated NTG mice (\(P < 0.005\); Table 2 and Fig. 3A), and the rate of pressure development throughout early systole in dobutamine-treated TG\(^{302A}\) mice was lower when compared to the rate of pressure development observed in dobutamine-treated NTG mice (Fig. 3B), demonstrating that Ser\(^{302}\) phosphorylation is required to accelerate pressure development following β-adrenergic stimulation. The acceleration in pressure development in NTG mice following dobutamine increased early systolic developed pressure levels compared to baseline (Fig. 3), but the impaired acceleration in pressure development

---

**Table 1. LV morphology and in vivo cardiac performance measured by echocardiography.**

<table>
<thead>
<tr>
<th></th>
<th>NTG</th>
<th>TG(^{35A})</th>
<th>TG(^{302A})</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>27.5 ± 0.5</td>
<td>26.2 ± 1.3</td>
<td>26.9 ± 0.6</td>
</tr>
<tr>
<td>LV mass/BW</td>
<td>3.8 ± 0.2</td>
<td>5.8 ± 0.4*</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>418 ± 8</td>
<td>431 ± 13</td>
<td>425 ± 10</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>0.88 ± 0.01</td>
<td>1.13 ± 0.03*</td>
<td>0.91 ± 0.02*</td>
</tr>
<tr>
<td>PWs (mm)</td>
<td>1.18 ± 0.03</td>
<td>1.39 ± 0.1*</td>
<td>1.23 ± 0.02*</td>
</tr>
<tr>
<td>IVRT (ms)</td>
<td>18.1 ± 1.5</td>
<td>28.5 ± 2.1*</td>
<td>19.4 ± 1.4*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>75.1 ± 2.6</td>
<td>62.0 ± 2.2*</td>
<td>73.1 ± 2.2*</td>
</tr>
</tbody>
</table>

*\(P < 0.05\), different compared to NTG.
in TG<sup>S302A</sup> mice prevented an increase in developed pressure, resulting in developed pressure levels that were similar to NTG mice before dobutamine treatment (Fig. 3). Additionally, maximal systolic pressure was lower in TG<sup>S302A</sup> mice following dobutamine treatment compared to NTG controls (P < 0.005; Table 2). The impaired acceleration in pressure development and decreased maximal systolic pressure observed in TG<sup>S302A</sup> mice were also observed in TG<sup>3SA</sup> mice, consistent with our previous study (7). There were no differences in the rate or magnitude of pressure development following dobutamine treatment between TG<sup>3SA</sup> and TG<sup>S302A</sup> mice (Table 2), suggesting that phosphorylation of Ser<sup>273</sup> and Ser<sup>282</sup> cannot compensate for the loss of Ser<sup>302</sup> phosphorylation following β-adrenergic stimulation. Similar to previous reports of individual MyBP-C Ser residue phospho-ablation (16), Ser<sup>302</sup> phospho-ablation did not disrupt normal acceleration of diastolic pressure relaxation because dobutamine accelerated relaxation to a similar extent in TG<sup>S302A</sup> and NTG mice (Table 2), in contrast to TG<sup>3SA</sup> mice, which displayed slowed relaxation following dobutamine treatment.

### Basal myofilament contractile function

The effects of Ser<sup>302</sup> phospho-ablation on myofilament contractile properties were assessed in skinned myocardium isolated from TGS302A hearts (Fig. 4, A to D). Stretch activation experiments were performed to measure dynamic strain-induced XB behavior. Figure 4 illustrates a

![Fig. 3. Analysis of the β-adrenergic acceleration in pressure development.](http://advances.sciencemag.org/)

**Table 2.** Left ventricular hemodynamic function measured by P-V loop analysis. HR, heart rate; P<sub>max</sub>, maximal systolic pressure; EDP, end diastolic pressure; dP/dt<sub>max</sub>, maximum rate of pressure development; τ, time constant of pressure relaxation; DOB, dobutamine. Values are expressed as means ± SEM. n = 9 for NTG, 8 for TG<sup>3SA</sup>, and 10 for TG<sup>S302A</sup>.

