Supplemental material for Wang et al. 2012
Cardiac myosin isoforms exhibit differential rates of MgADP release and MgATP binding detected by myocardial viscoelasticity.

Supplemental Methods

Euthyroid rats were used as controls in examination of tension-pCa relationships presented in this supplement. In all measures of viscoelasticity and analyses of t_{on}, the hyperthyroid rats expressing ~100% α-MyHC were used as controls against the PTU-fed rats expressing ~100% β-MyHC.

Skinned Myocardial Strips

Solution concentrations (mmol/L) were formulated by solving equations describing ionic equilibria according to Godt and Lindley [1].

Sinusoidal length perturbations of amplitude 0.125% strip length were applied at 0.125-100 Hz as previously described [2, 3]. The elastic and viscous moduli, $E(\omega)$ and $V(\omega)$, respectively, were measured from the in-phase and out-of-phase portions of the tension response to length perturbation. A complex modulus, $Y(\omega)$, was defined as $E(\omega) + iV(\omega)$, where $i = \sqrt{-1}$. The fitting of the frequency dependence of the complex moduli to a mathematical model (Equation S1) provided estimates of six model parameters (A, k, B, $2\pi b$, C, $2\pi c$), and myosin t_{on} as was calculated as $(2\pi c)^{-1}$ [3].

$$Y(\omega) = A(i\omega)^k - B\left(\frac{i\omega}{2\pi b + i\omega}\right) + C\left(\frac{i\omega}{2\pi c + i\omega}\right) \qquad \text{Equation S1.}$$

The A-term in Equation S1 reflects the viscoelastic mechanical response of passive elements in the muscle. In our interpretation, parameter A represents the combined mechanical stiffness of the parallel elastic elements, the myofilaments and the number of strongly bound crossbridges, and k describes the degree of the viscoelastic response as elastic ($k \rightarrow 0$) versus viscous ($k \rightarrow 1$). The B- and C-terms reflect enzymatically driven myosin crossbridge formation in activated muscle. Parameters B and C reflect the number of crossbridges formed × their mean stiffness (or total myosin crossbridges available × duty ratio

× mean stiffness), and the rate parameters $2\pi b$ and $2\pi c$ reflect crossbridge kinetics sensitive to biochemical perturbations known to affect enzymatic activity, most notably concentrations of MgATP, MgADP and P_i [2, 4, 5]. According to Kawai et al. [2], the parameter $2\pi b$ refers to the sum of characteristic rates of myosin isomerization and P_i-dependent crossbridge detachment. According to Palmer [6], $2\pi b$ refers to the characteristic rate at which crossbridge time-on is modified by strain on the myosin head. The parameter $2\pi c$ is equal to the myosin detachment rate, termed g by Huxley [7], the reciprocal of the mean myosin crossbridge lifetime or time-on, t_{on} [3].

Supplemental Results

Supplemental Table 1 provides animal characteristics including age, body weight and heart weight that may differ due to hypothyroidism. There was a slight reduction in heart mass for WKY_{PTU} and $SvEv_{PTU}$ compared to control animals. Table 2 provides characteristics of transgenic mice, which were comparable to controls.

