Altered Crossbridge Kinetics in the αMHC^{403/+} Mouse Model of Familial Hypertrophic Cardiomyopathy

Edward Blanchard, Christine Seidman, J.G. Seidman, Martin LeWinter, David Maughan

Abstract—A mutation in the cardiac β-myosin heavy chain, Arg403Gln (R403Q), causes a severe form of familial hypertrophic cardiomyopathy (FHC) in humans. We used small-amplitude (0.25%) length-perturbation analysis to examine the mechanical properties of skinned left ventricular papillary muscle strips from mouse hearts bearing the R403Q mutation in the α-myosin heavy chain (αMHC^{403/+}). Myofibrillar disarray with variable penetrance occurred in the left ventricular free wall of the αMHC^{403/+} hearts. In resting strips (pCa 8), dynamic stiffness was ≈40% greater than in wild-type strips, consistent with elevated diastolic stiffness reported for murine hearts with FHC. At pCa 6 (submaximal activation), strip isometric tension was ≈3 times higher than for wild-type strips, whereas at pCa 5 (maximal activation), tension was marginally lower. At submaximal calcium activation the characteristic frequencies of the work-producing (b) and work-absorbing (c) steps of the crossbridge were less in αMHC^{403/+} strips than in wild-type strips (b=11±1 versus 15±1 Hz; c=58±3 versus 66±3 Hz; 27°C). At maximal calcium activation, strip oscillatory power was reduced (0.53±0.25 versus 1.03±0.18 mW/mm²; 27°C), which is partly attributable to the reduced frequency b, at which crossbridge work is maximum. The results are consistent with the hypothesis that the R403Q mutation reduces the strong binding affinity of myosin for actin. Myosin heads may accumulate in a preforce state that promotes cooperative activation of the thin filament at submaximal calcium but blunts maximal tension and oscillatory power output at maximal calcium. The calcium-dependent effect of the mutation (whether facilitating or debilitating), together with a variable degree of fibrosis and myofibrillar disorder, may contribute to the diversity of clinical symptoms observed in murine FHC. (Circ Res. 1999;84:475-483.)

Key Words: cardiomyopathy ■ myosin mutation ■ mouse ■ crossbridge kinetics

Familial hypertrophic cardiomyopathy (FHC) is a human autosomal dominant disease characterized by ventricular hypertrophy, with myocyte and myofibrillar disarray. The disorder is often devastating, resulting in heart failure and premature death, although many individuals survive to old age.1–3 Affected individuals usually experience shortness of breath, angina, and arrhythmia, but many individuals are remarkably asymptomatic.

The heterogeneous effects of FHC mutations are reflected in the diversity of clinical assessments of ventricular function.1–5 FHC is a disease of the sarcomere, with genetic point mutations causing replacement of 1 amino acid for another in β-myosin heavy chain (β-MHC).1,6 myosin essential light chain,7 myosin regulatory light chain,7 myosin binding protein C,8 α-tropomyosin,9 or troponin T.9 Each mutation probably affects the 3-dimensional shape of the mutant protein and its interaction with regulatory ligands and other sarcomeric proteins in different and complex ways, resulting in an inconsistent clinical picture of cardiac function for the general FHC population.

The Arg403Gln (R403Q) missense mutation in the β-MHC is one of the most extensively characterized FHC mutations.10–13 The R403Q missense mutation lies on the globular head of β-MHC near the actin binding interface9 and causes a decrease in affinity for actin, a depression of actin-activated myosin ATPase activity, and a slower actin velocity motility assays.8,10–12,14 A decreased power output at all loads and a higher stiffness-to-force ratio has also been reported for maximally activated human soleus-skinned fibers containing a β-MHC R403Q mutation.13

Recently a murine homolog of the R403Q myosin mutation has been generated using targeted recombination.15 Homozygous (αMHC^{403/403}) mice die within a week or 2 after birth. Heterozygous (αMHC^{403/+}) mice survive for at least 1 year, although their hearts exhibit histopathology and dysfunction that resemble human FHC, including compromised exercise...
capacity. Spindler et al. used 1 of the αMHC<sup>403Q<sup>−/−</sup> lines to obtain additional mechanical and energetic information from hearts perfused with the Langendorff method. Although no evidence of systolic dysfunction was apparent, diastolic function was significantly impaired (decreased rate of left ventricular relaxation, increased end-diastolic pressure).<sup>3</sup> Nuclear magnetic resonance measurements showed a reduced availability of high-energy phosphates (lower phosphocreatine (PCr) content, higher inorganic phosphate content) that may have contributed to the observed diastolic dysfunction. Spindler et al. hypothesized that altered actin-myosin binding kinetics of the R403Q crossbridges underlie the decreased rate of left ventricular relaxation and increased end-diastolic pressure.