<table>
<thead>
<tr>
<th>Group</th>
<th>HR (beats/min)</th>
<th>P&lt;sub&gt;max&lt;/sub&gt; (mmHg)</th>
<th>EDP (mmHg)</th>
<th>dP/dt&lt;sub&gt;max&lt;/sub&gt; (mmHg/s)</th>
<th>τ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>− DOB NTG</td>
<td>456 ± 11</td>
<td>95.3 ± 3.7</td>
<td>6.1 ± 0.9</td>
<td>7,487 ± 512</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>TG&lt;sup&gt;3SA&lt;/sup&gt;</td>
<td>451 ± 11</td>
<td>87.7 ± 3.1</td>
<td>6.2 ± 1.1</td>
<td>7,216 ± 290</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>TG&lt;sup&gt;S302A&lt;/sup&gt;</td>
<td>470 ± 5</td>
<td>92.9 ± 3.4</td>
<td>6.4 ± 0.6</td>
<td>6,690 ± 401</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td>+ DOB NTG</td>
<td>537 ± 7</td>
<td>85.0 ± 2.6</td>
<td>4.8 ± 0.6</td>
<td>13,962 ± 919</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>TG&lt;sup&gt;3SA&lt;/sup&gt;</td>
<td>531 ± 9</td>
<td>87.0 ± 1.9</td>
<td>3.7 ± 0.2</td>
<td>8,725 ± 337</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>TG&lt;sup&gt;S302A&lt;/sup&gt;</td>
<td>553 ± 6</td>
<td>96.9 ± 3.7</td>
<td>6.3 ± 0.6</td>
<td>7,141 ± 652</td>
<td>8.8 ± 0.6</td>
</tr>
</tbody>
</table>

*P < 0.05, different versus the corresponding baseline group (without dobutamine). †P < 0.05, different versus the corresponding NTG group.

---

typical stretch activation response in skinned myocardium isolated from NTG, TG\textsuperscript{3SA}, and TG\textsuperscript{S302A} hearts and highlights the parameters assessed in the resultant force transients following the imposed stretch. Under basal conditions, the rate of delayed force development following the imposed stretch (that is, \(k_{df}\)) and the magnitude of XB recruitment (that is, \(P_{df}\)) were not different between the NTG and TG\textsuperscript{S302A} groups, whereas TG\textsuperscript{3SA} myocardium displayed diminished \(P_{df}\) (Table 3). However, the rate of force decay (\(k_{rel}\)) in TG\textsuperscript{S302A} myocardium was slower than that in NTG myocardium (\(P = 0.01\)) and was not different from TG\textsuperscript{3SA} skinned myocardium (Table 3). Additionally, \(P_{2}\) values in TG\textsuperscript{S302A} skinned myocardium were higher than those in NTG myocardium (\(P = 0.03\)) and were similar to TG\textsuperscript{3SA} values, indicating that the magnitude of strain-induced XB detachment was lower in the TG\textsuperscript{3SA} and TG\textsuperscript{S302A} groups. No differences in Ca\textsuperscript{2+}-activated maximal force (\(F_{\text{max}}\)), Ca\textsuperscript{2+}-independent force (\(F_{\text{min}}\)), Ca\textsuperscript{2+} sensitivity of force development (pCa\textsubscript{50}), or cooperativity of force development (\(n_{H}\)) were observed between the NTG, TG\textsuperscript{3SA}, and TG\textsuperscript{S302A} groups (Table 4), indicating that differences in dynamic XB behavior were not due to changes in Ca\textsuperscript{2+}-mediated thin filament activation.

Myofilament contractile function following PKA treatment

Following PKA treatment, both TG\textsuperscript{S302A} and TG\textsuperscript{3SA} skinned myocardium displayed slower rates of stretch-induced delayed force development (\(k_{df}\)) compared to NTG myocardium (Table 3 and Fig. 4D). PKA treatment accelerated \(k_{df}\) by \(\sim 65 \pm 17\% (P < 0.005)\) in NTG myocardium but had no effect on \(k_{df}\) in TG\textsuperscript{S302A} or TG\textsuperscript{3SA} myocardium, demonstrating that Ser\textsuperscript{302} phospho-ablation impairs PKA-mediated accelerations in the rate of XB recruitment to the same extent as complete MyBP-C phospho-ablation. Following PKA incubation, \(P_{3}\) was increased by \(\sim 43 \pm 9\% (P < 0.005)\) and \(\sim 25 \pm 6\% (P = 0.005)\) in the NTG and TG\textsuperscript{S302A} groups but not in the TG\textsuperscript{3SA} group (Fig. 5A). TG\textsuperscript{S302A} skinned myocardium also displayed an impaired magnitude of XB recruitment (\(P_{df}\)) following PKA treatment compared to NTG myocardium (Table 3). Whereas \(P_{df}\) was increased by PKA incubation by \(\sim 84 \pm 14\% (P < 0.005)\), \(\sim 61 \pm 12\% (P < 0.005)\), and \(\sim 61 \pm 9\% (P < 0.005)\) in the NTG, TG\textsuperscript{3SA}, and TG\textsuperscript{S302A} groups (Fig. 5B), respectively (Table 3), \(P_{df}\) was higher in the NTG group compared to the TGS\textsuperscript{302A} group (\(P < 0.005\)), demonstrating that Ser\textsuperscript{302} phospho-ablation blunts the PKA-mediated enhancement in the overall magnitude of XB recruitment.