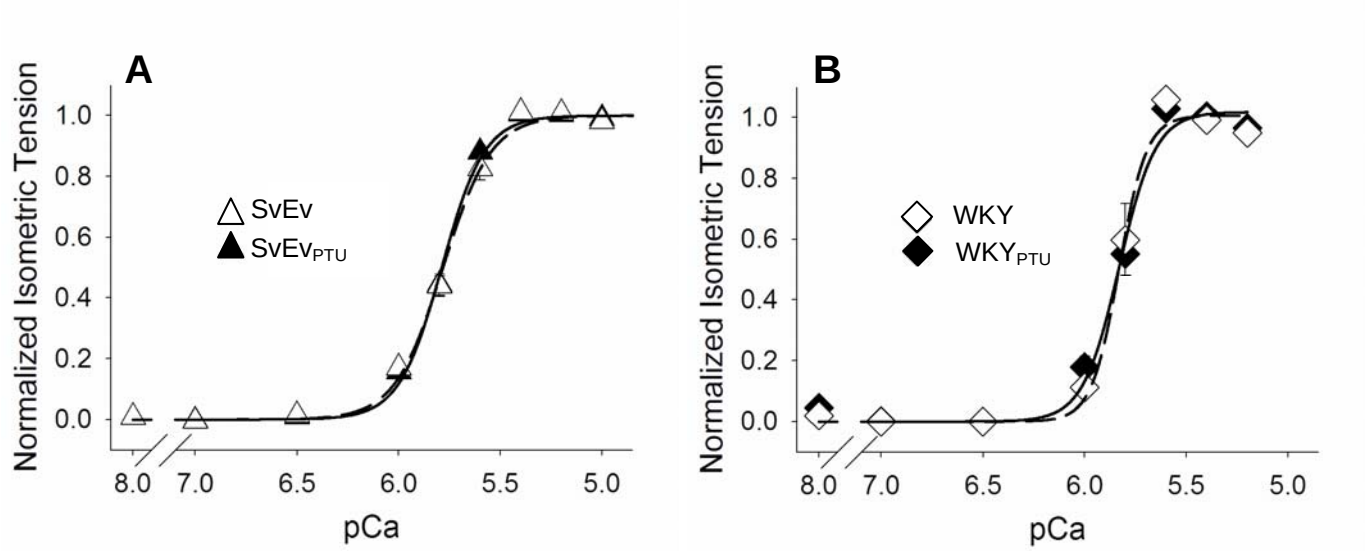
Supplemental Table 1: Animal characteristics. Hypothyroidism due to PTU diet resulted in reduced ventricular mass in addition to upregulating expression of β-MyHC. **P*<0.05, †*P*<0.01 by t-test against nonPTU-fed controls. WKY = rat strain Wistar-Kyoto. SvEv = mouse strain 129/SvEv.

	WKY (n=4)	WKY_{PTU} (4)	SvEv (7)	$SvEv_{PTU}$ (6)
Age (wks)	29.4±0.9	29.4±0.4	29.1±1.2	29.7±1.2
LV / Body mass (mg/g)	4.48±1.3	3.16±1.9 †	3.35±1.2	3.07±1.3
RV+LV / Body mass (mg/g)	5.57±1.7	3.89±2.5 †	4.33±1.4	3.80±1.7 *

Supplemental Table 2: Animal characteristics of transgenic mice and controls. Transgenic mice expressing ~70% β-MyHC ($FVB_{\beta TG}$) exhibited no difference in ventricular mass per body weight compared to FVB controls of similar age.

	FVB (n=8)	$FVB_{\beta TG}$ (4)
Age (wks)	6.1±0.2	6.0±0.4
LV / Body mass (mg/g)	3.82±0.12	3.92±0.23
RV+LV / Body mass (mg/g)	5.49±0.15	5.81±0.49

Supplemental Figure 1 presents the tension-pCa relationships for SvEv and $SvEv_{PTU}$ mouse myocardium (replotted from Fig. 2B) and for WKY and WKY_{PTU} rat myocardium after alkaline phosphatase (AP) treatment. These data illustrate that thin filament calcium sensitivity was similar between the hypothyroid and euthyroid animals after AP treatment. Supplemental Table 3 presents the characteristics of the tension-pCa relationships in rat myocardium.

