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RESEARCH ARTICLE

Cardiac Excitation and Contraction

Cardiac myosin binding protein-C phosphorylation accelerates β-cardiac myosin detachment rate in mouse myocardium

Bertrand C. W. Tanner,[1] Michael J. Previs,[1] Yuan Wang,[1] Jeffrey Robbins,[2] and Bradley M. Palmer[1]

[1]*Department of Molecular Physiology and Biophysics, University of Vermont, Burlington, Vermont and* [2]*Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio*

Abstract

Cardiac myosin binding protein-C (cMyBP-C) is a thick filament protein that influences sarcomere stiffness and modulates cardiac contraction-relaxation through its phosphorylation. Phosphorylation of cMyBP-C and ablation of cMyBP-C have been shown to increase the rate of MgADP release in the acto-myosin cross-bridge cycle in the intact sarcomere. The influence of cMyBP-C on Pi-dependent myosin kinetics has not yet been examined. We investigated the effect of cMyBP-C, and its phosphorylation, on myosin kinetics in demembranated papillary muscle strips bearing the β-cardiac myosin isoform from nontransgenic and homozygous transgenic mice lacking cMyBP-C. We used quick stretch and stochastic length-perturbation analysis to characterize rates of myosin detachment and force development over 0–12 mM Pi and at maximal (pCa 4.8) and near-half maximal (pCa 5.75) Ca^{2+} activation. Protein kinase A (PKA) treatment was applied to half the strips to probe the effect of cMyBP-C phosphorylation on Pi sensitivity of myosin kinetics. Increasing Pi increased myosin cross-bridge detachment rate similarly for muscles with and without cMyBP-C, although these rates were higher in muscle without cMyBP-C. Treating myocardial strips with PKA accelerated detachment rate when cMyBP-C was present over all Pi, but not when cMyBP-C was absent. The rate of force development increased with Pi in all muscles. However, Pi sensitivity of the rate force development was reduced when cMyBP-C was present versus absent, suggesting that cMyBP-C inhibits Pi-dependent reversal of the power stroke or stabilizes cross-bridge attachment to enhance the probability of completing the power stroke. These results support a functional role for cMyBP-C in slowing myosin detachment rate, possibly through a direct interaction with myosin or by altering strain-dependent myosin detachment via cMyBP-C-dependent stiffness of the thick filament and myofilament lattice. PKA treatment reduces the role for cMyBP-C to slow myosin detachment and thus effectively accelerates β-myosin detachment in the intact myofilament lattice.

NEW & NOTEWORTHY Length perturbation analysis was used to demonstrate that β-cardiac myosin characteristic rates of detachment and recruitment in the intact myofilament lattice are accelerated by Pi, phosphorylation of cMyBP-C, and the absence of cMyBP-C. The results suggest that cMyBP-C normally slows myosin detachment, including Pi-dependent detachment, and that this inhibition is released with phosphorylation or absence of cMyBP-C.

cardiac; myofilament; myosin binding protein-C; step response; stretch activation

INTRODUCTION

The NH_2-terminus of cardiac myosin binding protein-C (cMyBP-C) binds to several functionally relevant sarcomeric proteins: actin (1–3), myosin S2 (1, 4), and myosin regulatory light chain (5). The NH_2-terminus also bears a phosphorylation motif that affects its preferred binding partner and thereby can modify sarcomeric mechanics. Protein kinase A (PKA) phosphorylation of the NH_2-terminus of cMyBP-C diminishes its binding affinity to actin (6–8) and myosin S2 (4), which in turn leads to a faster actin-filament sliding velocity in vitro (6, 8) and a faster rate of force development after a quick stretch in skinned myocardial strips (9, 10). Thus, cMyBP-C phosphorylation has been identified as a significant molecular regulator of myosin function that can enhance cardiac contractility in response to β-adrenergic stimulation in part by enhancing myosin cross-bridge characteristic rates of detachment and recruitment. Functional mechanics of skinned mouse myocardium lacking cMyBP-C (11–13) also show faster rates of force development (14–16), higher frequencies of oscillatory work production (17), and faster loaded shortening velocities (14). These similarities in the functional consequences of phosphorylated cMyBP-C and the loss of cMyBP-C on myosin-dependent rates suggest that cMyBP-C phosphorylation effectively eliminates interactions between the NH_2-terminus of cMyBP-C and other myofilament proteins within the sarcomere, as the binding data suggest (4, 7, 8). This concept is perhaps illustrated best

Correspondence: B. M. Palmer (bmpalmer@uvm.edu).
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in Stelzer et al. (9) where the two conditions, phosphorylation and absence of cMyBP-C, result in force responses after a quick stretch that are nearly superimposable. Nevertheless, it must be noted that chronic phosphorylation of cMyBP-C, as mimicked in the transgenic AllP^{+} and t3SD mice (18, 19), does not lead to a cardiomyopathy like that observed in mice lacking cMyBP-C and could be cardioprotective in vivo.

Although myosin detachment rate is determined principally by MgADP release rate, inorganic phosphate (Pi) can influence detachment by reversing the myosin power stroke and detaching the force-producing myosin cross-bridge (20–24). We previously reported increased Pi sensitivity of the frequency of oscillatory work production in mouse myocardium lacking cMyBP-C and expressing predominantly β-cardiac myosin (17). That observation suggested that cMyBP-C slows Pi-dependent myosin cross-bridge detachment at least in the β-cardiac myosin background. In light of the similar functional consequences between phosphorylation and removal of cMyBP-C previously reported, we hypothesized that PKA-phosphorylated cMyBP-C would effectively remove its influence on Pi-dependent detachment in β-cardiac myosin, thereby resulting in greater sensitivity of cross-bridge detachment rate to Pi.

Herein, we present characteristics of Pi-dependent β-cardiac myosin cross-bridge detachment due to cMyBP-C phosphorylation in skinned mouse myocardial strips and compare these characteristics with those when cMyBP-C is absent. The rates of cross-bridge detachment rate and force development were measured using a step length change and stochastic length perturbation analysis techniques, thus allowing a direct comparison of the PKA effects on these rate parameters with previous studies (9, 10, 15, 17, 25). These measurements were also performed at maximal and submaximal Ca^{2+} activation to compare with previous observations. With our study design, a significant statistical interaction detected by repeated measures ANOVA involving the absence of cMyBP-C would indicate that myosin-sensitive mechanics are differentially affected by the presence or absence of cMyBP-C.

METHODS

Animal Models

All procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Vermont College of Medicine and the University of Cincinnati Children's Hospital and complied with the *Guide for the Use and Care of Laboratory Animals*, published by the National Institutes of Health. Adult male mice were acquired from the University of Cincinnati and were either nontransgenic (NTG) or homozygous transgenic lacking cMyBP-C (*t/t*) due to truncated mutation of the MYBPC3 gene (13). To mitigate the added difficulty of inferring cross-bridge detachment rates due to the absence of cMyBP-C or due to a mixture of α- and β-cardiac myosin (25), all mice were fed an iodine-deficient 0.15% propylthiouracil diet for at least 12 wk (Harlan Teklad, Indianapolis, IN), resulting in hypothyroidism and a complete shift to β-cardiac myosin expressed in the LV (17, 26, 27). Mouse populations are referred to as NTG_{β} (n = 5) and t/t_{β} (n = 6) to denote the β-cardiac myosin background. The two populations were of comparable age (NTG_{β} 33.2 ± 2.0 wk vs. t/t_{β} 34.5 ± 1.3 wk, P = 0.589 by two-tailed t test). The t/t_{β} mice were smaller by body mass (NTG_{β} 33.1 ± 2.0 g vs. t/t_{β} 27.5 ± 1.2 g, P = 0.033) while also having larger LVs. (NTG_{β} 75 ± 5 mg vs. t/t_{β} 89 ± 4 mg, P = 0.039).

Solutions for Skinned Myocardial Strips

Chemicals and reagents were obtained from Sigma Corp. (St. Louis, MO) unless otherwise noted. Solution concentrations (mmol/L) were formulated by solving equations describing ionic equilibria according to Godt and Lindley (28). The types of solutions are as follows: relaxing solution: pCa 8.0 (pCa = $-\log_{10}[Ca^{2+}]$), 5 EGTA, 5 MgATP, 1 Mg^{2+}, 0 Pi, 35 phosphocreatine, 300 U/mL creatine kinase (CK), 200 ionic strength, pH 7.0; maximal activation solution: same as relaxing with pCa 4.8 and 0 or 16 Pi; half-maximal activation solution: same as activating at pCa 5.75; skinning solution: same as relaxing without CK, with 1% Triton X-100 wt/vol and 50% glycerol wt/vol; storage solution: same as skinning without Triton, with 10 μg/mL leupeptin; alkaline phosphatase (AP) solution: same as relaxing solution with 6 U/mL recombinant AP from *Escherichia coli* (P-4252, Sigma); cAMP-dependent PKA solution: same as relaxing solution with 1 U/μL recombinant PKA from *E. coli* (V516A, Promega, Madison, WI).

cMyBP-C Phosphorylation and Mass Spectrometry

Phosphorylated amino acids were identified at four specific sites within cMyBP-C isolated from NTG_{β} expressing wild-type MyBP-C, and the degree of phosphorylation was quantified by label-free liquid chromatography-mass spectrometry (LC-MS). An explanation of these methods is provided elsewhere (29).