In addition to enhancing the rate of delayed force development, PKA treatment also accelerated the rate and magnitude of force decay following stretch. In NTG myocardium, PKA treatment accelerated the rate of force decay (\(\sim 80 \pm 13\% \text{ increase in } k_{rel}; P < 0.005\)) and produced a greater magnitude of force decay (that is, more negative \(P_{2}\) values). However, the PKA-mediated acceleration in force decay was...
blunted in TGS302A myocardium (~45 ± 8% increase in $k_{\text{rel}}$; $P < 0.005$) and resulted in $k_{\text{rel}}$ values similar to pre-PKA $k_{\text{rel}}$ values in NTG skinned myocardium (Table 3), suggesting that Ser302 phosphorylation is required for complete acceleration of the rate of XB detachment following PKA treatment. PKA incubation also decreased $P_2$ values in the TGS302A group but to a lesser extent than in the NTG group (Table 3). PKA treatment did not accelerate $k_{\text{rel}}$ (Fig. 5C) or decrease $P_2$ amplitude in TG3SA myocardium when compared to the NTG group, as previously reported (5).

PKA treatment similarly decreased the pCa50 in all the groups, and no differences were observed in $F_{\text{max}}$, $F_{\text{min}}$, and $n_\text{H}$ between groups before or following PKA treatment (Table 4). Therefore, our data indicate that Ser302 phospho-ablation does not affect the Ca$^{2+}$-sensitivity of force generation and that the impaired acceleration in force decay and delayed force development in TG302A skinned myocardium are due to a diminished PKA-mediated enhancement of XB kinetics in the absence of Ser302 phosphorylation.

### DISCUSSION

Although augmented ventricular function during β-adrenergic stimulation requires MyBP-C phosphorylation, it is unclear how Ser302 phosphorylation contributes to enhanced myofilament and whole-heart contractile function. Therefore, to define the effects of Ser302 phosphorylation on in vitro and in vivo contractile function, we generated novel TG mice expressing nonphosphorylatable MyBP-C Ser302 (that is, TGS302A). Our findings show that Ser302 phospho-ablation did not disrupt PKA-mediated phosphorylation of neighboring MyBP-C Ser residues (that is, 273 and 282) or other sarcomeric proteins and did not induce significant maladaptive cardiac remodeling or altered basal ventricular function. However, Ser302 phospho-ablation largely prevented β-adrenergic enhancement of the rate of systolic pressure development and decreased maximal systolic pressure following β-adrenergic stimulation, demonstrating that Ser302 phosphorylation is the predominant MyBP-C residue responsible for regulation of cardiac systolic β-adrenergic reserve. Measurements of dynamic XB behavior revealed that loss of β-adrenergic reserve at the whole-heart level was mirrored by the loss of PKA-mediated accelerations in cooperative XB recruitment into the force-generating states at the myofilament level. Collectively, our data show that MyBP-C Ser302 phosphorylation is required to enhance force and pressure generation during enhanced β-adrenergic signaling and uniquely equips the heart with a functional reserve to meet higher circulatory demands during β-adrenergic stimulation.

### Table 3. Dynamic stretch-activation parameters measured in NTG, TG3SA, and TGS302A skinned myocardium. Twelve preparations isolated from four hearts were used for all the groups. P1, XB stiffness; $P_2$, magnitude of XB detachment; P3, the new steady-state force attained in response to the imposed stretch in ML; $P_{\text{df}}$, magnitude of XB recruitment; $P_0$, prestretch isometric force; $k_{\text{rel}}$, rate of XB detachment; $k_{\text{df}}$, rate of XB recruitment. Traces in the top row highlight the portion of the stretch activation trace that the particular parameter represents. Values are expressed as means ± SEM.