To gain further insight into the functional consequences of the R403Q myosin defect at the sarcomere level, we used the αMHC<sup>403Q<sup>−/−<sup> mouse model to examine isometric force, oscillatory power, and crossbridge kinetics derived from dynamic stiffness measurements in skinned left ventricular papillary muscle strips. At submaximal calcium activation (pCa 6), isometric tension of αMHC<sup>403Q<sup>−/− strips is significantly greater than that of wild-type mice, whereas at maximal calcium activation (pCa 5), isometric tension and oscillatory power are less. Crossbridge rate constants are depressed, consistent with the hypothesis that the R403Q mutation reduces the strong binding affinity of myosin for actin. The kinetic data suggest that myosin heads accumulate in a preforce state that promotes cooperative activation of the thin filament at submaximal calcium but blunts tension and oscillatory power output at maximal calcium.

Materials and Methods

Animals, Dissection, and Preparation for Ultrastructural Analysis

Fourteen heterozygous αMHC<sup>403Q/+<sup> mutant mice<sup>15<sup> and 15 αMHC<sup>+/+<sup> wild-type (control) mice were used in the study (males, 24 to 36 weeks old). The mice, treated according to the guidelines of the Animal Care and Use Committee of the University of Vermont, were euthanized by cervical dislocation and their hearts removed. Twelve mutant hearts and 13 wild-type hearts earmarked for mechanical studies (see below) were placed immediately in a standard Krebs solution<sup>14<sup> containing 30 mmol/L 2,3-butanedione monoxime (BDM) and gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. BDM protects the myocardial tissue from cutting injury. The remaining hearts (2 mutant and 2 wild type) were fixed, stained with osmium tetroxide, and embedded using conventional methods.<sup>19</sup> Thin sections were contrasted with lead and uranyl acetate, and micrographs were taken on a scanning transmission electron microscope (JEOL 1210) operated at 60 kV.

Fibrosis was quantified in 3 mutant and 2 wild-type hearts. Fixed hearts were cut transversely at the midventricular level, and the apical portions (containing sections of the free wall and papillary muscles) were embedded in glycolmethacrylate. Sections were cut at 2 μm and stained with Mason’s trichrome stain for collagen. An Olympus BX-50 microscope (Olympus Corp) was used to view the slides. Images (magnification ×400) were collected with a true-color video camera (DXC-960 MD/LLP; Sony) and camera adapter (CMA-D2; Sony) coupled with an Olympus viewing screen and remote control unit (RM-930; Sony). Images were stored, manipulated, and analyzed with a Sun SPARCstation 5 computer (Sun Microsystems) using IMIX/IMAGIST Version 8 software (Integrated Microanalyzer for Imaging Software, Princeton Gamma-Tech). The TrueColor module of the imaging software was used for area-extraction analysis. Trichrome-stained collagen was imaged in blue, and all other material in red. Fibrosis was defined as the area in blue divided by the total area imaged (blue plus red), expressed as a percentage.

Strip Preparation

Left ventricular papillary muscles were dissected in BDM-Ringer’s solution to yield thin strips (diameter, ~0.125 mm; length, ~1.5 mm), which were tethered with silk, transferred to a vessel containing relaxing solution, and stretched just taut. Relaxing solution contained 5 mmol/L MgATP, 40 mmol/L PCr, 240 U/mL creatine kinase (CK), 1 mmol/L free Mg<sup>2+</sup>, 0.11 mmol/L CaCl<sub>2</sub>, 5 mmol/L EGTA, and 20 mmol/L BES buffer (pH 7.0); ionic strength was adjusted to 190 mmol/L with added sodium methane sulfonate. The strips were demembranated (skinned) by adding 1% wt/vol Triton X-100, incubated overnight at 4°C, transferred to detergent-free relaxing solution (with 50% wt/vol glycerol and 10 μg/mL leupeptin), and stored at ~20°C. Strips were used within 1 week of dissection (generally the day after dissection).