Skinned Myocardial Strips

Skinned myocardial strips were studied as previously described (25, 27). Papillary muscles from the LV were dissected to yield at least two thin strips (~140 μm diameter) with longitudinally oriented parallel fibers, skinned for 2 h at room temperature, and stored at −20°C. Aluminum T-clips were attached to the ends of a strip ~300 μm apart. Immediately before mechanical analysis, all strips were incubated for 10 min in AP solution at room temperature. Some strips received a second incubation in PKA solution for 1 h at room temperature. Strips were mounted between a piezoelectric motor and a strain gauge, lowered into a 30 μL droplet of relaxing solution maintained at 17°C, chosen to ease future use of Q_{10} to infer rates at body temperature, and stretched to 2.2 μm sarcomere length measured by digital Fourier transform. Strips were mounted at pCa 8.0 and then activated at pCa 4.8. Activating solution containing 16 mM Pi was then exchanged to raise Pi concentration from 0 to 12 mM. Strips were then activated at pCa 5.75, and a second Pi titration performed. Calcium activation at pCa 5.75 was chosen to provide a submaximal calcium activation near or below 50% activation. Our previously measured tension-pCa curves between two similar genotypes also fed 6-propyl-2-thiouracil (PTU) (17) and both to have similar pCa_{50} ~5.65. PKA would be expected to lower the calcium sensitivity of

the thin filament; therefore, pCa 5.75 is expected to be near or below 50% activation. Recorded forces were normalized to cross-sectional area to calculate isometric tension (T), with individual recordings of developed tension (T_{dev}) calculated by subtracting the relaxed tension (T_{min}) value: $T_{dev} = T - T_{min}$.

Mechanics Analysis

Step length changes of amplitude 1% strip length were applied under increasing Pi conditions. Estimates of rates of force release ($k_{f,rel}$) and subsequent force development ($k_{f,dev}$) were calculated from the half-time of the decaying ($t_{1,2}$, *phase 3*) and rising ($t_{2,3}$, *phase 3*) force transients following step length change (15, 30):

$$k_{f,rel} = \frac{\ln(2)}{t_{1,2}}, \text{ and}$$
$$k_{f,dev} = \frac{\ln(2)}{t_{2,3}}. \quad (1)$$

The force release rate, $k_{f,rel}$, reflects the rate of myosin cross-bridge detachment apparent in *phase 2* (31–33). The force development rate, $k_{f,dev}$, reflects the rate of myosin cross-bridge recruitment in *phase 3* (31).

Stochastic length perturbations were also applied at each Pi condition for a period of 40 s as previously described (34), using an amplitude distribution with a standard deviation of 0.1% strip length that covered the frequency range 0.25–250 Hz. Elastic and viscous moduli as a function of angular frequency (ω), $E(\omega)$, and $V(\omega)$ were measured from the in-phase and out-of-phase portions of the tension response to length perturbation (25). A complex modulus, $Y(\omega)$, was defined as $E(\omega) + iV(\omega)$, where $i = \sqrt{-1}$. Fitting Eq. *1* to the entire frequency range of moduli values provided estimates of six model parameters (A, k, B, $2\pi b$, C, $2\pi c$).

$$Y(\omega) = A(i\omega)^k - B\left(\frac{i\omega}{2\pi b + i\omega}\right) + C\left(\frac{i\omega}{2\pi c + i\omega}\right). \quad (2)$$

The A term in Eq. *1* reflects the viscoelastic mechanical response of passive elements in the muscle. The B and C terms of Eq. *1* reflect enzymatically driven myosin cross-bridge formation in activated muscle. Parameters $2\pi b$ and $2\pi c$ reflect cross-bridge-dependent rates that are sensitive to biochemical perturbations affecting enzymatic activity, such as MgATP, MgADP, or Pi concentrations (35). The B term reflects force generation, such that $2\pi b$ describes cross-bridge recruitment rate and should be directly related to rate of force development, $k_{f,dev}$ (31). The rate $2\pi b$ has also been described as the rate of myosin isomerization including Pi-dependent cross-bridge detachment (32, 36, 37). C term reflects processes pertaining to cross-bridge detachment or force decay, such that $2\pi c$ describes to the rate of cross-bridge detachment and should be related to rate of force release, $k_{f,rel}$ (33).

Statistical Analysis

All values are means ± SE, using n = 9, 8, 10, and 10 individual skinned myocardial strips for NTG_β, $NTG_{\beta,PKA}$, t/t_β, and $t/t_{\beta,PKA}$, respectively. Constrained nonlinear least squares fitting of Eq. *2* to moduli were performed using sequential quadratic programming methods in MATLAB (v 7.9.0, The MathWorks, Natick, MA). Statistical analyses were performed using SPSS (v. 27, IBM, Chicago, IL), using repeated measures ANOVA with Pi concentration as the repeated measure. The sensitivity of measured variables to Pi was calculated as the slope of a linear regression between the variable value and Pi conditions 0–5 mM. This sensitivity was then subject to two-way ANOVA to ascertain whether the absence of cMyBP-C and/or PKA differentially affected the sensitivity of these variables to Pi. The terms $2\pi b$ and $2\pi c$ are expected to directly represent the same physiological processes as $k_{f,dev}$ and $k_{f,rel}$, respectively, and were compared by correlation. Statistical significance is reported at $P < 0.05$ and $P < 0.01$ levels.

RESULTS

Myosin Isoform and Protein Phosphorylation Status

Hypothyroidism resulted in 100% expression of β-cardiac myosin in both the NTG_β and t/t_β groups (Fig. 1*A*). The application of PKA following alkaline phosphatase treatment clearly enhanced phosphorylation status of troponin-I, as indicated by Pro-Q diamond phospho-staining (Fig. 1*B*). PKA-mediated phosphorylation of cMyBP-C was quantified at four phosphorylation sites, Ser-273, Ser-282, Ser-302, and Ser-307, by LC-MS (Table 1). PKA treatment resulted in elevated overall phosphorylation status from 50% to 62% taken as an average phosphorylation of these four sites, and most notably, phosphorylation of Ser-307 was elevated from 3% to 27%.

Developed Tension Dependence on cMyBP-C, Pi, and PKA

The absence of cMyBP-C led to a reduced maximum developed tension (T_{dev}) at pCa 4.8 (Table 2, P = 0.020) as seen by overall lower maximum T_{dev} in the t/t_β compared with NTG_β when Pi and PKA conditions are controlled. Similar trends for a reduction in maximum T_{dev} with the loss of cMyBP-C in the β-cardiac myosin background have been reported previously (17, 25).

Increasing concentrations of Pi would be expected to reduce T_{dev} by biasing the myosin cross-bridge to a prepower state and reducing the number of cross-bridges in the force-producing postpower stroke state (22, 23). We likewise found maximum T_{dev} depressed with increasing Pi at maximum calcium activation (Fig. 2). Statistical analysis verified a highly significant Pi main effect independent of absence of cMyBP-C at both pCa 4.8 and 5.75 (Table 2, $P < 0.01$). We did not find a significant Pi × MYBPC3 interaction for maximum developed tension or a significant MYBPC3 main effect for Pi sensitivity $\Delta T_{dev}/\Delta Pi$ (Table 2). Therefore, the effects of Pi on maximum developed tension were not significantly dependent upon cMyBP-C.

PKA is known to reduce the sensitivity of the thin filament to calcium activation via phosphorylation of TnI and would be expected to reduce maximal developed tension (38). We found PKA treatment reduced maximal developed tension values in both the NTG_β with cMyBP-C and the t/t_β without cMyBP-C (Fig. 2) as affirmed by the highly significant PKA main effects (Table 2, $P < 0.01$). It is noteworthy that the drop in maximum T_{dev} with PKA was on the order of 20%,

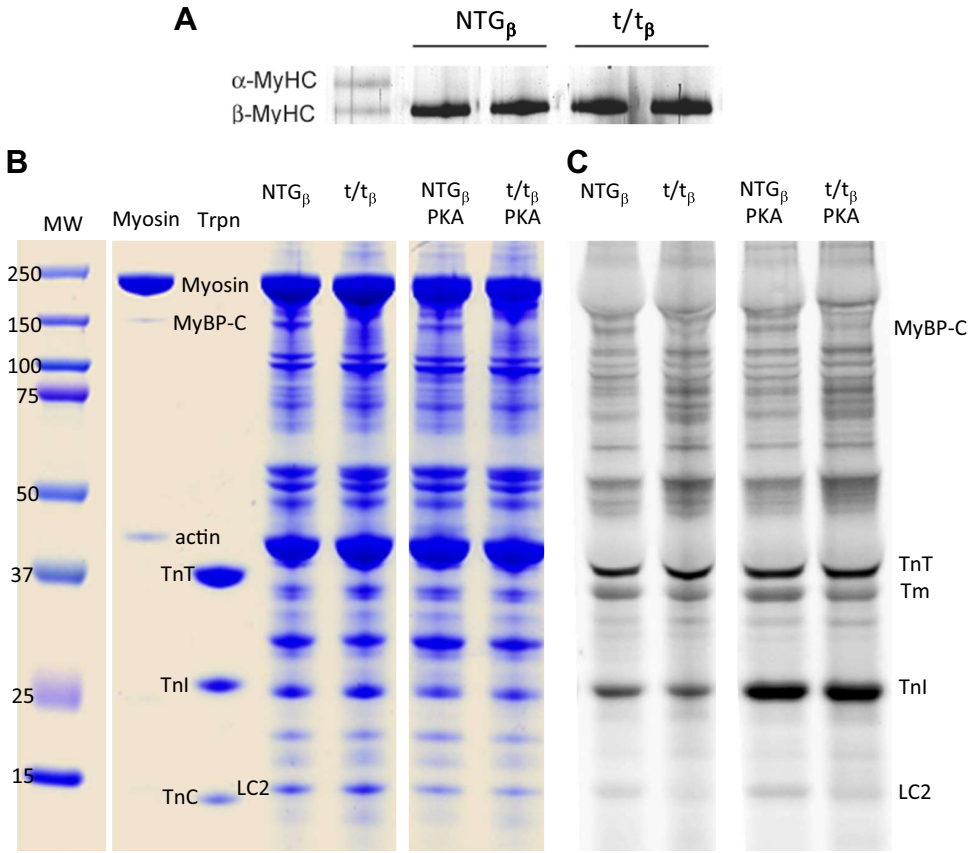

Figure 1. Cardiac myosin isoforms and PKA-induced phosphorylation of sarcomeric proteins. *A*: mice were hypothyroid after 12 wk of PTU diet and expressed 100% β-cardiac myosin in the LV at time of harvest. *B*: gels show comparable protein loads. The lane marked myosin provides a reference for myosin and its contaminants of MyBP-C and actin. The lane loaded with isolated troponin (Trpn) with its three components provides reference for TnT, TnC, and particularly phosphorylatable TnI at ~24 kDa. *C*: Pro-Q diamond phospho-stain demonstrates that PKA treatment led to significant phosphorylation of TnI in both genotypes. Phosphorylation of cMyBP-C was not as visually apparent and was quantified instead by mass spectrometry (see Table 1). PKA, protein kinase A; PTU, 6-propyl-2-thiouracil.

which we attribute to pretreatment with AP. The AP-treated muscle would possess dephosphorylated myofilament proteins TnI, titin, and myosin regulatory light chain.