<table>
<thead>
<tr>
<th>Group</th>
<th>P1 ($P_1/P_0$)</th>
<th>P2 ($P_2/P_0$)</th>
<th>P3 ($P_3/P_0$)</th>
<th>$P_{\text{df}}$</th>
<th>$k_{\text{rel}}$ (s$^{-1}$)</th>
<th>$k_{\text{df}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>− PKA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTG</td>
<td>0.558 ± 0.013</td>
<td>−0.026 ± 0.010</td>
<td>0.189 ± 0.012</td>
<td>0.215 ± 0.021</td>
<td>497.33 ± 40.51</td>
<td>5.24 ± 0.51</td>
</tr>
<tr>
<td>TG3SA</td>
<td>0.564 ± 0.031</td>
<td>0.053 ± 0.015*</td>
<td>0.175 ± 0.012</td>
<td>0.123 ± 0.016*</td>
<td>314.38 ± 25.85*</td>
<td>5.31 ± 0.34</td>
</tr>
<tr>
<td>TGS302A</td>
<td>0.537 ± 0.018</td>
<td>0.016 ± 0.014*</td>
<td>0.183 ± 0.013</td>
<td>0.167 ± 0.020</td>
<td>355.36 ± 34.10*</td>
<td>6.15 ± 0.51</td>
</tr>
<tr>
<td><strong>+ PKA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTG</td>
<td>0.466 ± 0.011†</td>
<td>−0.106 ± 0.011†</td>
<td>0.265 ± 0.017†</td>
<td>0.371 ± 0.023†</td>
<td>874.41 ± 77.10†</td>
<td>7.85 ± 0.44†</td>
</tr>
<tr>
<td>TG3SA</td>
<td>0.513 ± 0.026</td>
<td>0.013 ± 0.013*</td>
<td>0.203 ± 0.016*</td>
<td>0.190 ± 0.023*†</td>
<td>346.91 ± 37.46*</td>
<td>4.85 ± 0.35*</td>
</tr>
<tr>
<td>TGS302A</td>
<td>0.521 ± 0.017</td>
<td>−0.024 ± 0.019*†</td>
<td>0.227 ± 0.016†</td>
<td>0.251 ± 0.019*†‡</td>
<td>509.89 ± 52.27*†‡</td>
<td>6.20 ± 0.50*‡</td>
</tr>
</tbody>
</table>

*Different versus the corresponding NTG group. †Different versus the corresponding (−PKA) group, $P < 0.05$. ‡Different versus the corresponding TG3SA group.
Ser^{302} phosphorylation is required for complete cooperative recruitment of XBs during submaximal Ca^{2+} activation

MyBP-C Ser^{302} phosphorylation is relatively lower than Ser^{273} and Ser^{282} under basal conditions but is highly phosphorylated following PKA incubation (16, 23); therefore, it is expected that MyBP-C Ser^{302} phosphorylation substantially contributes to enhanced contractile performance following β-adrenergic stimulation. Our data show that TG^{302A} skinned myocardium displayed a slowed rate and diminished decreases in both the rate and magnitude of XB detachment in TGS^{302A} to the NTG skinned myocardium, indicating that Ser^{302} phosphorylation is required for complete cooperative XB recruitment into the force-generating XB pool. This finding is in agreement with an earlier study, which showed that selective phosphorylation of Ser^{302} by PKD accelerates the rate of force development and relaxation in wild-type fibers but these responses are diminished in TG^{35A} fibers, indicating that phospho-ablation of MyBP-C depresses CaMKIIδ-mediated enhancements in contractile function (21). However, under normal physiological conditions, the immediate/short-term demand for increased cardiac inotropy is primarily met by PKA activation, which provides an acute boost in contractility due to phosphorylation of all three Ser residues, whereas the need for prolonged increases in cardiac contractile function [that is, prolonged increases in pacing frequency (21)] may be met by increased CaMKII activation, which phosphorylates only a subset of Ser residues (24). Thus, it is likely that PKA-mediated Ser^{302} phosphorylation is the main contributor to β-adrenergic stimulation–mediated enhancements in cardiac contractility under normal physiological conditions.

The magnitude of cooperative XB recruitment and binding to the thin filaments is influenced both by Ca^{2+} binding to TnC and by XB binding to actin. Ca^{2+} binding to TnC rapidly turns on the thin filament regulatory units and opens up the myosin binding sites on actin by displacing the tropomyosin to facilitate XB binding, which allows for propagation of XB-mediated cooperative XB recruitment along neighboring thin filament regulatory units (25). Cooperative XB-mediated recruitment of additional XBs is further facilitated by PKA phosphorylation of MyBP-C (26), which accelerates XB transitions to force-bearing states and enhances the probability of actomyosin interactions by displacing and orienting the XBs away from the thick filament backbone toward the actin sites (27) and also by reducing XB on time, which allows for accelerated XB turnover and recruitment of additional XBs to vacated myosin binding sites on actin (26).

The mechanism by which MyBP-C phosphorylation enhances cooperative XB recruitment may be due to enhanced thin filament activation upon binding of phosphorylated MyBP-C to actin, which induces displacement of tropomyosin to the open state (28), or due to reduced binding of MyBP-C to myosin S2, which facilitates XB binding to actin (29–32), or both. Thus, at submaximal Ca^{2+} activations, MyBP-C phosphorylation not only accelerates the rate of force development but also expands the spread of thin filament activation beyond regulatory units that have already been activated by Ca^{2+}, thereby shifting the net equilibrium of myosin XBs toward the force-bearing states and greatly enhancing myocardin force generation (26). In contrast, a reduction in MyBP-C Ser^{302} phosphorylation would be expected to slow cooperative XB recruitment and turnover, thereby disrupting the equilibrium of XB transitions into the force-generating pool, which in turn limits systolic pressure generation during β-adrenergic stimulation. However, as the level of Ca^{2+}-activation increases, myofilament force generation becomes less reliant on cooperative XB recruitment because more thin filament regulatory units are directly activated by Ca^{2+}, and thus, the modulatory influence of MyBP-C phosphorylation on XB behavior diminishes (5, 12).