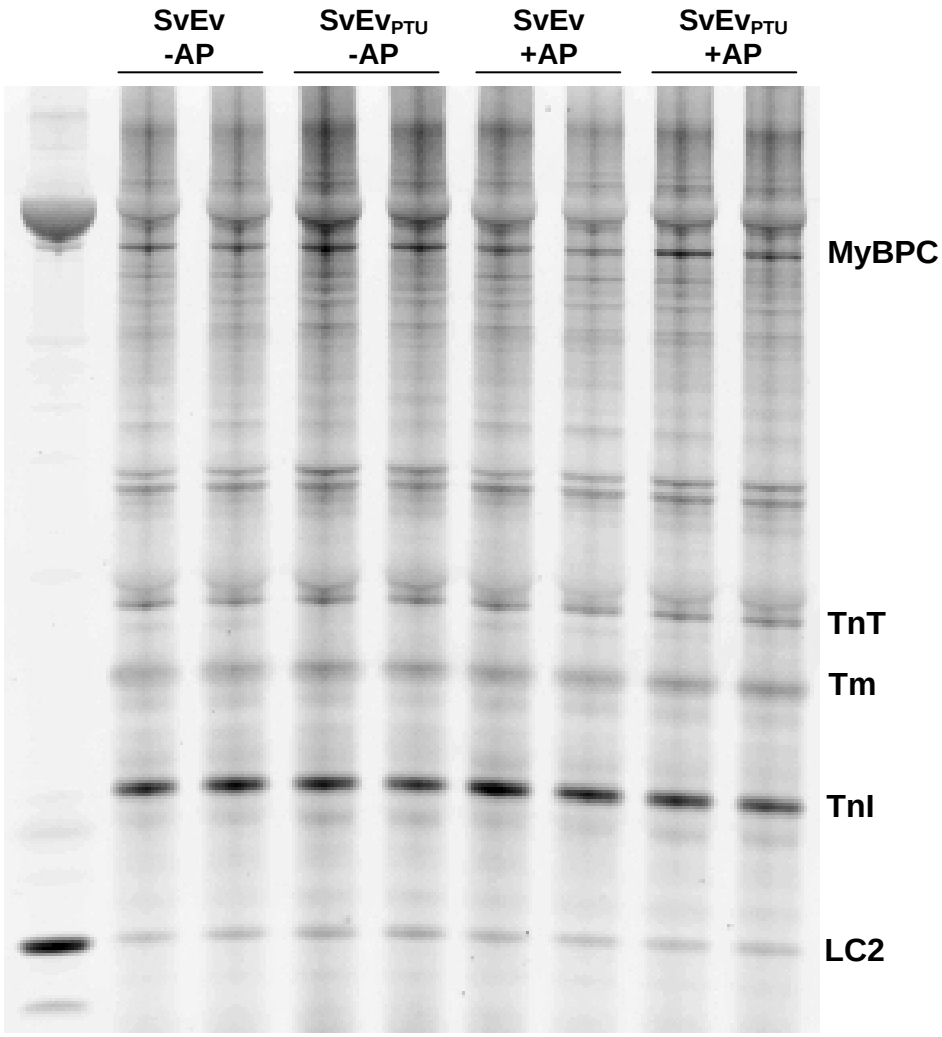


Supplemental Figure 1: Tension-pCa relationships for skinned mouse (**A**) and rat (**B**) myocardium after AP treatment are not different between PTU-fed hypothyroid animals and euthyroid controls. These data suggest that AP treatment normalizes any differences in thin filament calcium sensitivity that may arise due to PTU treatment.

Supplemental Table 3: Characteristics of tension-pCa relationships due to PTU diet in rat myocardium treated with AP treatment. Differences reported at #$P<0.10$, *$P<0.05$, †$P<0.01$ by t-test against nonPTU-fed controls.

	WKY (n=4)	WKY_{PTU} (4)
T_{min} ($mN.mm^{-2}$)	2.48±1.07	2.43±0.62
T_{max} ($mN.mm^{-2}$)	14.7±3.72	16.57±1.28
T_{dev} ($mN.mm^{-2}$)	12.23±2.94	14.43±0.90
$[Ca^{2+}]_{50}$ (μM)	1.46±0.87	1.47±0.38
pCa_{50}	5.84±0.03	5.83±0.01
n_{Hill}	9.09±1.67	5.43±0.81

Phospho-stain by Pro-Q diamond (Supplemental **Figure 2**) did not reveal visually significant differences in protein phosphorylation due to PTU diet or AP treatment. More specific tests for phosphorylation reduction by alkaline phosphatase are presented in the main text.