A significant Pi × PKA interaction was detected for maximum developed tension, indicating that PKA treatment reduced the sensitivity of isometric tension to Pi. This is evident in the shallower slope of the Pi-reduced tension after PKA treatment in Fig. 2. This slope, indicative of the sensitivity of maximum T_{dev} to Pi and given as $\Delta T_{dev}/\Delta$Pi in Table 2, demonstrated a significant PKA main effect, meaning that the loss of T_{dev} with Pi was significantly reduced after PKA treatment. Upon noting the lack of a significant Pi × PKA × MYBPC3 interaction for T_{dev} and a lack of PKA × MYBPC3 interaction for $\Delta T_{dev}/\Delta$Pi, the Pi × PKA interaction for maximum T_{dev} was affected similarly both with and without cMyBP-C, suggesting that the effect of PKA on TnI and not cMyBP-C was likely the underlying cause of PKA treatment enhancing the Pi sensitivity of T_{dev}.

Step Response Sensitivity to cMyBP-C, Pi, and PKA

The effects of Pi and PKA on β-cardiac myosin cross-bridge rates of detachment and recruitment in the presence and absence of cMyBP-C were measured from the force response following a 1% length step change (Fig. 3*A*). Strain, defined as the fractional change in length, was rapidly applied at time t_0. The typical stress response included a rapid rise until t_1 (the end of *phase 1*), a decay from t_1–t_2 (*phase 2*), and finally a slower rise from t_2–t_3 (*phase 3*) (30).

The rates of force release and development, $k_{f,rel}$ and $k_{f,dev}$, were consistently higher in the t/t_β myocardium lacking

Table 1. *Percentage of phosphorylated peptides as observed by mass spectrometry after AP and subsequent PKA treatments*

Phosphorylated Amino Acid	Phosphopeptide Observed	%Phosphorylation	
		AP	PKA
Ser-273	^{272}TS$_p$LAGAGR^{279} ^{271}RTS$_p$LAGAGR^{279}	55 ± 11	68 ± 11 (13)
Ser-282	^{281}TS$_p$DSHEDAGTLDFSSLLK^{298} ^{280}RTS$_p$DSHEDAGTLDFSSLLK^{298}	84 ± 8	91 ± 5 (7)
Ser-302	^{299}KRDS$_p$FR^{304}	56 ± 12	63 ± 12 (7)
Ser-307	^{305}RDS$_p$KLEAPAEEDVWEILR^{322}	3 ± 2	27 ± 6 (24)
Average		50 ± 2	62 ± 13 (12)

Values are means ± SD (%change in phosphorylation due to PKA treatment). Average refers to average phosphorylated percentage and associated SD calculated as square root of the sum of variances to reflect error propagation. AP, alkaline phosphatase; PKA, protein kinase A.

Table 2. *Results for maximum developed tension (T_{dev}) at pCa 4.8*

T_{dev} (kPa)	NTG_{β}	$NTG_{\beta,PKA}$	t/t_{β}	$t/t_{\beta,PKA}$	ANOVA
n	9	8	10	10	
Pi (mM) = 0	25.33 ± 1.60	14.98 ± 2.37	17.52 ± 1.76	13.38 ± 1.26	Main effects
1	21.49 ± 1.21	13.90 ± 2.41	16.34 ± 1.64	12.67 ± 1.11	*MYBPC3, †Pi, †PKA
2	20.34 ± 1.01	13.14 ± 2.23	15.10 ± 1.45	11.68 ± 1.03	
3	19.20 ± 1.03	11.98 ± 2.07	13.87 ± 1.27	10.66 ± 0.92	Interactions
4	18.38 ± 0.80	11.23 ± 1.89	13.05 ± 1.19	9.90 ± 0.91	*Pi×PKA
5	17.74 ± 0.72	10.53 ± 1.71	12.36 ± 1.20	9.09 ± 0.79	
8	16.37 ± 0.43	9.22 ± 1.46	10.72 ± 1.11	7.63 ± 0.72	
12	14.18 ± 0.73	7.88 ± 1.22	9.05 ± 1.01	6.02 ± 0.68	
$\Delta T_{dev}/\Delta Pi$	−1.38 ± 0.17	−0.90 ± 0.18	−1.05 ± 0.16	−0.88 ± 0.14	*PKA

Values are means ± SE; *n*, number of strips. Statistical results reflect repeated-measures ANOVA and demonstrate statistical significance for the main effects of MYBPC3, Pi, PKA, and for interactions Pi × MYBPC3, PKA × MYBPC3, Pi × PKA, Pi × PKA × MYBPC3. The sensitivity of T_{dev} to Pi was calculated by linear regression using values measured at 0–5 mM Pi and denoted here as $\Delta T_{dev}/\Delta Pi$. Two-way ANOVA was then used to determine whether $\Delta T_{dev}/\Delta Pi$ was influenced by the absence of cMyBP-C and/or PKA. *$P < 0.05$, †$P < 0.01$. NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

cMyBP-C compared with NTG_{β} bearing cMyBP-C when the comparison accounts for Pi and PKA conditions as indicated by a highly significant MYBPC3 effect at both maximal and submaximal calcium activations (Tables 3 and 4, MYBPC3 main effect $P < 0.01$). Values for $k_{f,rel}$ and $k_{f,dev}$ were noticeably higher in t/t_{β} versus NTG_{β} and in $t/t_{\beta,PKA}$ versus $NTG_{\beta,PKA}$ comparisons over all Pi (Fig. 4). These results demonstrate faster force transient dynamics when cMyBP-C is absent from the sarcomere as reported previously (14–17, 25).

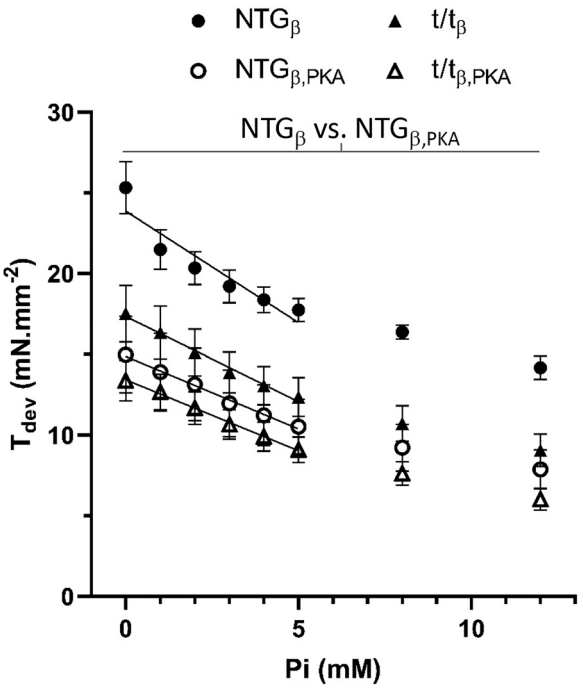

Figure 2. Effects of Pi and protein kinase A (PKA) on maximum developed isometric tension in skinned myocardial strips. *A*: maximum developed isometric tension (T_{dev}) from NTG_{β} and t/t_{β} myocardial strips at pCa 4.8 was reduced with PKA and with increasing Pi. The Pi sensitivity of T_{dev} was calculated as $\Delta T_{dev}/\Delta Pi$ over 0–5 mM Pi and depicted as the slope of the line drawn over the data points. This Pi sensitivity was reduced (shallower slope) with PKA treatment but was not found to be dependent upon presence or absence of cMyBP-C (see Table 2). Means ± SE. $P < 0.01$ for NTG_{β} vs. $NTG_{\beta,PKA}$. cMyBP-C, cardiac myosin binding protein-C; NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

Pi effects and Pi × MYBPC3 interaction.

The rates $k_{f,rel}$ and $k_{f,dev}$ were highly sensitive to Pi at both maximal and submaximal calcium activations (Tables 3 and 4, Pi main effect $P < 0.01$). This sensitivity to Pi can be seen in the dramatic shortening of the durations in *phases 2* and *3* as Pi is elevated from 0 to 12 mM (Fig. 3, *B–E*). The calculated values for $k_{f,rel}$ and $k_{f,dev}$ were clearly enhanced with increasing Pi (Fig. 4) and therefore consistent with results reported by others that myosin-dependent force dynamics are enhanced with Pi availability (22, 39, 40).

A significant Pi × MYBPC3 interaction was observed for both $k_{f,rel}$ and $k_{f,dev}$ (Tables 3 and 4, $P < 0.01$). These interactions may be better recognized upon considering the Pi sensitivity of the variables, $\Delta k_{f,rel}/\Delta Pi$ and $\Delta k_{f,dev}/\Delta Pi$. These Pi sensitivities were consistently higher in the t/t_{β} lacking cMyBP-C compared with NTG_{β} bearing cMyBP-C as demonstrated by a significant MYBPC3 main effect for both $\Delta k_{f,rel}/\Delta Pi$ and $\Delta k_{f,dev}/\Delta Pi$ under both calcium activation conditions (Tables 3 and 4) and can be recognized in Fig. 4, *A* and *B*, where values for t/t_{β} show a greater rate of rise with Pi compared with NTG_{β} independent of the PKA condition. These results suggest that the Pi-sensitive β-myosin detachment rate and recruitment rate are slowed by the presence of cMyBP-C.

PKA effects and PKA × MYBPC3 interaction.