MyBP-C Ser^{302} phosphorylation is required to enhance systolic pressure development

Although all three M-domain MyBP-C residues are targets of PKA phosphorylation, Ser^{302} phosphorylation is observed at low levels in vivo and is lower than the other two PKA-targeted sites, Ser^{273} and Ser^{282} (16, 23). Because the pool of MyBP-C that can be phosphorylated at Ser^{302} following β-adrenergic stimulation (~95% of total MyBP-C) is larger than for Ser^{273} and Ser^{282}, loss of phosphorylation at Ser^{302} would be expected to reduce the recruitable pool of force-generating XBs to a greater extent than loss of phosphorylation at the other PKA sites in response to β-adrenergic signaling. The rate of pressure development and maximal developed pressure were not different between TG^{35A} and TG^{302A} mice following dobutamine treatment, suggesting

Table 4. Steady-state contractile parameters measured in NTG, TG^{35A}, and TG^{302A} skinned myocardium. F_{max}, Ca^{2+}-independent force measured at pCa 9.0; F_{max}, Ca^{2+}-activated maximal force measured at pCa 4.5; n_{Hill} coefficient of the force-pCa relationship; pCa_{50}, pCa required for the generation of half-maximal force. Values are expressed as means ± SEM.

<table>
<thead>
<tr>
<th>Group</th>
<th>F_{min} (mN/mm²)</th>
<th>F_{max} (mN/mm²)</th>
<th>n_{Hill}</th>
<th>pCa_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>− PKA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTG</td>
<td>1.21 ± 0.13</td>
<td>17.21 ± 2.05</td>
<td>2.50 ± 0.15</td>
<td>5.86 ± 0.02</td>
</tr>
<tr>
<td>TG^{35A}</td>
<td>1.48 ± 0.18</td>
<td>20.44 ± 2.47</td>
<td>2.66 ± 0.27</td>
<td>5.88 ± 0.01</td>
</tr>
<tr>
<td>TG^{302A}</td>
<td>1.18 ± 0.20</td>
<td>17.46 ± 2.48</td>
<td>2.37 ± 0.15</td>
<td>5.89 ± 0.01</td>
</tr>
<tr>
<td>+ PKA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG^{35A}</td>
<td>1.17 ± 0.21</td>
<td>20.77 ± 3.54</td>
<td>2.80 ± 0.20</td>
<td>5.77 ± 0.02</td>
</tr>
<tr>
<td>TG^{302A}</td>
<td>1.07 ± 0.23</td>
<td>16.25 ± 2.39</td>
<td>2.42 ± 0.17</td>
<td>5.78 ± 0.02</td>
</tr>
</tbody>
</table>

*Different versus the corresponding (−PKA) group; 12 preparations isolated from four hearts were used for all the groups.
that phosphorylation of Ser\(^{302}\) was needed to recruit the reserve force-generating XBs to enhance pressure development above basal levels. Previous studies have suggested that in addition to the known PKA phosphorylation residues Ser\(^{273}\), Ser\(^{282}\), and Ser\(^{302}\), a fourth residue, Ser\(^{307}\), may also be a target of PKA phosphorylation (9–13). However, TG\(^{35\text{SA}}\) hearts did not exhibit systolic enhancements following dobutamine treatment despite expressing a phosphorylatable Ser\(^{307}\) residue, suggesting that Ser\(^{307}\) is unlikely to be involved in modulating the molecular and in vivo behaviors that we measured in this study. Together, our data suggest that Ser\(^{302}\) is the primary MyBP-C residue that modulates the positive inotropic response to enhanced \(\beta\)-adrenergic stimulation.

By preventing the recruitment of additional XBs following \(\beta\)-adrenergic stimulation, Ser\(^{302}\) phospho-ablation slowed pressure development and reduced maximal systolic pressure development without disrupting basal systolic pressure development. Loss of phosphorylation at Ser\(^{302}\) did not produce pathological cardiac hypertrophy or notable changes in basal unstressed ventricular function, perhaps because the primary role of Ser\(^{302}\) phosphorylation is to promote greater XB recruitment at higher workloads when \(\beta\)-adrenergic signaling is enhanced. This is also consistent with the observation that myocardial force generation is unaltered at baseline but cannot be enhanced by PKA or CaMKII (which targets Ser\(^{36}\)) in myocardium expressing nonphosphorylatable MyBP-C (21) because the reserve pool of recruitable XBs is reduced by Ser\(^{302}\) phospho-ablation.