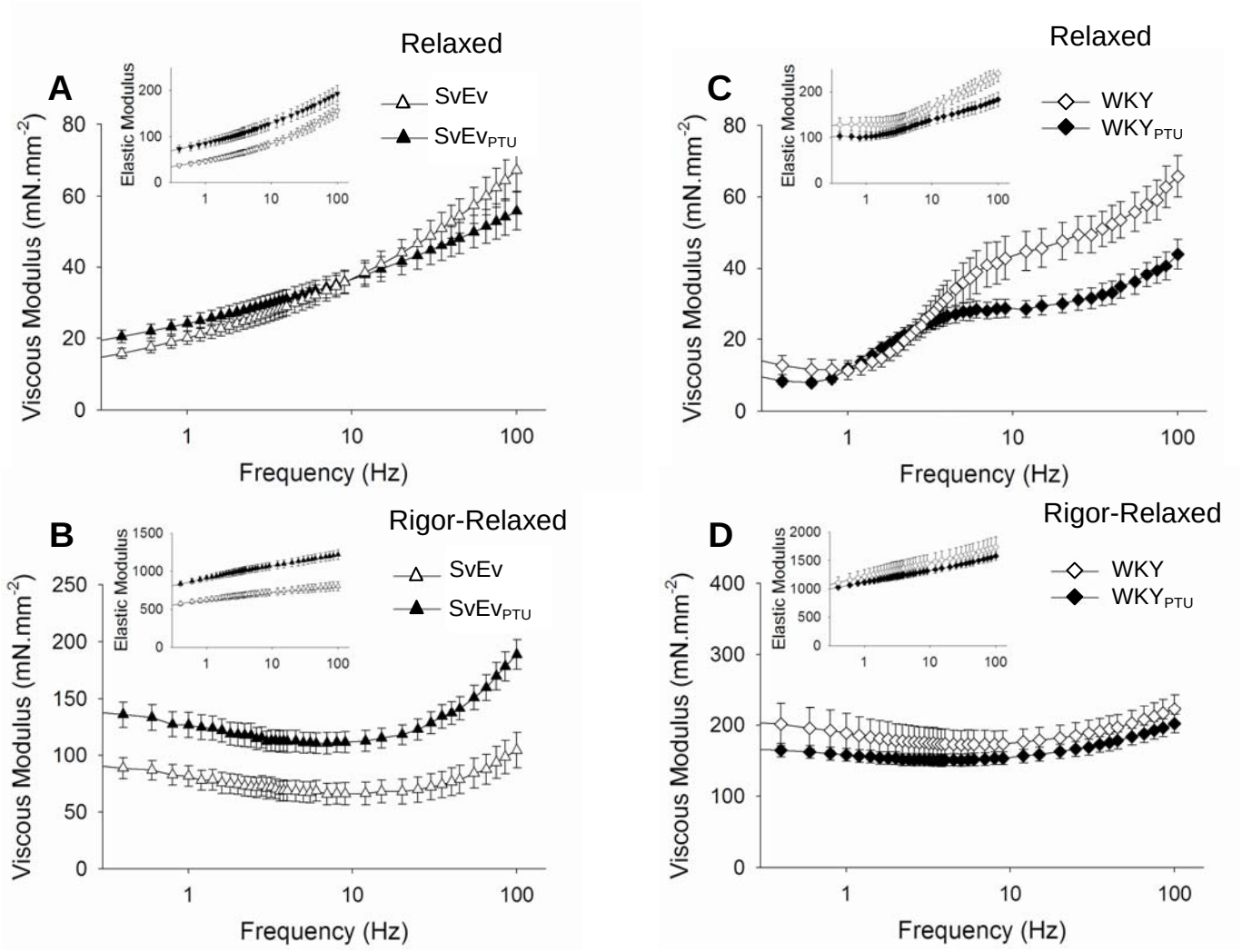


Supplemental Figure 2: Phosphorylation of troponin-I (TnI), troponin-T (TnT), tropomyosin (Tm), myosin regulatory light chain (LC2) and myosin binding protein-C (MyBP-C) detected by Pro-Q diamond stain were not different between groups of mice or between treatments with and without alkaline phosphatase (AP). The first lane is of skeletal muscle myosin standard.

Supplemental Figure 3A illustrates the elastic and viscous moduli under relaxed (pCa 8) conditions. The $SvEv_{PTU}$ strips demonstrated a higher elastic modulus at all frequencies, which reflects a higher stiffness that is not dependent upon myosin crossbridge formation. Because hypothyroidism by PTU diet is known to cause an increased density of collagen in the myocardium, we attribute the higher elastic modulus in the $SvEv_{PTU}$ strips to collagen or any other sarcomeric protein that may have been differentially expressed and resides mechanically parallel with the longitudinal axis of the sarcomere.