The tethered strips were placed in relaxing solution in a second vessel, where small aluminum clips were used to isolate a uniform segment (~0.7 mm in length). The clipped segment was cut free and transferred to a 30-μL drop of relaxing solution in a glass-bottom aluminum chamber filled with mineral oil. The strip was attached to a strain gauge and piezoelectric motor, described elsewhere.<sup>19</sup> Analogue displacement and tension signals were monitored by a thermal strip chart recorder and digital storage oscilloscope. Oil temperature was maintained at 27°C or 37°C (±0.5°C) by a Peltier-effect thermoelectric recorder (Cambion; Cambridge Thermionic Corp).

The skinned strips were stretched (incrementally, by 0.05-μm steps per sarcomere) to a sarcomere spacing of ~2.2 μm (estimated with an inverted microscope and filar micrometer). Strip tension (mN/mm<sup>2</sup>) was calculated by dividing force by the cross-sectional area obtained by multiplying the width imaged from the top by the width imaged from the side (using a small mirror). The mean diameter (average of top and side dimensions) of mutant strips (133±6 μm) was slightly larger than that of wild-type strips (119±3 μm; P<0.05).

The skinned strips were activated incrementally by Ca<sup>2+</sup> by exchanging equal volumes of relaxing solution for activating solution (pCa 4.5) to attain pCa values of 7, 6, 5.75, 5.5, and 5. Activating solution had the same ionic composition as relaxing solution, except the total concentration of CaCl<sub>2</sub> was 5.03 mmol/L (pCa 4.5). Solutions were formulated by solving equations describing the ionic equilibria.<sup>20</sup>

Sinusoidal Analysis and Strip Viscoelasticity

Small-amplitude sinusoidal length perturbation analysis (sinusoidal analysis) was used to model strip viscoelastic properties and to obtain information about R403Q-induced changes in crossbridge kinetics. Sinusoidal length perturbations of 0.25% strip length (peak-to-peak) were applied at 42 discrete frequencies (0.125 to 100 Hz) using a microcomputer and 16-bit data acquisition board (DT2838, Data Translation Inc.). The length and force signals from the servomotor and strain gauge were digitized, and the elastic and viscous components of dynamic stiffness were calculated from the change in tension and length at each frequency (Figure 1, left and caption). Nyquist diagrams were constructed (Figure 1, right) by plotting the viscous modulus versus the elastic modulus at each frequency. Details of the method have been described previously.<sup>21,22</sup>

Statistics

Significance of differences between αMHC<sup>403Q/+<sup> (mutant) and αMHC<sup>+/+<sup> (wild-type) groups for any specific parameter was assessed using an unpaired Student t test. Data are presented as mean±SEM, unless otherwise indicated.

Results

Morphometric Analysis

Electron micrographs of left-ventricular sections from the αMHC<sup>403Q<sup>−/−<sup> mouse hearts (Figure 2B and 2C) displayed...
sarcomeres that appeared to be identical to those of wild-type hearts (Figure 2A). In 1 of 2 mutant mouse hearts examined (Figure 2B), myofibrils were disarrayed, especially where myofibrils attached to the intercalated disk (arrow). The disarray is similar to that reported previously on both the light\(^1\) and electron\(^2\) microscopic levels. In the other mutant mouse heart (Figure 2C), all areas examined appeared to be normal. These results indicate variable penetrance of the morphological phenotype, reminiscent of the diversity of ventricular myocyte disarray and left atrial enlargement in male mice reported previously.\(^15\)

We assessed the extent and regional variability of fibrosis in representative hearts from both mutant and wild-type