It was proposed that Ca\(^{2+}\) is released fromryanodine receptors near the Z line in myocytes and diffuses toward the center of the sarcomere (13): first, activating thin filament regulatory units in the region of the sarcomere that does not contain MyBP-C and, subsequently, activating the thin filament in MyBP-C–containing regions. Increased Ca\(^{2+}\) release following \(\beta\)-adrenergic stimulation would be expected to increase XB recruitment, in part, by increasing thin filament activation closer to the center of the sarcomere, in the region of the sarcomere regulated by MyBP-C. Thus, MyBP-C phospho-ablation would limit the recruitment of additional XBs in this region of the sarcomere following \(\beta\)-adrenergic signaling, thereby reducing the pool of recruitable XBs compared to the myofilaments expressing phosphorylatable MyBP-C. MyBP-C phosphorylation– and Ca\(^{2+}\)-dependent regulation of XB recruitment could also explain the temporal pattern of enhanced pressure development following \(\beta\)-adrenergic signaling (Fig. 3). The recruitment of XBs not regulated by MyBP-C would produce an initial increase in pressure as cytosolic Ca\(^{2+}\) levels increase, followed by dobutamine-mediated acceleration of pressure development (observed after several milliseconds) due to recruitment of newly available XBs following MyBP-C phosphorylation to force-generating states.

Previously, it has been suggested that cardiac thick filaments harbor a subpopulation of super-relaxed (SRX) myosin heads that are characterized by substantially slower adenosine 5\(^{-}\)-triphosphate (ATP) turnover rates (33, 34) and thereby equip the cardiac contractile apparatus with a reserve of inactive myosin heads that can potentially be recruited into the force-bearing state under conditions of increased cardiac stress (35). Previous findings demonstrate that MyBP-C modulates the SRX state of myosin heads via its ability to interact with and position the myosin heads closer to the thick filament backbone and that a loss of MyBP-C decreases the number of SRX myosin heads (36). Thus, ablation of Ser\(^{302}\) phosphorylation will potentially maintain the interactions of MyBP-C–SRX heads and possibly prevent them from being recruited toward the myosin binding sites on actin filaments, effectively reducing the number of XBs that can be recruited during thin filament activation. This suggests that a possible role of MyBP-C is to regulate the recruitable pool of XBs to fine-tune force generation during a cardiac twitch, with enhanced \(\beta\)-adrenergic signaling accelerating the initial rate of pressure development at higher workloads by increasing XB recruitment through MyBP-C Ser\(^{302}\) phosphorylation. MyBP-C dephosphorylation limits this recruitable pool and prevents enhanced contraction in response to increased pacing or \(\beta\)-adrenergic signaling (7, 21), and loss of this regulation leads to altered timing of the cardiac cycle and a mismatch between ejection and filling (7).

CONCLUSIONS
Dephosphorylation of MyBP-C, and reduced Ser\(^{302}\) phosphorylation (37, 38), in particular, has been documented in conditions of chronic human heart failure (HF) (37, 39), including patients exhibiting HF with preserved EF (HFP EF) (40), a detrimental condition caused by a complex interplay of deficits in both diastolic and systolic reserves (41). Notably, cardiac dysfunction in HFP EF is often not apparent at rest but becomes noticeable during stress or increased workloads, suggesting that an inability to modulate cardiac output leads to exercise intolerance in HFP EF patients (41). Myocardial samples isolated from HF patients also display desensitization of \(\beta\)-adrenergic signaling pathway (42) that produces a reduction in PKA-mediated phosphorylation of myofilament contractile proteins, including MyBP-C (37, 43). Recent evidence demonstrates that the loss of MyBP-C phosphorylation directly blunts enhanced myocardial force generation and ventricular pressure development in response to increased workloads (7, 21). Here,
we show that abolishing Ser\(^{302}\) phosphorylation greatly diminishes the cardiac β-adrenergic reserve by blunting PKA-mediated enhancements in XB kinetics and dobutamine-mediated enhancements in systolic pressure generation, similar to the effects of abolishing all three PKA phosphorylation residues. These results establish MyBP-C Ser\(^{302}\) as a critical modulator of cardiac output at higher workloads and suggest that therapeutic strategies designed to boost Ser\(^{302}\) phosphorylation could be beneficial for contractile function and enhance cardiac output in HF patients with reduced cardiac β-adrenergic reserve.