The rate of force release, $k_{f,rel}$, was highly sensitive to PKA at both maximal and submaximal calcium activations (Table 3, $P < 0.01$). This rate, which characterizes the steepness of *phase 2*, is thought to reflect the myosin detachment rate (31, 33). Our findings suggest that PKA enhances myosin detachment rate and can be seen clearly as higher values for $k_{f,rel}$ in the $NTG_{\beta,PKA}$ group bearing phosphorylated myofilament proteins including cMyBP-C compared with the NTG_{β} group in Fig. 4, *A* and *C*. Conversely, any effect of PKA was not apparent in the t/t_{β} lacking cMyBP-C. In fact, the significant PKA × MYBPC3 interaction for $k_{f,rel}$ at pCa 5.75 (Fig. 4*C*) indicates that the presence of cMyBP-C in the NTG_{β} group was statistically more sensitive to the effects of PKA over the entire range of Pi conditions. These results suggest that PKA enhances myosin detachment rate over the entire range of Pi when cMyBP-C is present.

The effect of PKA on the rate of force development, $k_{f,dev}$, was subtler than that on $k_{f,rel}$. There was a significant PKA

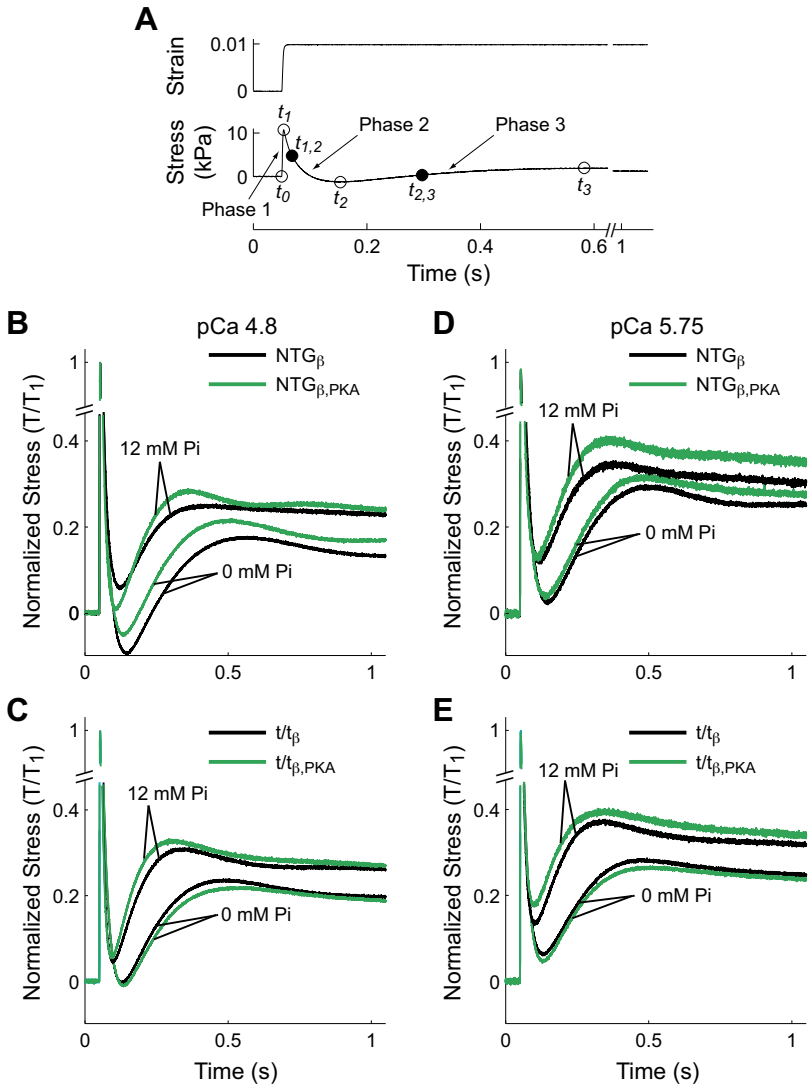

Figure 3. Step stretch response of skinned mouse myocardium and summary of effects of Pi and protein kinase A. *A*: measured values of strain and stress are plotted against time for a step length stretch of 1% muscle length. Open circles illustrate time points (t_1–t_3) bounding *phases 1–3*. Filled circles represent the time of half-maximal force for *phase 2* ($t_{1,2}$, the phase of relaxation or cross-bridge detachment) and *phase 3* ($t_{2,3}$, the phase of force redevelopment or cross-bridge recruitment). *B*: representative stress responses for NTG$_\beta$ bearing cMyBP-C at maximally activated pCa 4.8. Temporal characteristics of the stress response were significantly shortened by Pi and PKA. *C*: representative stress responses for t/t_β lacking cMyBP-C at maximally activated pCa 4.8. When compared against *A*, responses when cMyBP-C is absent were faster than those when cMyBP-C was present. Pi significantly shortened temporal characteristics of the response, but PKA did not significantly influence these characteristics when cMyBP-C was absent. *D*: stress responses for NTG$_\beta$ at pCa 5.75, near-half maximally activation, in the presence of cMyBPC. Again, Pi significantly accelerated the response. PKA also accelerated the response although more subtly and can be seen by a shorter time to the nadir between *phases 2* and *3*. *E*: stress responses for t/t_β at pCa 5.75 were accelerated by Pi, whereas PKA did not. cMyBP-C, cardiac myosin binding protein-C; NTG$_\beta$, nontransgenic; t/t_β, transgenic lacking cMyBP-C; PKA, protein kinase A.

main effect on $k_{f,dev}$ only at maximum calcium activation (Table 4, P = 0.025). The enhancement of $k_{f,dev}$ by PKA can be visualized in Fig. 4*B* where values in both NTG$_{\beta,PKA}$ and $t/t_{\beta,PKA}$ groups are generally higher than their respective non-PKA counterparts over the entire range of Pi conditions.

***Pi* × *PKA* and *Pi* × *PKA* × *MYBPC3* interactions.** Whether PKA activity modifies the Pi sensitivity of myosin-dependent rate constants can be answered with the Pi × PKA interaction statistic. We found a highly significant Pi × PKA interaction for the rate of force release, $k_{f,rel}$, at maximum calcium activation (Table 3, $P < 0.01$), which indicated that PKA modifies the Pi sensitivity of myosin detachment rate. As can be visualized in Fig. 4*A*, values for $k_{f,rel}$ in the PKA-treated myocardium were more sensitive to Pi and rose higher with each successively increasing Pi condition compared with non-PKA-treated myocardium either bearing or lacking cMyBP-C. This sensitivity, quantified as $\Delta k_{f,rel}/\Delta$Pi, similarly demonstrated a PKA main effect at pCa 4.8 affirming that PKA-mediated phosphorylation of the myofilaments enhances Pi-dependent myosin detachment rate.

The presence or absence of cMyBP-C did not affect the PKA effect on Pi sensitivity, as indicated by the lack of a Pi × PKA × MYBPC3 interaction for $k_{f,rel}$ (Table 3, P = 0.853). These two results for $k_{f,rel}$, a significant Pi × PKA interaction without a significant Pi × PKA × MYBPC3 interaction, suggest that the effect of PKA on enhancing the Pi sensitivity of myosin detachment rate is not mediated by cMyBP-C.

Although we did not find a significant Pi × PKA interaction for the rate of force development, $k_{f,dev}$, at maximum calcium activation (Table 4), we did find a significant three-way interaction Pi × PKA × MYBPC3 (Table 4, P = 0.032). As can be visualized in Fig. 4*B*, values for $k_{f,dev}$ in the PKA-treated $t/t_{\beta,PKA}$ myocardium lacking cMyBP-C were more sensitive to Pi compared with AP-treated t/t_β myocardium, whereas conversely values for $k_{f,dev}$ in the PKA-treated NTG$_{\beta,PKA}$ myocardium bearing cMyBP-C were less sensitive to Pi compared with AP-treated NTG$_\beta$

Table 3. *Results for rates of force release, $k_{f,rel}$, at maximal calcium activation pCa 4.8 and submaximal pCa 5.75*

$k_{f,rel}$ (s^{-1}) at pCa 4.8	NTG_{β}	$NTG_{\beta,PKA}$	t/t_{β}	$t/t_{\beta,PKA}$	ANOVA
n	9	8	10	10	
Pi (mM) = 0	46.96 ± 1.16	56.86 ± 2.74	63.31 ± 2.40	61.70 ± 1.26	Main effects
1	49.01 ± 2.06	62.68 ± 2.76	67.39 ± 2.19	70.74 ± 2.47	†MYBPC3, †Pi, †PKA
2	50.39 ± 2.15	64.55 ± 3.70	71.23 ± 2.38	77.41 ± 2.78	
3	51.50 ± 2.24	69.78 ± 3.73	78.09 ± 2.05	82.30 ± 2.26	Interactions
4	55.78 ± 2.40	74.02 ± 4.40	80.59 ± 2.31	87.29 ± 2.99	†Pi × MYBPC3
5	60.05 ± 2.12	76.68 ± 3.90	85.63 ± 2.52	92.70 ± 2.64	†Pi × PKA
8	61.17 ± 2.72	80.35 ± 3.29	93.04 ± 2.41	99.86 ± 3.19	
12	63.60 ± 3.35	86.86 ± 3.25	96.71 ± 3.25	106.46 ± 3.54	
$\Delta k_{f,rel}/\Delta$Pi	2.15 ± 0.24	3.27 ± 0.48	4.35 ± 0.37	5.51 ± 0.30	†MYBPC3, †PKA
$k_{f,rel}$ (s^{-1}) at pCa 5.75					
Pi (mM) = 0	50.59 ± 1.87	64.13 ± 3.88	73.30 ± 3.31	70.59 ± 2.45	Main effects
1	52.22 ± 1.84	65.87 ± 2.21	76.84 ± 3.33	72.80 ± 2.45	†MYBPC3, †Pi, ₊PKA
2	54.76 ± 2.41	66.53 ± 2.20	80.43 ± 3.67	79.32 ± 2.93	
3	57.71 ± 2.26	68.76 ± 2.31	83.87 ± 2.73	82.54 ± 2.60	Interactions
4	60.26 ± 2.18	75.74 ± 2.12	88.13 ± 3.46	84.86 ± 2.46	†PKA × MYBPC3
5	63.81 ± 2.19	79.94 ± 2.70	89.95 ± 3.24	89.34 ± 2.60	
8	66.37 ± 2.35	85.50 ± 2.96	93.21 ± 3.45	95.84 ± 3.19	
12	69.76 ± 2.42	83.84 ± 3.60	93.66 ± 3.74	94.29 ± 2.19	
$\Delta k_{f,rel}/\Delta$Pi	2.64 ± 0.25	2.68 ± 0.54	3.45 ± 0.33	3.81 ± 0.37	*MYBPC3

Values are means ± SE; *n*, number of strips. The sensitivity of $k_{f,rel}$ to Pi, $\Delta k_{f,rel}/\Delta$Pi, was calculated as the slope of linear regression using values measured at 0–5 mM Pi. Statistical results reflect repeated-measures ANOVA. Two-way ANOVA was then used to determine whether $\Delta k_{f,rel}/\Delta$Pi was influenced by absence of cMyBP-C and/or PKA. $^{*}P < 0.05$, $\dagger P < 0.01$. NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

myocardium. This result, while statistically significant, appears a subtle effect of cMyBP-C phosphorylation on the Pi sensitivity of this characteristic of force development. More specifically, PKA phosphorylation of cMyBP-C reduced the Pi sensitivity of force development characterized by $k_{f,dev}$.