![Figure 2. Ultrastructure of left ventricular myocardium from control (A) and heterozygous αMHC\(^{403/+}\) (B and C) mouse hearts at magnification \(×2000\) (longitudinal sections). Inserted in the upper left corner of each panel is a section at higher magnification \(×6000\), illustrating the absence of myocardial disruption at the level of the sarcomere. Myofibrillar disarray was evident in the heart illustrated in panel B but not in the heart illustrated in panel C. Note the severe disruption of the attachment site of the sarcomere to the intercalated disk (B, arrow).](image)

strains. Two of 3 mutant hearts examined showed evidence of fibrosis (blue-stained collagen), but the extent of fibrosis varied considerably from region to region in both the papillary and free wall muscles. In sections from 1 mutant heart, fibrosis was 4.41±1.44\% (n=14 regions sampled) for papillary muscle and 1.13±0.76\% (n=18) for ventricular wall. In another, fibrosis was absent in the papillary muscle but present in the ventricular wall (1.13±3.25\%; n=18). In the third mutant heart, there was no evidence of fibrosis. Sections from both wild-type hearts also exhibited little (<0.6\%) or no fibrosis.

### Calcium Dependency of Isometric Tension and Oscillatory Power

Figure 3 illustrates the calcium dependency of isometric force and oscillatory power output (per cross-sectional area) in αMHC\(^{403/+}\) and wild-type strips at 27°C. After steady-state isometric tension reached a maximal level at each calcium concentration, small-amplitude (0.25\%, peak to peak) sinusoidal length perturbations were used to generate oscillatory work. At or near saturating calcium concentrations (pCa 5 to 5.75), αMHC\(^{403/+}\) strips tended to generate less isometric tension and oscillatory power than wild-type strips (Figure 3A and 3C; Table 1), although values were highly variable within each group. Differences between group means were marginal (0.06<P<0.08).

At submaximal calcium concentration (pCa 6), αMHC\(^{403/+}\) strip tension was significantly higher (∼2.5 times; P<0.001) than wild-type tension, but oscillatory power was not significantly different from wild-type power (Figure 3A and 3C; Table 1); at the lowest calcium concentrations (pCa 7 to 8), there was no difference between mutant and wild-type strips (Figure 3A and 3C; Table 1). Similar results were obtained at 37°C (Table 1).

Tensions were normalized with respect to their values at pCa 5, and the Hill equation (Figure 3, caption) was fit by least squares to data from each strip. The αMHC\(^{403/+}\) strips required less calcium (∼1/3 μmol/L) to achieve half activation than control strips (Figure 3B; Table 1); that is, calcium sensitivity increased. The pCa-tension relationship is very steep (Hill coefficient, n, is ≈6), indicating a highly cooperative activation mechanism. Thus, over a limited range of calcium concentrations, small differences in [Ca\(^{2+}\)] make large differences in tension and power output. The αMHC\(^{403/+}\) strips appeared to have slightly steeper pCa-tension relationships (ie, higher Hill coefficient n) than wild-type strips, but the difference was not significant (Table 1).

We confirmed the adequacy of the PCr/CK MgATP-regenerating system by examining the PCr and CK dependency of the frequency of maximum oscillatory power output (f\(_{\text{max}}\)) under conditions of maximal activation (pCa 5; [CK], 240 U/mL; perturbation amplitude, 0.25\%). Since f\(_{\text{max}}\) declines sharply with substrate depletion (data not shown), f\(_{\text{max}}\) is a sensitive indicator of a lack of [MgATP] in the core of the strip. For wild-type strips at 40 mmol/L PCr, f\(_{\text{max}}\) was 13±1 Hz at 27°C and 15±1 Hz at 37°C. At 33 mmol/L PCr, f\(_{\text{max}}\) was 12±1 Hz at 27°C and 14±1 Hz at 37°C, a reduction of ∼5% at both temperatures, which suggests that 33 mmol/L PCr represents a slight substrate limitation. At 20 mmol/L
TpxCa 5, and the resulting pCa-tension relationship is fitted by least squares to the Hill equation as follows: $T_x/T_{max} = x^n/(a^n + x^n)$, where $T_x$ is the isometric tension at $[Ca^{2+}] = x$ (in mol/L), $T_{max}$ is the maximum isometric tension generated at $[Ca^{2+}] = 10^{-5}$ mol/L, $a = [Ca^{2+}]$ at half-maximal tension ($pCa_{50} = -\log a$), and $n$ is the Hill coefficient (cooperativity index). Net power was calculated at the frequency of maximum power output (6 to 12 Hz; see text), pCa-log $[Ca^{2+}]$ (in mol/L). Significance levels, mutant versus wild type, are as follows: ***P<0.001, *P=0.05 to 0.075. Data shown are mean±SEM.