Supplemental Figure 3B illustrates higher elastic and viscous moduli in the $SvEv_{PTU}$ strips under rigor conditions minus the moduli detected under relaxed conditions. These rigor-relaxed moduli reflect the myosin crossbridge-dependent stiffness of the myocardial strips. The myosin crossbridge-dependent stiffness of the $SvEv_{PTU}$ strips reflected in both the elastic and viscous moduli was 30-50% higher at all frequencies compared to SvEv strips. The myosin crossbridge-dependent stiffness has been attributed to the lever arm and S2 region not incorporated into the myosin rod [8-10]. These data would suggest that these portions of the myosin molecule are significantly more stiff in the β-MyHC compared to α-MyHC, or that there are significantly greater myofilament cross-sectional density after PTU treatment. We do not have evidence to support or refute the latter.

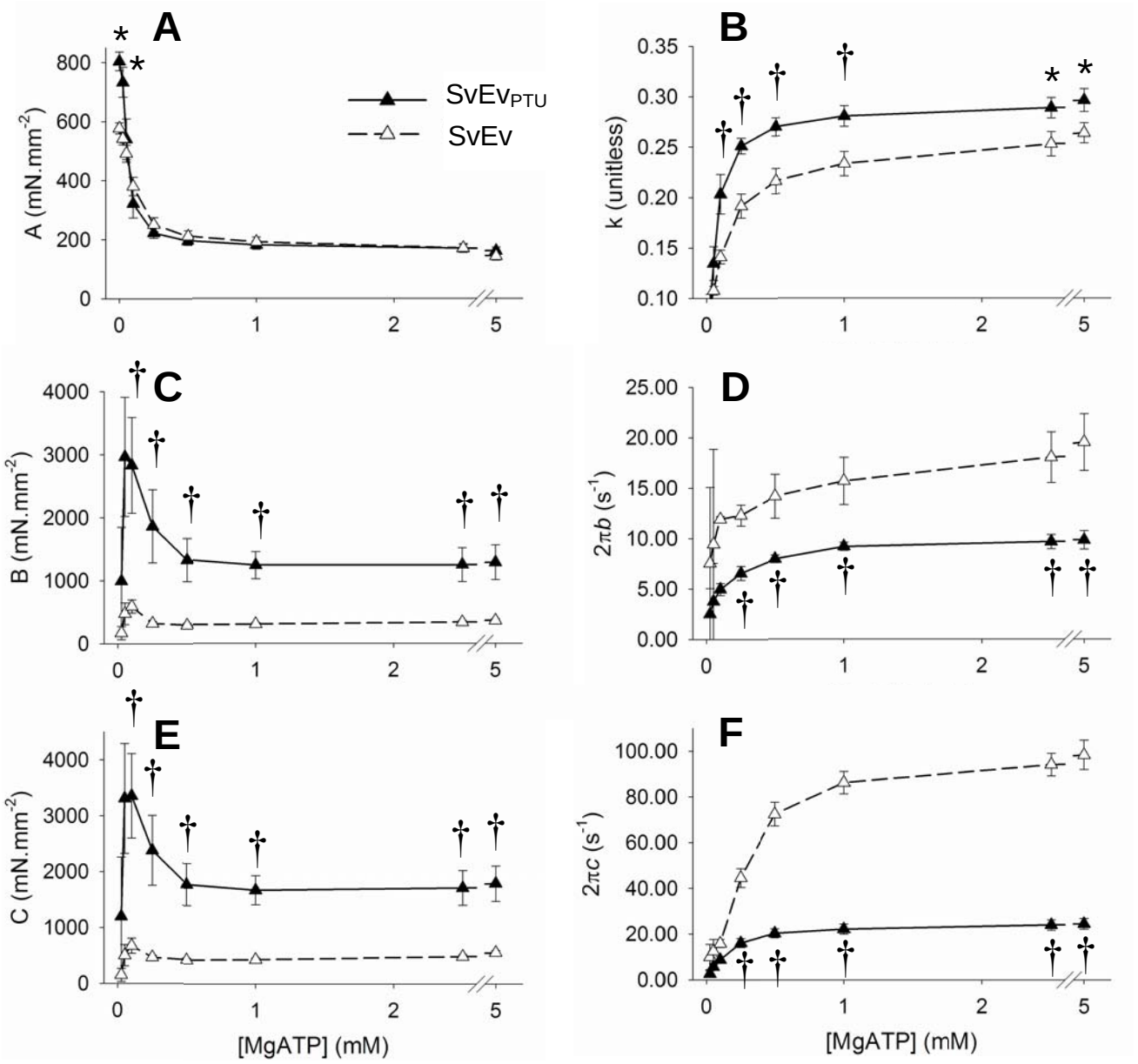
Comparable measurements for relaxed (Figure 3C) rat myocardium indicate that WKY was slightly stiffer high frequencies than WKY_{PTU} myocardium under relaxed conditions. Relaxed-rigor (Figure 3D) moduli values suggest no differences in crossbridge-dependent stiffness for WKY vs. WKY_{PTU}.