Complex Modulus Sensitivity to cMyBP-C, Pi, and PKA

Example elastic and viscous moduli are presented in Fig. 5. All moduli were fit to Eq. *2* to assess cross-bridge rate of force development $2\pi b$ and cross-bridge detachment rate $2\pi c$ at pCa 4.8 and pCa 5.75. We present below results and statistical tests for $2\pi b$ and $2\pi c$.

The rates $2\pi b$ and $2\pi c$ were higher in myocardium lacking cMyBP-C compared with that bearing cMyBP-C as indicated by significant MYBPC3 main effects (Tables 5 and 6, $P < 0.05$). These effects can be visualized in Fig. 5, *B* and *D*, where the peak frequency of the viscous moduli, which best corresponds to $2\pi c$, was higher in t/t_{β} compared with NTG_{β}.

Pi effects and Pi × MYBPC3 interaction.

Moduli were also shifted to higher frequencies with higher concentrations of Pi as indicated by a highly significant Pi main effect for $2\pi b$ and $2\pi c$ (Tables 5 and 6, $P < 0.01$). Furthermore, the moduli of the myocardium lacking cMyBP-C were more sensitive to the effects of Pi than the that bearing cMyBP-C as

Table 4. *Results for rate of force development, $k_{f,dev}$, at maximal pCa 4.8 and submaximal pCa 5.75*

$k_{f,dev}$ (s^{-1}) at pCa 4.8	NTG_{β}	$NTG_{\beta,PKA}$	t/t_{β}	$t/t_{\beta,PKA}$	ANOVA
n	9	8	10	10	
Pi (mM) = 0	5.59 ± 0.24	6.12 ± 0.39	6.69 ± 0.41	6.37 ± 0.21	Main Effects
1	5.37 ± 0.37	6.45 ± 0.25	6.80 ± 0.43	7.57 ± 0.41	MYBPC3, †Pi, *PKA
2	5.98 ± 0.41	6.75 ± 0.27	7.19 ± 0.42	8.22 ± 0.53	
3	6.02 ± 0.44	7.12 ± 0.33	7.84 ± 0.41	9.08 ± 0.48	Interactions
4	6.69 ± 0.70	7.45 ± 0.31	8.20 ± 0.50	9.62 ± 0.58	Pi × MYBPC3
5	6.94 ± 0.31	8.07 ± 0.46	8.87 ± 0.51	9.91 ± 0.48	*Pi × PKA × MYBPC3
8	7.51 ± 0.44	8.23 ± 0.38	9.60 ± 0.50	10.67 ± 0.66	
12	8.42 ± 0.61	8.09 ± 0.27	9.82 ± 0.49	11.52 ± 0.59	
$\Delta k_{f,dev}/\Delta$Pi	0.33 ± 0.07	0.40 ± 0.05	0.52 ± 0.06	0.68 ± 0.05	†MYBPC3, *PKA
$k_{f,dev}$ (s^{-1}) at pCa 5.75					
Pi (mM) = 0	5.48 ± 0.24	6.10 ± 0.42	6.30 ± 0.39	6.48 ± 0.37	Main effects
1	5.90 ± 0.30	6.36 ± 0.44	6.93 ± 0.40	6.81 ± 0.71	†MYBPC3, †Pi
2	6.78 ± 0.53	6.36 ± 0.24	7.50 ± 0.36	8.38 ± 0.59	
3	6.81 ± 0.35	7.09 ± 0.36	7.80 ± 0.42	8.60 ± 0.48	
4	7.28 ± 0.20	7.38 ± 0.20	8.22 ± 0.36	9.05 ± 0.81	
5	7.53 ± 0.28	7.98 ± 0.44	8.80 ± 0.47	9.10 ± 0.45	
8	8.48 ± 0.51	8.24 ± 0.63	9.83 ± 0.62	10.10 ± 0.45	
12	8.69 ± 0.31	7.93 ± 0.57	9.67 ± 0.50	10.51 ± 0.98	
$\Delta k_{f,dev}/\Delta$Pi	0.39 ± 0.03	0.23 ± 0.10	0.48 ± 0.04	0.64 ± 0.07	†MYBPC3 *PKA × MYBPC3

Values are means ± SE; *n*, number of strips. The sensitivity of $k_{f,dev}$ to Pi, $\Delta k_{f,dev}/\Delta$Pi, was calculated as the slope by linear regression using values measured at 0–5 mM Pi. Statistical results reflect repeated-measures ANOVA. Two-way ANOVA was then applied to $\Delta k_{f,dev}/\Delta$Pi. $^{*}P < 0.05$, $\dagger P < 0.01$. NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

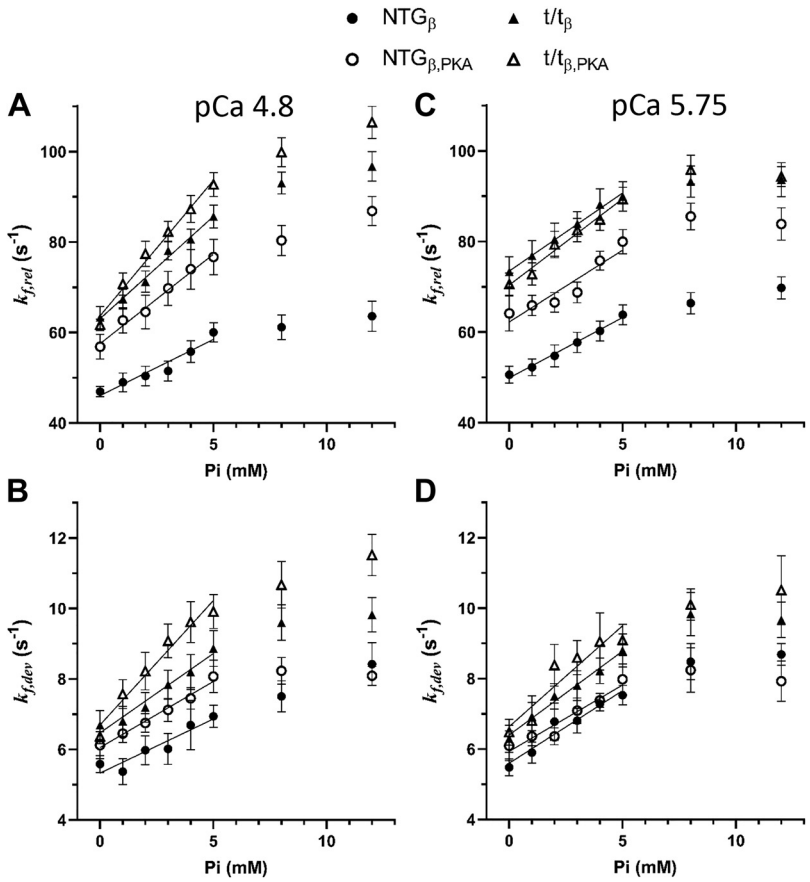

Figure 4. Effects of Pi and PKA on rates of force release and force development from step responses. *A*: the rate of force release, $k_{f,rel}$, at pCa 4.8 was significantly higher in the t/t_β and $t/t_{\beta,PKA}$ compared with NTG$_\beta$ and NTG$_{\beta,PKA}$ and was accelerated with increasing Pi. PKA also accelerated $k_{f,rel}$ in both populations, but significantly more so in the NTG$_\beta$ bearing cMyBP-C than in the t/t_β lacking cMyBP-C. *B*: rate of force development, $k_{f,dev}$, at pCa 4.8 demonstrated similar trends to $k_{f,rel}$ including enhanced in the t/t_β and $t/t_{\beta,PKA}$ lacking cMyBP-C. The rate $k_{f,dev}$ was also accelerated by Pi and PKA. *C*: at submaximal calcium activation, pCa 5.75, $k_{f,rel}$ was enhanced by lack of cMyBP-C and also by Pi and PKA. The enhancement of $k_{f,rel}$ by PKA was dependent upon the presence of cMyBP-C and implicates cMyBP-C as the primary mediator of PKA effects on β-cardiac myosin cross-bridge detachment rate. *D*: at pCa 5.75, $k_{f,dev}$ was also enhanced by lack of cMyBP-C and increasing Pi, but not by PKA. Means ± SE. cMyBP-C, cardiac myosin binding protein-C; NTG$_\beta$, nontransgenic; t/t_β, transgenic lacking cMyBP-C; PKA, protein kinase A.

indicated by a highly significant Pi × MYBPC3 interaction for both 2π*b* and 2π*c* at both pCa 4.8 and pCa 5.75 (Tables 5 and 6, $P < 0.01$). As an example of these results, the peak in the viscous modulus in the NTG$_\beta$ (Fig. 5*C*) was shifted from ~2 to ~4 Hz with 12 mM Pi, whereas the peak in the t/t_β (Fig. 5*D*) was shifted from ~5 to ~10 Hz. These data demonstrate that the frequency of the peak viscous modulus, which reflects the myosin detachment rate, was shifted to higher frequencies by Pi, and more so when cMyBP-C was absent from the sarcomere.