**MATERIALS AND METHODS**

**Ethical approval and animal treatment protocols**

Experiments were conducted according to the procedures laid out in the *Guide for the Care and Use of Laboratory Animals* [National Institutes of Health (NIH) Publication No. 85-23, Revised 1996] and as per the guidelines of the Institutional Animal Care and Use Committee at the Case Western Reserve University. NTG wild-type mice expressing full-length MyBP-C and TG mice expressing nonphosphorylatable MyBP-C with Ser-to-Ala substitutions at Ser\(^{273}\), Ser\(^{282}\), and Ser\(^{302}\) (that is, TG\(^{SAA}\)) (6, 16) were used as control groups. TG mice expressing MyBP-C with Ser-to-Ala substitution at Ser\(^{302}\) (that is, TG\(^{S302A}\)) were generated on a MyBP-C-null background such that there is no endogenous Ser\(^{302}\) phosphorylation. All mouse lines were of the SV/129 strain, and adult mice (3 to 6 months old) of both sexes were used for this study.

**Determination of sarcomeric protein phosphorylation and MHC expression**

Western blot and Pro-Q Diamond phosphoprotein stain (Life Technologies) were used to assess myofilament protein phosphorylation, as described previously (14, 44). On the day of the experiment, cardiac myofibrils were prepared by briefly homogenizing frozen mouse ventricular tissue for 15 s using a handheld homogenizer (PowerGen 500, Thermo Fisher Scientific) in relaxing solution that contains protease and phosphatase inhibitors (PhosSTOP and cComplete ULTRA Tablets, Roche Applied Science). Myofibrils were chemically skinned for 15 min using 1% Triton X-100, centrifuged at 10,000 g for 5 min, and resuspended in fresh relaxing solution on ice until further use. For PKA treatment, 100 μg of myofibrils isolated from hearts of each mouse line was incubated with the catalytic subunit of bovine PKA (Sigma-Aldrich) to a final concentration of 0.15 U PKA/μg myofibrils for 1 hour at 30°C, and the reaction was stopped by the addition of Laemmli buffer (16). Control myofibrils were incubated under the same conditions without PKA. For Western blots, myofibrils were separated on 4 to 20% tris-glycine gels (Lonza) and stained with Pro-Q phosphoprotein stain (Life Technologies) in relaxing solution, followed by detergent skinning using 1% Triton X-100 (Thermo Scientific) for 1 hour. Multicellular ventricular preparations measuring ~100 μm in width and ~400 μm in length were selected for the mechanical experiments. The composition of Ca\(^{2+}\) activation solutions prepared for the experiments was based on a computer program (45) and established stability constants (46). Ca\(^{2+}\) solutions contained the following: 14.5 mM creatine phosphate, 7 mM EGTA, and 20 mM imidazole. The maximal activating solution (pCa 4.5; pCa = −log [Ca\(^{2+}\)]\(_{\text{free}}\)) also contained 65.45 KCl, 7.01 CaCl\(_2\), 5.27 MgCl\(_2\), 4.81 ATP, whereas the relaxing solution (pCa 9.0) contained 72.45 mM KCl, 0.02 mM CaCl\(_2\), 5.42 mM MgCl\(_2\), 4.76 mM ATP. The pH and the ionic strength of the Ca\(^{2+}\) solutions were set to 7.0 and 180 mM, respectively. A range of pCa solutions (pCa 6.3 to 5.4) containing varying amounts of [Ca\(^{2+}\)]\(_{\text{free}}\) were then prepared by mixing appropriate volumes of pCa 9.0 and 4.5 stock solutions, and the mechanical experiments were performed at 23°C.

**Experimental setup for measurement of dynamic and steady-state contractile properties in skinned myocardium**

Detergent-skinned multicellular ventricular preparations were attached between a motor arm (312C, Aurora Scientific Inc.) and a force transducer (403A, Aurora Scientific Inc.), as described previously (47). Changes in the motor arm position and force transducer signals were sampled at 2000 Hz using a custom-built sarcomere length (SL) control software program developed by Campbell and Moss (48). For all mechanical measurements, SL of the ventricular preparations was set to 2.1 μm (16). Force-pCa relationships were determined by incubating the skinned myocardial preparations in pCa solutions ranging from pCa 6.3 to 4.5. The apparent cooperativity of force generation was estimated from the steepness of Hill plot transformation of the force-pCa relationships (44). The force-pCa data were fit using the equation

\[ P/P_0 = \frac{[Ca^{2+}]^{n_H}}{(k_{\text{ass}} + [Ca^{2+}]^{n_H})} \]

where \(n_H\) is the Hill coefficient and \(k\) is the pCa needed to elicit half-maximal force (that is, pCa\(_{50}\)) (44).
Stretch activation experiments to measure dynamic XB contractile parameters