Supplemental Figure 3. Viscoelastic characteristics of mouse and rat myocardial strips under relaxed and rigor conditions with AP treatment. **A**. Under relaxed conditions, mouse myocardium of the SvEv$_{PTU}$ was considerably stiffer than that of SvEv, although not more viscous. This may have been due to an increased collagen content in the PTU-fed mice. **B**. Under rigor conditions, the contribution of myosin crossbridges to stiffness is maximized. Elastic and viscous moduli measured under relaxed conditions were subtracted from those measured under rigor conditions and represent a crossbridge-dependent stiffness of the myocardium. The β-MyHC crossbridges in the PTU-fed mice were significantly stiffer and more viscous compared to α-MyHC crossbridges in the euthyroid controls. **C**. Under relaxed conditions, rat myocardium of the WKY was slightly more stiff (higher elastic modulus at higher frequencies) compared to WKY$_{PTU}$, but not more viscous. The most conspicuous attribute of the rat myocardium under relaxed conditions was the evidence for myosin crossbridge formation. The characteristic dips and shoulders indicate the formation of cycling crossbridges even when thin filament activation would be considered negligible. **D**. Rigor conditions suggest that crossbridge-dependent stiffness is not different between α- and β-MyHC in the rat.

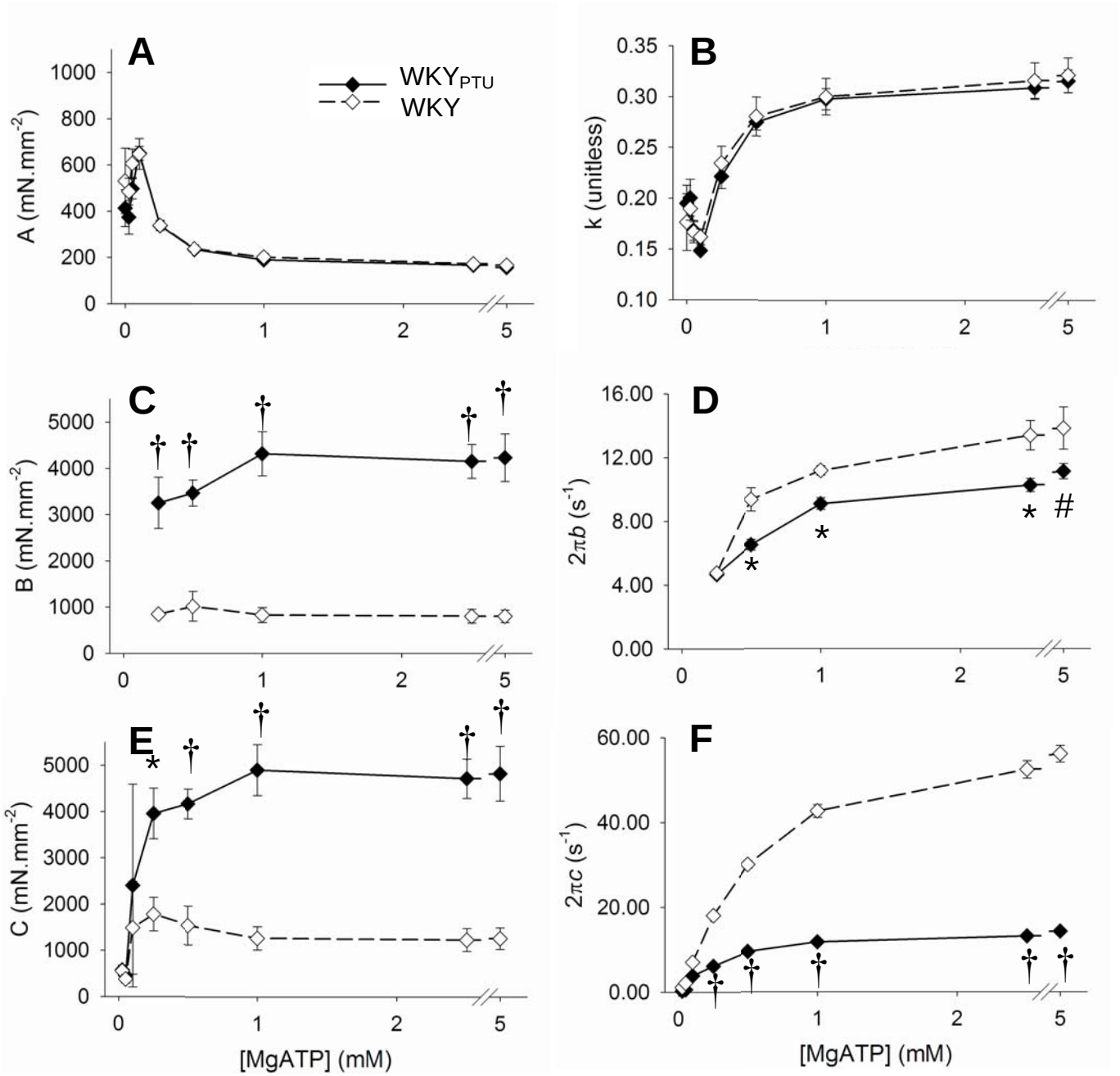
Supplemental Figure 4 presents estimates of the model parameter in Equation S1 for mouse myocardium. The magnitude *A*, which represents myocardial stiffness, was significantly higher in the $SvEv_{PTU}$ myocardium compared to SvEv at the lowest MgATP concentrations less than 0.1 mM (Supplemental Figure 4A). As MgATP concentrations rose, the magnitude parameters *A*, *B* and *C* were reduced (Supplemental Figures 4A, C and E), which likely reflects a concomitant reduction in the duty ratio as MgATP became more plentiful and myosin crossbridge time-on was reduced.