PCr, $f_{iso}$ was 10±1 Hz at 37°C and 7.5±1 Hz at 27°C, a drop of ~22% and ~50%, respectively. The more severe drop suggests greater substrate limitations at 20 mmol/L PCr, especially at 37°C. Similar parameter values were obtained when CK concentration was doubled, from 240 to 580 U/mL, indicating that the lower [CK] is adequate. Similar results were obtained from mutant strips, although $f_{iso}$ of mutant strips (10±1 Hz, n=12) tended to be lower than that of wild-type strips (12±1 Hz, n=12; P=0.068). These results suggest that 40 mmol/L PCr and 240 U/mL CK constitutes an adequate MgATP regenerating system at 27°C, with slightly less regenerating capacity at 37°C. This conclusion was supported by the absence of any correlation between $f_{iso}$ and strip diameter (range, 77 to 163 μm) at 40 mmol/L PCr and 240 U/mL CK.

Figure 3. Isometric tension (A), normalized tension (B), and oscillatory power (B) as functions of calcium concentration. Control (●) and αMHC403/1 (□) strips from left ventricular papillary muscle at 27°C. In panel B, isometric tension is expressed as a fraction of that at pCa 5, and the resulting pCa-tension relationship is fitted by least squares to the Hill equation as follows: $T_x/T_{max} = x^n/(a^n + x^n)$, where $T_x$ is the isometric tension at $[Ca^{2+}] = x$ (in mol/L), $T_{max}$ is the maximum isometric tension generated at $[Ca^{2+}] = 10^{-5}$ mol/L, $a = [Ca^{2+}]$ at half-maximal tension ($pCa_{50} = -\log a$), and $n$ is the Hill coefficient (cooperativity index). Net power was calculated at the frequency of maximum power output (6 to 12 Hz; see text), pCa-log $[Ca^{2+}]$ (in mol/L). Significance levels, mutant versus wild type, are as follows: ***P<0.001, *P=0.05 to 0.075. Data shown are mean±SEM.

Strip Viscoelasticity and Crossbridge Model Parameters
Sinusoidal length perturbation analysis was used to investigate actomyosin crossbridge function. The force response to a small-amplitude sinusoidal length change is also sinusoidal (Figure 1, left), although the amplitude and phase relationship of the force response to the length change varies dramatically with frequency in calcium-activated strips. The resulting dynamic stiffness plot (Figure 1, right) exhibits components that have counterparts in the time domain of step-length perturbation analysis (see Appendix, part 1), but, because of the high signal-to-noise ratio, the unique signatures of the individual components in the frequency domain (see below), and the ease of extracting information about oscillatory work and power, we chose to use the sinusoidal length perturbation method to probe crossbridge function.

In the frequency domain (sinusoidal analysis), the expression for complex stiffness (tension divided by fractional change in length) is $\chi(f) = A \infty l/\alpha^b - B \infty l/(b+i\alpha) + C \infty l/(c+i\alpha)$, for $f<1$ kHz, where $i=\sqrt{-1}$, $\alpha = 1$ Hz (by definition), and $k$ is a unitless exponent proportional to the phase angle $\theta$ of the Nyquist plot (Figure 1). Coefficients $A$, $B$, and $C$ (in N/m²) define the magnitude of the complex stiffness of components (or processes) $A$, $B$, and $C$ (note that $B$ and $C$ are