This differential sensitivity to Pi due to the presence or absence of cMyBP-C is affirmed with the statistical analysis of Δ2π*b*/ΔPi and Δ2π*b*/ΔPi (Tables 5 and 6). The Pi sensitivity of the rate of force development, Δ2π*b*/ΔPi, was significantly enhanced by the absence of cMyBP-C at both maximal and submaximal calcium activations (Table 5, MYBPC3 main effect $P < 0.01$). The Pi sensitivity of the myosin detachment rate, Δ2π*c*/ΔPi, was significantly enhanced by the absence of cMyBP-C at maximal calcium activation (Table 6, MYBPC3 main effect $P < 0.05$).

These results collectively suggest that, whereas cMyBP-C plays a role in slowing cross-bridge detachment rate and associated rate of force development, cMyBP-C also suppresses Pi-dependent enhancement of cross-bridge detachment rate and rate of force development.

PKA effects and PKA × MYBPC3 interaction.

PKA treatment enhanced the frequency characteristic 2π*c* at pCa 4.8 (Table 4, PKA main effect $P = 0.043$). This is a subtle effect at 0 Pi but is clearer at 12 mM Pi where the peak frequency of the viscous modulus is higher in PKA-treated myocardium (Fig. 5, *B* and *D*). We did not observe a significant PKA × MYBPC3 interaction for 2π*c* with this data set, which would have implicated a cMyBP-C dependence in the PKA effect on 2π*c*.

Pi × PKA and Pi × PKA × MYBPC3 interactions.

Although PKA treatment did not result in statistically significant effects on 2π*b*, PKA treatment did enhance the Pi sensitivity of 2π*b* as indicated by a significant Pi × PKA interaction at pCa 4.8 (Table 4, $P = 0.014$). This sensitivity represented as Δ2π*b*/ΔPi demonstrated a significant PKA effect, affirming that the Pi-dependent enhancement of the rate of force development was further elevated by PKA. This effect is also illustrated in Fig. 6*A* where the values of 2π*b* are enhanced with increasing Pi and more so following treatment with PKA.

Although we did not find a Pi × PKA interaction for 2π*c*, we did find a significant Pi × PKA × MYBPC3 interaction for 2π*c* at pCa 5.75 (Table 6, $P < 0.01$), which indicates that PKA phosphorylation of cMyBP-C influences the sensitivity of myosin detachment rate to Pi. The Pi sensitivity of myosin detachment rate, Δ2π*c*/ΔPi, demonstrated a PKA × MYBPC3 interaction, which indicates that cMyBP-C influences the PKA-dependent changes to Pi-sensitive myosin detachment. The values of Δ2π*c*/ΔPi among the four populations examined suggest that, when cMyBP-C is present, there is virtually no change in Pi

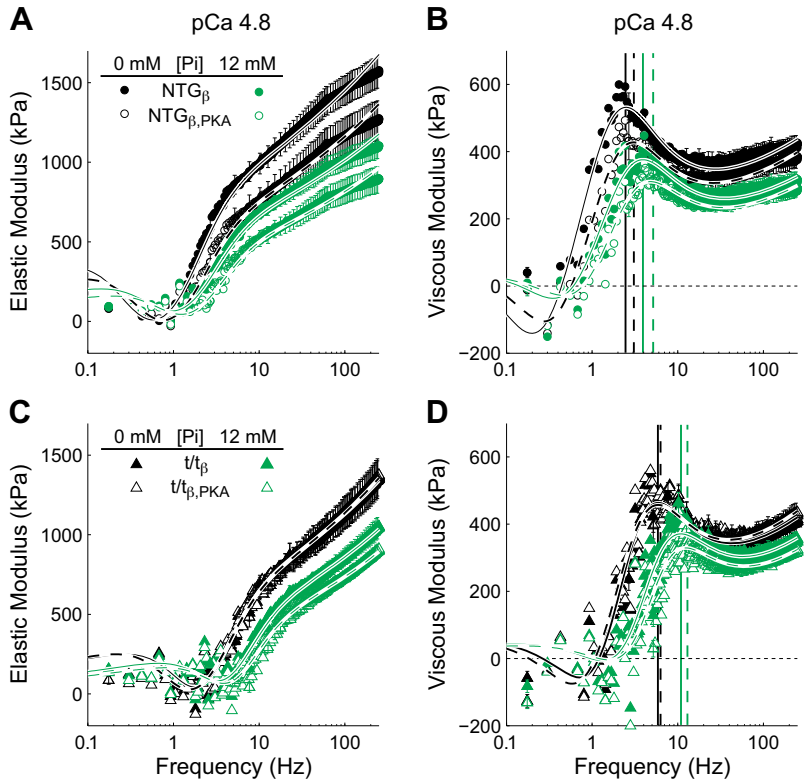

Figure 5. Effect of cMyBP-C, Pi, and PKA on elastic and viscous moduli measured by stochastic length perturbation analysis at pCa 4.8. Elastic (*A* and *C*) and viscous (*B* and *D*) moduli plotted against oscillatory frequency for NTG_{β} and t/t_{β} mice. Black and green lines indicate 0 and 12 mM Pi conditions, respectively. Solid and dashed lines over the data represent fits to Eq. *2* after AP or PKA treatment, respectively. Vertical lines in *B* and *D* indicate the frequencies of the peak viscous moduli and are black for 0 mM Pi, green for 12 mM Pi, solid for AP, and dashed for PKA. Characteristics of the moduli were shifted to higher frequencies with lack of cMyBP-C in the t/t_{β}, increasing Pi, and PKA treatment. AP, alkaline phosphatase; cMyBP-C, cardiac myosin binding protein-C; NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

sensitivity of myosin detachment caused by PKA, and when cMyBP-C is absent, there is a significant enhancement of the Pi sensitivity of myosin detachment caused by PKA. This result is illustrated in Fig. 6*D* where values for $2\pi c$ are shown to rise with increasing Pi but increase more so after PKA treatment in the t/t_{β} lacking cMyBP-C and less so after PKA treatment in the NTG_{β} bearing cMyBP-C. These data indicate that PKA phosphorylation of cMyBP-C does not affect the Pi sensitivity of the myosin detachment rate characterized by $2\pi c$.

DISCUSSION

This study examined the role of cMyBP-C phosphorylation by PKA in Pi-sensitive β-cardiac myosin-dependent mechan-

Table 5. *Results for rate of force development, 2πb, at maximal pCa 4.8 and submaximal pCa 5.75*

$2\pi b$ (s^{-1}) at pCa 4.8	NTG_{β}	$NTG_{\beta,PKA}$	t/t_{β}	$t/t_{\beta,PKA}$	ANOVA
n	9	8	10	10	
Pi (mM) = 0	7.98 ± 0.59	7.98 ± 0.63	10.98 ± 0.61	8.64 ± 0.62	Main effects
1	8.27 ± 0.54	9.60 ± 0.63	12.79 ± 0.79	11.49 ± 0.89	†MYBPC3, †Pi
2	8.46 ± 0.60	10.87 ± 0.73	14.51 ± 0.79	13.15 ± 1.10	
3	9.23 ± 0.62	11.66 ± 1.03	16.54 ± 0.83	15.91 ± 1.31	Interactions
4	9.73 ± 0.84	12.61 ± 0.99	18.18 ± 0.84	18.60 ± 1.29	†Pi × MYBPC3
5	9.90 ± 0.84	13.70 ± 1.12	19.32 ± 0.84	19.78 ± 1.59	*Pi × PKA
8	10.72 ± 1.38	14.36 ± 1.28	21.70 ± 1.24	23.12 ± 1.63	
12	12.71 ± 1.71	15.32 ± 1.40	23.48 ± 1.60	26.37 ± 1.69	
$\Delta 2\pi b/\Delta Pi$	0.55 ± 0.10	0.92 ± 0.18	1.62 ± 0.17	2.23 ± 0.33	†MYBPC3, *PKA
$2\pi b$ (s^{-1}) at pCa 5.75					
Pi (mM) = 0	8.01 ± 0.52	7.95 ± 0.84	9.85 ± 0.86	7.47 ± 0.66	Main effects
1	8.09 ± 0.54	9.71 ± 1.09	12.05 ± 1.04	8.92 ± 0.85	†MYBPC3, †Pi
2	9.58 ± 0.55	9.75 ± 1.34	13.17 ± 0.86	10.45 ± 0.92	
3	9.48 ± 0.72	10.31 ± 1.51	14.57 ± 1.11	11.85 ± 1.01	Interactions
4	10.42 ± 0.61	11.66 ± 1.59	15.71 ± 1.42	12.84 ± 0.91	†Pi × MYBPC3
5	11.15 ± 0.66	12.20 ± 1.71	17.51 ± 1.35	13.09 ± 1.32	
8	12.41 ± 1.00	15.00 ± 1.81	19.85 ± 1.37	16.43 ± 1.45	
12	13.31 ± 1.26	16.88 ± 2.03	21.78 ± 1.64	16.61 ± 1.51	
$\Delta 2\pi b/\Delta Pi$	0.68 ± 0.07	0.79 ± 0.19	1.41 ± 0.23	1.18 ± 0.20	†MYBPC3

Values are means ± SE; *n*, number of strips. The sensitivity of 2πb to Pi was calculated and denoted here as $\Delta 2\pi b/\Delta Pi$. Statistical results reflect repeated-measures ANOVA. Two-way ANOVA was then applied to $\Delta 2\pi b/\Delta Pi$. *$P < 0.05$, †$P < 0.01$. NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

Table 6. *Results for rate of myosin detachment rate, 2πc, at maximal pCa 4.8 and submaximal pCa 5.75*