The parameter *k* was higher in the $SvEv_{PTU}$ than in the SvEv over nearly all MgATP concentrations (Supplemental Figure 4B). The higher *k* reflects a more viscous quality to the viscoelastic stiffness in the $SvEv_{PTU}$ myocardium compared to that of the SvEv. The characteristics rates $2\pi b$ and $2\pi c$ were significantly lower in the $SvEv_{PTU}$ compared to SvEv at nearly all MgATP concentrations (Supplemental Figures 4D and 4F). The lower rate $2\pi b$ in the $SvEv_{PTU}$ would reflect a lower rate of force redevelopment as might occur after a quick stretch [3]. The lower rate $2\pi c$ in the $SvEv_{PTU}$ would reflect a lower myosin crossbridge off-rate. The lower myosin detachment rate for the $SvEv_{PTU}$ myocardium suggests a longer myosin crossbridge time-on in the β-MyHC compared to α-MyHC.



Supplemental Figure 4: Complex modulus model parameters vs. MgATP found in mouse myocardium. **A**. The myocardial viscoelastic stiffness, reflected in magnitude *A*, was greater in the $SvEv_{PTU}$ under near rigor conditions, i.e., when MgATP < 0.05 mM. **B**. The parameter *k* was higher in the $SvEv_{PTU}$ than in SvEv and therefore demonstrated a greater viscous quality in the $SvEv_{PTU}$ myocardium compared to SvEv. **C and E**. The magnitudes *B* and *C* reflect the total number of myosin crossbridges available × duty ratio × mean stiffness. Only the duty ratio would be dependent upon MgATP. The dependency of *B* and *C* upon MgATP is consistent with a reduced duty ratio with increasing MgATP. **D and F**. The rate parameter $2\pi b$, which may refer to the rates at which the power stroke occurs and is reversed, was significantly lower in the $SvEv_{PTU}$ compared to SvEv. The myosin crossbridge off-rate, $2\pi c$, is dramatically reduced in the $SvEv_{PTU}$ compared to SvEv across all MgATP concentrations, and reflects the significantly reduced myosin crossbridge off-rate in the mouse β-MyHC compared to α-MyHC.