**TABLE 1. Comparison of Isometric Tension and Oscillatory Power:** αMHC403/1 Versus Wild Type

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature, °C</th>
<th>at pCa 6, mN/mm²</th>
<th>at pCa 5, mN/mm²</th>
<th>pCa₅₀</th>
<th>n</th>
<th>at pCa 6, mW/mm²</th>
<th>at pCa 5, mW/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>37</td>
<td>1.64 (0.38)</td>
<td>12.96 (0.82)</td>
<td>5.83  (0.03)</td>
<td>6.68 (0.63)</td>
<td>0.71 (0.06)</td>
<td>2.13 (0.30)</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>1.01 (0.21)</td>
<td>11.07 (0.69)</td>
<td>5.78  (0.02)</td>
<td>5.96 (0.45)</td>
<td>0.24 (0.06)</td>
<td>1.03 (0.18)</td>
</tr>
<tr>
<td>αMHC403/1</td>
<td>37</td>
<td>2.98 (0.44)</td>
<td>10.32 (1.39)</td>
<td>5.94  (0.02)</td>
<td>7.52 (0.75)</td>
<td>0.88 (0.23)</td>
<td>1.12 (0.31)</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>2.59 (0.48)</td>
<td>9.14 (1.15)</td>
<td>5.93  (0.02)</td>
<td>6.43 (0.73)</td>
<td>0.30 (0.09)</td>
<td>0.53 (0.25)</td>
</tr>
</tbody>
</table>

*Maximum (steady-state) isometric tension generated at the pCa (= -log [Ca²⁺]). The Hill equation (see Figure 3 legend) was fitted to individual experiments to yield pCa₅₀ values and Hill coefficients (n) used in the statistical comparison of groups. Mean (±SEM); n=12 for each case, 1 mN/mm² = 1 kN/m².†Maximum oscillatory power generated using length perturbations $\Delta L/L₀ = 0.00125$ (ie, ~2 nm/half sarcomere) at the pCa indicated, where $\Delta L/L₀$ is the fractional change in strip length. Power = $\pi f E_i \Delta L/L₀ f^2$, where $f$ is the frequency of the length perturbation (s⁻¹), and $E_i$ is the viscous modulus (mN/mm²).‡P<0.05, §P<0.001, ¶P=0.05–0.075, significance levels, mutant vs wild type.
of opposite sign), and kinetic parameters $b$ and $c$ (in Hz) define the characteristic frequencies of processes $B$ and $C$, respectively. Maximum oscillatory work production is produced by the crossbridges at frequency $b$; maximum oscillatory work absorption, at frequency $c$. The process A term $A_i (2\pi f/\alpha)^b$ plots as a straight line in the Nyquist diagram (see Appendix, part 2), whereas the process $B$ and $C$ terms $[Bi/(b+i\alpha)]$ and $[Ci/(c+i\alpha)]$ plot as semicircles of opposite sign (Figure 4). These distinctive features allowed us to readily distinguish processes $A$, $B$, and $C$ and to derive model parameters from curve fits of $y(f)$ to the data (Table 2 and Figure 5).

Figure 4 (top panels) shows Nyquist plots of $y(f)$ fit to average data from $\alpha$MHC403/ (left) and wild-type (right) strips. (An exemplar fit to data from 1 strip is illustrated in Figure 1 [right]) Components $A$, $B$, and $C$ deconvolved from each Nyquist plot and averaged are shown in Figure 4 (bottom).

Parameter $A$ is a measure of the amplitude of the viscoelastic response of passive elements of the strip, on a per cross-sectional area basis. At 27°C, $A$ at pCa 8 to 6 was greater in $\alpha$MHC403/ than in wild-type strips, but at pCa 5.75 to 5, $A$ was not significantly different (Table 2). At 37°C, $A$ of $\alpha$MHC403/ was marginally greater at pCa 8 and 7.

Values for parameter $B$ and $C$ reflect both the number and unitary stiffness of cycling crossbridges. At pCa 5.5 and 5, $B$ and $C$ of $\alpha$MHC403/ strips were $\approx 20\%$ less than that of wild-type strips (although the reduction was highly variable), whereas at pCa 6, $B$ and $C$ were $\approx 50\%$ more (Figure 5, 27°C). Similar differences between mutant and wild-type strips were observed at 37°C (data not shown). In general, the calcium dependency of $B$ (Figure 5A) and $C$ (Figure 5B) mimics the pCa-tension relationship (Figure 3A).