$2\pi c$ (s^{-1}) at pCa 4.8	NTG_{β}	$NTG_{\beta,PKA}$	t/t_{β}	$t/t_{\beta,PKA}$	ANOVA
n	9	8	10	10	
Pi (mM) = 0	15.75 ± 1.08	20.24 ± 1.44	20.63 ± 1.43	21.35 ± 1.66	Main effects
1	16.77 ± 1.56	21.29 ± 1.54	21.17 ± 1.01	24.36 ± 1.91	*MYBPC3, †Pi, *PKA
2	17.98 ± 1.69	22.07 ± 1.71	22.28 ± 0.92	26.08 ± 1.82	
3	19.17 ± 1.70	23.82 ± 1.76	23.33 ± 0.80	27.76 ± 1.84	Interactions
4	20.63 ± 1.74	24.55 ± 1.85	24.62 ± 0.77	28.80 ± 2.01	†Pi × MYBPC3
5	21.98 ± 1.70	26.30 ± 1.90	26.39 ± 0.95	31.07 ± 1.96	
8	24.20 ± 1.78	29.02 ± 1.87	28.99 ± 1.19	34.25 ± 2.04	
12	27.07 ± 1.84	31.06 ± 1.81	32.16 ± 1.75	37.81 ± 1.95	
Δ2πc/ΔPi	1.15 ± 0.12	1.20 ± 0.18	1.32 ± 0.13	1.71 ± 0.17	*MYBPC3
$2\pi c$ (s^{-1}) at pCa 5.75					
Pi (mM) = 0	17.11 ± 1.34	19.56 ± 1.08	22.33 ± 1.55	24.99 ± 1.72	Main effects
1	19.01 ± 1.65	20.92 ± 1.12	23.22 ± 1.51	27.46 ± 2.09	†MYBPC3, †Pi
2	19.74 ± 1.64	22.62 ± 1.00	24.40 ± 1.49	29.60 ± 2.45	
3	22.14 ± 1.83	24.67 ± 1.19	25.84 ± 1.58	31.20 ± 2.68	Interactions
4	23.25 ± 1.86	26.10 ± 1.52	27.79 ± 1.81	33.03 ± 2.55	†Pi × MYBPC3
5	24.75 ± 1.89	26.71 ± 1.25	29.24 ± 1.85	35.77 ± 2.73	†Pi × PKA × MYBPC3
8	27.63 ± 2.06	27.95 ± 1.44	31.46 ± 1.81	38.87 ± 3.09	
12	29.44 ± 2.23	28.49 ± 1.59	34.43 ± 2.04	42.71 ± 2.97	
Δ2πc/ΔPi	1.56 ± 0.13	1.52 ± 0.16	1.48 ± 0.13	2.29 ± 0.31	*PKA × MYBPC3

Values are means ± SE; *n*, number of strips. The sensitivity of 2πc to Pi was calculated and denoted here as Δ2πc/ΔPi. Statistical results reflect repeated-measures ANOVA. Two-way ANOVA was then applied to Δ2π*b*/ΔPi. *$P < 0.05$, †$P < 0.01$. NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

ics, as reflected in rates of detachment and recruitment, in an intact myofilament lattice. Our results confirmed that β-cardiac myosin detachment rate and recruitment rate are enhanced when cMyBP-C is removed from the sarcomere (14–17). The most straightforward interpretation includes a role for cMyBP-C in normally slowing myosin cross-bridge detachment rate by inhibiting MgADP release and extending the cross-bridge lifetime (15, 17, 34). We also found that β-cardiac myosin kinetics were elevated with increasing Pi concentrations in myocardial strips both with and without cMyBP-C.

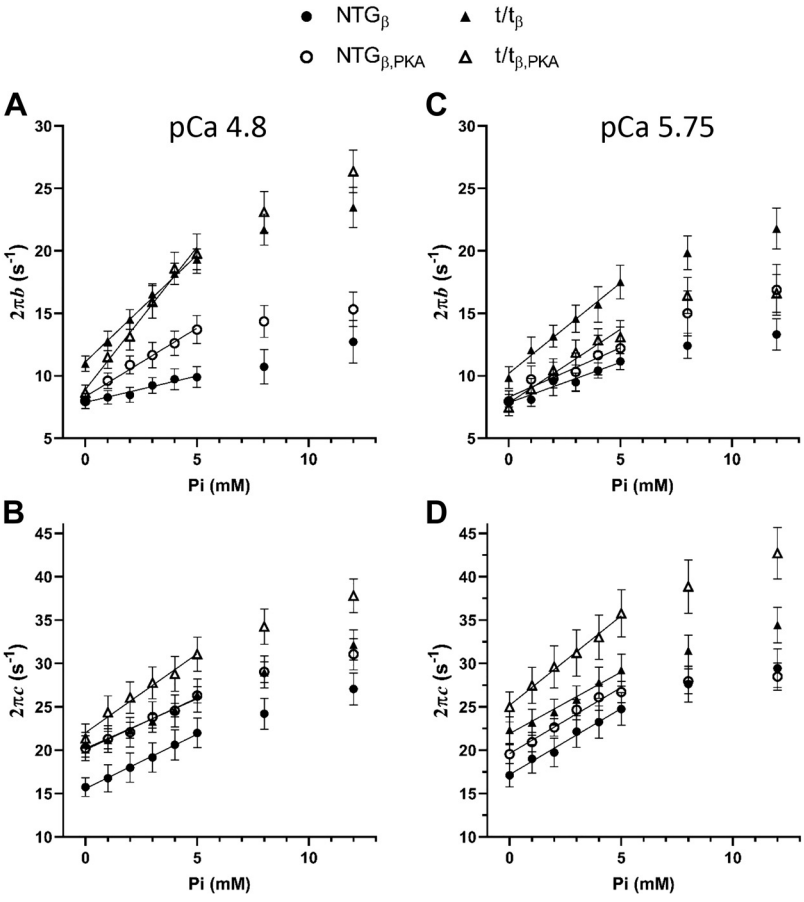

Figure 6. Effect of cMyBP-C, Pi, and PKA on myosin cross-bridge kinetics in skinned myocardial strips. *A*: the rate of force development (2π*b*) at maximum calcium activation (pCa 4.8) was significantly enhanced by a lack of cMyBP-C and by increasing Pi. The sensitivity of 2π*b* to Pi was furthermore enhanced by the absence of cMyBP-C. These data suggest that cMyBP-C inhibits β-cardiac myosin cross-bridge kinetics including those dependent upon Pi. PKA also enhanced 2π*b* at pCa 4.8 although subtly. *B*: myosin cross-bridge detachment rate (2π*c*) at pCa 4.8 was enhanced by a lack of cMyBP-C and by increasing Pi. The Pi sensitivity of 2π*c* was further enhanced by a lack of cMyBP-C. *C* and *D*: results for 2π*b* and 2π*c* at submaximal calcium activation mirrored those at pCa 4.8 with enhancement due to lack of cMyBP-C and with increasing Pi. The sensitivity of 2π*b* and 2π*c* to Pi was further enhanced when cMyBP-C was absent. Means ± SE. cMyBP-C, cardiac myosin binding protein-C; NTG_{β}, nontransgenic; t/t_{β}, transgenic lacking cMyBP-C; PKA, protein kinase A.

This finding was expected as it recapitulates reports of elevated myosin cross-bridge detachment rate or myosin-dependent muscle function, e.g., velocity of shortening, with increasing Pi (22, 41). The mechanism by which Pi influences the force-producing myosin cross bridge, however, is still debated and may include Pi-induced inhibition of myosin cross-bridge formation (42, 43), reversal of the myosin power stroke (20, 23, 24), and detachment of the myosin cross-bridge without reversal (21). Despite not knowing the specific action of Pi on the myosin cross bridge, we found that the absence of cMyBP-C further augmented the sensitivity of β-cardiac myosin to Pi. Our data provide evidence in support of the idea that cMyBP-C in the intact sarcomere normally inhibits the Pi effects on myosin detachment rate, likely through suppressing Pi-dependent detachment or reversal, in addition to its slowing MgADP release rate.

We also found that PKA treatment led to a faster rate of β-cardiac myosin cross-bridge detachment, as reflected in the rate $k_{f,rel}$ and $2\pi c$, both with and without cMyBP-C, but was significantly more pronounced in the presence of cMyBP-C. This result was consistent across a range of Pi concentrations and agrees with the enhanced rate of force release observed following the quick stretch attributable to PKA phosphorylation of cMyBP-C at low Pi (9, 10, 40, 44). Our results at both maximal and submaximal activations substantiate the idea that Pi sensitivity of β-cardiac myosin cross-bridge detachment rate, reflected in $k_{f,rel}$ and $2\pi c$, in the absence of cMyBP-C is mimicked by PKA-mediated phosphorylation of cMyBP-C. Thus, PKA-phosphorylation of cMyBP-C effectively removes cMyBP-C inhibition of Pi-dependent power stroke reversal.

We found cMyBP-C phosphorylation also enhances the rate of force development, $k_{f,dev}$, across the full range of Pi examined at maximum calcium activation (Figs. 4*B*). However, we did not detect any effect of PKA phosphorylation of cMyBP-C on Pi sensitivity of $k_{f,dev}$ and its frequency analog $2\pi b$. Other studies in an α-cardiac myosin background, most notably Stelzer et al. (9, 10) and Tong et al. (45), do report PKA-induced enhancement of the rate of force development due to cMyBP-C. Differences may reside in the cardiac myosin isoform, but further confirmation in the β-cardiac myosin is warranted.

Inferring cMyBP-C Function

How can cMyBP-C slow myosin detachment in the intact sarcomere? Given that cMyBP-C is not a kinase or phosphatase and is not obviously some other regulator of protein post-translational modification, it is presumed that cMyBP-C must act through its binding to nearby sarcomeric structures. We have already demonstrated that the C-terminus of cMyBP-C affects longitudinal thick filament stiffness (46) and its NH_2-terminus effects transverse stiffness of the myofilament lattice (25). The concept that the lifetime, unitary force, or any other attribute of myosin cross-bridge can be affected by structural elements far from the myosin head is exemplified by the development of cardiomyopathies due to point mutations in cMyBP-C or in the myosin rod (47). Similarly, cMyBP-C may influence myosin cross-bridge force production by effectively providing a stabilizing scaffold on the lattice and thereby on the formed myosin cross-bridge. The result is an inhibition of MgADP release and Pi-dependent detachment and thereby prolongs the cross-bridge lifetime (33). A role for cMyBP-C in mechanically stabilizing myosin heads in a super-relaxed state has been described (48), where ablation of cMyBP-C removes this stabilization and myosin becomes more active. We would presume that phosphorylation of cMyBP-C would again mimic ablation, thereby releasing the super-relaxed myosin and increasing the active myosin population.