The stretch activation protocol used in these studies was described earlier (3, 16, 49). Skinned myocardial preparations were placed in CaCl2 solutions (pCa 6.1) that generate ~35% of the maximal force. Once the preparations attained a steady-state force, they were rapidly stretched by 2% of their initial ML, held at the new ML for 8 s, and then returned back to their initial ML. The key features of the stretch activation responses in cardiac muscle have been described earlier (50, 51), and various stretch activation parameters measured are illustrated in Fig. 4A. In brief, a sudden 2% stretch in ML causes an instantaneous spike in the force response (P1), which is due to the sudden strain of elastic elements within the strongly bound XB (phase 1). The force then rapidly decays (phase 2) because of the detachment of the strained XB into a non–force-bearing state, with a dynamic rate constant \( k_{rel} \) (an index of XB detachment). The lowest point of phase 2 (nadir) is indicated by P2 and is an index of the magnitude of XB detachment. Following phase 2, the preparations exhibit a gradual rise of force (phase 3), with a dynamic rate constant \( k_{rel} (\text{an index of the rate of XB recruitment}) \). The delayed force rise in phase 3 is due to the sudden stretch-mediated recruitment of new XB into the force-bearing state (50, 51). Stretch activation amplitudes, P3 and \( P_{30} \), were normalized to prestretch Ca2+-activated force where P3 was measured from prestretch steady-state force to the peak force value of the delayed force attained in phase 3, whereas \( P_{30} \) was measured as the difference between P3 and P2 values, as described previously (3, 4, 47).

\( k_{rel} \) was measured by fitting a single exponential equation to the time course of force decay using the equation: \( F(t) = a(1 + \exp(-k_{rel}t)) \), where “a” is the amplitude of the single exponential phase and \( k_{rel} \) is the rate constant of the force decay, as done earlier (5). \( k_{rel} \), which represents the rate of recruitment of all XB that give rise to the delayed force transient following the sudden stretch in ML (that is, \( P_{30} \)), was estimated by linear transformation of the half-time of force redevelopement (5), that is, \( k_{rel} = 0.693/t_{1/2} \), where \( t_{1/2} \) is the time (in milliseconds) taken from the nadir (that is, the point of force reuptake at the end of phase 2) to the point of half-maximal force in phase 3 of the force response, where maximal force is indicated by a plateau region of phase 3 (that is, P3) (Fig. 4A) (5).

Stretch activation experiments were repeated following incubation of the myocardial preparations with PKA. Because PKA treatment decreases myocardial Ca2+ sensitivity of force generation, we used a pCa solution with slightly higher [Ca2+]I (pCa 6.0) to closely match the activation levels before PKA treatment, as done in earlier studies (5, 16).

Data analysis

Data were analyzed using two-way analysis of variance (ANOVA), and multiple pairwise comparisons were made using Fisher’s least significant difference (Fisher’s LSD) method, as previously reported (44). One-way ANOVA was used for analyzing the data reported for echocardiography experiments. Values are reported as means ± SEM. The criterion for statistical significance was set at \( P < 0.05 \), and the asterisks in the figures and tables denote statistical significance using post hoc Fisher’s LSD comparisons.

REFERENCES AND NOTES


44. R. Mamidi, K. S. Gresham, J. E. Stelzer, Length-dependent changes in contractile dynamics are blunted due to cardiac myosin binding protein-C ablation. Front. Physiol. 5, 461 (2014).


Acknowledgments: Funding: This work was supported by the NIH (HL-1144770 and P30 EY011373 to J.E.S.) and the American Heart Association (16POST30730000 to R.M.) grants. Author contributions: R.M., K.S.G., and J.E.S. contributed to the conception and design of the experiments. R.M., K.S.G., and J.E.S. participated in performing the experiments, in acquiring, analyzing, and interpreting data, and in drafting and revising the manuscript. All authors approved the final version of the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper. Additional data related to this paper may be requested from the authors.

Submitted 6 October 2016
Accepted 2 February 2017
Published 10 March 2017
10.1126/sciadv.1602445

This article is published under a Creative Commons license. The specific license under which this article is published is noted on the first page.

For articles published under CC BY licenses, you may freely distribute, adapt, or reuse the article, including for commercial purposes, provided you give proper attribution.

For articles published under CC BY-NC licenses, you may distribute, adapt, or reuse the article for non-commercial purposes. Commercial use requires prior permission from the American Association for the Advancement of Science (AAAS). You may request permission by clicking here.

The following resources related to this article are available online at http://advances.sciencemag.org. (This information is current as of May 7, 2017):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:
http://advances.sciencemag.org/content/3/3/e1602445.full

This article cites 51 articles, 25 of which you can access for free at:
http://advances.sciencemag.org/content/3/3/e1602445#BIBL

Science Advances (ISSN 2375-2548) publishes new articles weekly. The journal is published by the American Association for the Advancement of Science (AAAS), 1200 New York Avenue NW, Washington, DC 20005. Copyright is held by the Authors unless stated otherwise. AAAS is the exclusive licensee. The title Science Advances is a registered trademark of AAAS.