Supplemental Figure 5 presents estimates of the model parameter in Equation 3 for rat myocardium. Note that the control rat was hyperthyroid due to thyroxine injection and expressed ~100% α-MyHC. The magnitude *A* and k were not different between WKY_{PTU} and WKY myocardium at all MgATP concentrations examined (Supplemental Figures 5A and B). In contrast to the mouse myocardium, as MgATP concentrations rose, the magnitude parameters *B* and *C* did not change much with MgATP concentration (Supplemental Figures 5C and E). Magnitudes *B* and *C* were consistently higher in the β-MyHC compared to α-MyHC. The characteristics rates $2\pi b$ and $2\pi c$ were significantly lower in the WKY_{PTU} compared to WKY at nearly all MgATP concentrations (Supplemental Figures 5D and F). As was the case with the mouse myocardium, the lower rate $2\pi b$ in the WKY_{PTU} would reflect a lower rate of force redevelopment after a quick stretch [2, 3, 11]. The lower rate $2\pi c$ in the WKY_{PTU} would reflect a lower myosin crossbridge off-rate and a longer t_{on} compared to WKY.



Supplemental Figure 5: Complex modulus model parameters vs. MgATP found in rat myocardium. **A and B**. The myocardial viscoelastic stiffness, reflected in magnitude *A*, and viscous vs elastic quality, reflected in *k*, were similar WKY_{PTU} and WKY for all MgATP concentrations examined. **C and E**. The magnitudes *B* and *C* reflect the total number of myosin crossbridges available × duty ratio × mean stiffness. Neither *B* nor *C* demonstrated significant dependence upon MgATP unless <0.025 mM MgATP. **D and F**. The rate parameter $2\pi b$, which may refer to the rates at which the power stroke occurs and is reversed, was significantly lower in the WKY_{PTU} compared to WKY controls. The myosin crossbridge off-rate, $2\pi c$, is dramatically reduced in the WKY_{PTU} compared to WKY, and reflects the significantly reduced myosin crossbridge off-rate in the rat β-MyHC compared to α-MyHC.

Supplemental References

[1] Godt RE, Lindley BD. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. J Gen Physiol. 1982 80(2): 279-97.
[2] Kawai M, Brandt PW. Sinusoidal analysis: a high resolution method for correlating biochemical reactions with physiological processes in activated skeletal muscles of rabbit, frog and crayfish. J Muscle Res Cell Motil. 1980 1(3): 279-303.
[3] Palmer BM, Suzuki T, Wang Y, Barnes WD, Miller MS, Maughan DW. Two-state model of acto-myosin attachment-detachment predicts C-process of sinusoidal analysis. Biophys J. 2007 93(3): 760-9.
[4] Kawai M, Halvorson HR. Role of MgATP and MgADP in the cross-bridge kinetics in chemically skinned rabbit psoas fibers. Study of a fast exponential process (C). Biophys J. 1989 55(4): 595-603.
[5] Maughan D, Moore J, Vigoreaux J, Barnes B, Mulieri LA. Work production and work absorption in muscle strips from vertebrate cardiac and insect flight muscle fibers. Advances in experimental medicine and biology. 1998 453: 471-80.
[6] Palmer BM. A strain-dependency of myosin off-rate must be sensitive to frequency to predict the B-process of sinusoidal analysis. Adv Exp Med Biol. 2010 682: 57-75.
[7] Huxley AF. Muscle structure and theories of contraction. Prog Biophys Biophys Chem. 1957 7: 255-318.
[8] Higuchi H, Yanagida T, Goldman YE. Compliance of thin filaments in skinned fibers of rabbit skeletal muscle. Biophys J. 1995 69(3): 1000-10.
[9] Schmid MF, Epstein HF. Muscle thick filaments are rigid coupled tubules, not flexible ropes. Cell Motil Cytoskeleton. 1998 41(3): 195-201.
[10] Tyska MJ, Warshaw DM. The myosin power stroke. Cell Motil Cytoskeleton. 2002 51(1): 1-15.
[11] Kawai M, Zhao Y. Cross-bridge scheme and force per cross-bridge state in skinned rabbit psoas muscle fibers. Biophys J. 1993 65(2): 638-51.