The many observations so far related to enhanced myosin-dependent rates through cMyBP-C phosphorylation (9, 10, 44, 45) suggest that the unbinding of the NH_2-terminus from actin and myosin S2 (4, 7, 8) is sufficient to augment stiffness of the myofilament lattice and influence myosin cross-bridge rate of MgADP release or Pi-dependent power stroke reversal. Based on data from Michalek et al. (49), the phosphorylation of the cMyBP-C cardiac-specific motif leads to a more stable and less extensible state of the C0-C3 fragment of cMyBP-C. The structural attributes of cMyBP-C after phosphorylation might then diminish its interaction with S2 or actin, whichever is slowing detachment rate, and thus result in mechanical characteristics that appear more like an effective loss of the NH_2-terminus of the molecule from the sarcomere. Because of the low MyBP-C:myosin ratio in the sarcomere C-zone and the absence of MyBP-C in the D-zone, we are currently biased against the idea that cMyBP-C phosphorylation affects myosin cross-bridge cycling through direct interactions with neighboring myosin molecules. Although our measurements cannot definitely rule out either of these possible mechanisms, we favor the idea of a S2-cMyBP-C interaction that indirectly stabilizes the β-cardiac myosin cross-bridge through a more stable myofilament lattice, in part because our findings were largely consistent between maximal and submaximal thin filament activations, and therefore relatively independent of Ca^{2+}-activation level.

The NH_2-terminus also plays a significant role in modulating actin velocity (2, 6, 8, 50) and must not be discounted as a contributing factor in the intact lattice. It is possible that an interaction of cMyBP-C NH_2-terminus with the thin filament would slow or inhibit movement of the thin filament relative to the thick filament and thereby influence detachment of any attached cross-bridge (2, 6, 8, 50). Although this viscous-like interaction is possible in settings of significant sarcomere shortening or lengthening, we suspect this possible structural influence in our measurements. In particular, the 0.125% amplitude of the stochastic length perturbation analysis translates to only ~1.2 nm movement of thin filaments relative to thick filaments in both directions, and therefore, the viscous-like load attributable to cMyBP-C would be negligible in our assays.

Phosphorylation of Other Sarcomeric Proteins

In addition to cMyBP-C, PKA also phosphorylates the sarcomeric proteins troponin-I (TnI) and titin. PKA-dependent phosphorylation of TnI at Ser-23/24 reduces myofilament Ca^{2+} sensitivity and increases cross-bridge cycling rate, which is thought to increase myocardial power output and facilitate faster relaxation during diastole (reviewed in 35 and 51). As noted earlier, PKA treatment of our myocardial

strips also led to faster rates of cross-bridge detachment, which could contribute to accelerated relaxation during diastole. However, we also observed that PKA treatment produced a minimal effect on cross-bridge rates of force development and detachment in the absence of cMyBP-C, and this minimal effect of PKA persisted at both pCa levels. These relatively small changes in PKA-dependent cross-bridge cycling kinetics in the absence of cMyBP-C suggest that phosphorylation of TnI and titin contributes minimally to β-cardiac myosin kinetics, as suggested previously for α-cardiac myosin (9, 10, 45). Thus, we attribute the PKA-dependent increases in cross-bridge cycling kinetics in the NTG_{β} measurements to cMyBP-C phosphorylation.

PKA-dependent phosphorylation of titin's N2B region reduces titin-dependent passive longitudinal stiffness of the sarcomere, which is thought to promote more rapid and complete ventricular filling during diastole (52–54). However, cMyBP-C is also responsible for ~50% of longitudinal stiffness of the thick filament and the sarcomere (46). Titin runs the whole length of the sarcomere, and the effective myofilament lattice stiffness would be ranked $NTG_{\beta} > NTG_{\beta,PKA} > t/t_{\beta} > t/t_{\beta,PKA}$, which interestingly coincides with the ranked order of increasing rates of cross-bridge detachment among these populations (Figs. 4 and 6). These data could be interpreted to suggest that the mechanical consequences of titin phosphorylation by PKA require the presence of cMyBP-C. More specifically, the reduced titin stiffness as occurs with PKA phosphorylation may influence myosin kinetics only when cMyBP-C is abundant enough to contribute to a relatively high baseline level of sarcomere stiffness from which sarcomere stiffness can be reduced. This argument would suggest that contributions to sarcomeric stiffness due to cMyBP-C ablation from the sarcomere are greater than those due to titin phosphorylation by PKA. As hearts do not function well without cMyBP-C, these data would support the idea that phosphorylation of titin upon β-adrenergic stimulation contributes to faster cross-bridge detachment that can accelerate diastolic relaxation.

Limitations

The use of hypothyroidism to induce β-myosin expression in rodent hearts presents limitations. The myocardium develops some fibrosis, and myocytes develop a degree of disarray not seen before hypothyroidism (27). Furthermore, hypothyroidism could possibly exacerbate the cardiomyopathy already established in the t/t. However, a transgenic mouse expressing β-myosin without cMyBP-C is not available. We believe the use of hypothyroidism is justified given the usefulness of the β-myosin background to be relevant to the myosin isoform found in the human ventricle.

We calculated rates of force development ($2\pi b$) and myosin cross-bridge detachment ($2\pi c$) using stochastic length perturbations, which provides a frequency spectrum of the force response. We have not thoroughly examined the limitations of each method, but we do recognize that $2\pi b$ of the stochastic length perturbation assay is estimated less precisely than $2\pi c$ due to the comparatively fewer number of frequencies represented in the spectrum near the *b*-frequency. Nevertheless, both measures were precise enough for statistical analysis and inference of underlying myosin function.

The rates $k_{f,dev}$ and $2\pi b$ should theoretically correspond to each other if the force response demonstrates characteristics of a linear system (Fig. 7*A*). Likewise, results for $k_{f,rel}$ and $2\pi c$ should also correspond (Fig. 7*B*). We found high correlation coefficients ($0.75 \leq R \leq 0.91$) for $k_{f,dev}$ versus $2\pi b$ and for $k_{f,rel}$ versus $2\pi c$. These correlations support the idea that the paired variables reflect similar phenomena. We also found that values of $k_{f,dev}$ and $k_{f,rel}$ from the quick stretch assay were ~2.5 times and 8 times larger than estimates of $2\pi b$ and $2\pi c$ from stochastic-length perturbation analysis. This discrepancy may have arisen from estimating $k_{f,dev}$ and $k_{f,rel}$ indirectly using time durations. It is noteworthy that the oscillations of *phase 4* in the raw data suggest that, whereas first-order rate constants $k_{f,dev}$ and $k_{f,rel}$ are reasonable characteristics, they do not describe the phenomenon thoroughly. Further work on fully characterizing the force response to length perturbations, whether in the time domain or frequency domain, is warranted.

Conclusions

Our results suggest that PKA-mediated phosphorylation of cMyBP-C in the context of an intact myofilament lattice of the sarcomere elicits a cMyBP-C structural conformation that

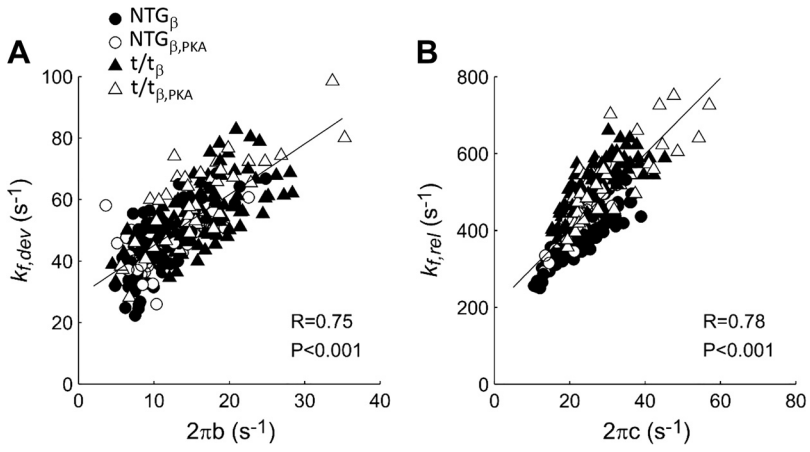

Figure 7. Comparison between cross-bridge rate constants from force response to a quick length step (Eq. *1*) and stochastic perturbation analysis (Eq. *2*). Rates of cross-bridge force development (*A*) and detachment (*B*) estimated step analysis are plotted against those estimated by stochastic perturbation analyses. Data represent estimated rates pooled from all measurements as pCa and Pi varied plus pre- and post-PKA treatment. Correlation coefficients (*R*) and *P* values are listed within each panel. Solid lines represent linear fits to the data. PKA, protein kinase A.

results in higher rates of β-cardiac myosin cross-bridge detachment, including Pi-dependent detachment, that would typically be inhibited or slowed at lower phosphorylation levels. These effects of cMyBP-C phosphorylation mimic those resulting from an absence of cMyBP-C. However, the influence of cMyBP-C phosphorylation specifically on the Pi dependency of myosin cross-bridge kinetics underlying the rate of force development does not fully mimic that of an absence of cMyBP-C. Although our use of mouse myocardium lacking cMyBP-C points to the phosphorylation of cMyBP-C as the primary PKA-sensitive influence on myosin cross-bridge kinetics, we recognize that it is still possible that effects of PKA-mediated phosphorylation on other proteins may require the presence of cMyBP-C to transmit their influence.

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Present address of B. C. W. Tanner: Dept. of Integrative Physiology and Neuroscience, Washington State Univ., Pullman, WA 99164.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.C.W.T., M.J.P., J.R., and B.M.P. conceived and designed research; B.C.W.T., M.J.P., and Y.W. performed experiments; B.C.W.T., M.J.P., and Y.W. analyzed data; B.C.W.T., M.J.P., Y.W., J.R., and B.M.P. interpreted results of experiments; B.C.W.T., M.J.P., and B.M.P. prepared figures; B.C.W.T. and B.M.P. drafted manuscript; B.C.W.T., M.J.P., J.R., and B.M.P. edited and revised manuscript; B.C.W.T., M.J.P., Y.W., J.R., and B.M.P. approved final version of manuscript.

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