The apparent rate constants characterizing the strain-induced transitions between crossbridge states underlying processes $B$ and $C$ (Figure 6) are the characteristic frequencies $b$ and $c$ multiplied by $2\pi$. Essentially, $2\pi b$ is the apparent rate constant of the work-producing step of the crossbridge cycle, and $2\pi c$ is the apparent rate constant of the work-absorbing step. At pCa 6 (submaximal calcium activation), the values of $2\pi b$ and $2\pi c$ were significantly less in $\alpha$MHC403/ than in wild-type strips (Figure 5C). The value of $2\pi b$ of $\alpha$MHC403/ strips was also significantly reduced at higher calcium concentration (pCa<6), although not quite to the same extent. Similar differences between mutant and wild-type strips were observed at 37°C (data not shown). The corresponding characteristic frequencies $b$ and $c$ for mutant versus wild-type strips at pCa 6 were as follows: $b=11.1 \pm 1$ versus $15.1 \pm 1$ Hz and $c=58.2 \pm 3$ versus $66.3 \pm 2$ Hz at 27°C, and $b=22.3 \pm 2$ versus $27.1 \pm 1$ Hz and $c=85.4 \pm 4$ versus $94.3 \pm 3$ Hz at 37°C (all $P<0.05$, mutant versus wild-type strips). These results demonstrate that kinetic processes directly attributable to crossbridge function are altered in the $\alpha$MHC403/ cardiac strips (see Appendix, part 3).

**Discussion**

We compared complex stiffness moduli of strips from $\alpha$MHC403/ and wild-type mouse hearts, which yielded infor-

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**Figure 4.** Nyquist diagrams, with averaged data (top) and deconvoluted components (bottom) of $\alpha$MHC403/ (left) and control (right) strips. Process $B$, which represents de novo force production, underlies the characteristic stretch activation response of striated muscle.29 Process $B$ power output is equal to $2\pi b B/2 (\Delta L/L_o)^2$ where $2\pi b$ is the apparent rate constant of process $B$ (s$^{-1}$), $B$ is the amplitude coefficient of process $B$ (kN m$^{-2}$), and $\Delta L/L_o$ is the fractional change in strip length (0.00125). See text for further explanation.

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**TABLE 2. Comparison of Passive Viscoelastic Parameter $A$: $\alpha$MHC403/ Versus Wild-Type Strips**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature, °C</th>
<th>n</th>
<th>pCa 8</th>
<th>pCa 7</th>
<th>pCa 6</th>
<th>pCa 5.75</th>
<th>pCa 5.5</th>
<th>pCa 5</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>27</td>
<td>12</td>
<td>123±18</td>
<td>136±21</td>
<td>121±19</td>
<td>192±25</td>
<td>262±28</td>
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<td></td>
<td>37</td>
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<td>126±30</td>
<td>108±20</td>
<td>129±31</td>
<td>199±45</td>
<td>257±42</td>
<td>305±51</td>
</tr>
<tr>
<td>$\alpha$MHC403/</td>
<td>27</td>
<td>12</td>
<td>177±28*</td>
<td>169±25*</td>
<td>168±25*</td>
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<td>169±29†</td>
<td>165±30</td>
<td>157±33</td>
<td>242±18</td>
<td>304±22</td>
<td>328±27</td>
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</table>

$p_{ca}$ data are given in kN/m$^2$ (mean±SEM). Passive component of dynamic stiffness is given by first term of $y(f)$, as follows: $A (2\pi f/\alpha)^b$, where $f$ is the frequency of the applied sinusoidal length perturbation, $f=1/2\pi$, $\alpha=1$ Hz. Parameter $k$ of $\alpha$MHC403/ was not significantly different from that of wild type ($k$=0.006–0.009). *$P<0.05$, †$P=0.050–0.075$, significance levels, mutant vs wild type.
formation about crossbridge kinetics at myocardial calcium concentrations (pCa 8 to 5) that encompass the physiological range (ie, pCa 7 to 6; probably higher in exercising mice). The frequency range used in the sinusoidal analysis (0.125 to 100 Hz) also encompassed the normal heart rate of the mouse (≈10 Hz, ie, 600 bpm; higher in exercising mice). To distinguish changes in passive and active properties of the strips and to characterize R403Q-induced changes in cross-bridge recruitment and kinetics, we partitioned the complex stiffness modulus into 3 components (or processes): A, B, and C. (In relaxed muscles, A reflects the change in passive force borne primarily by filaments connecting the thick filaments to the Z line when sarcomeres are stretched or released by an externally applied strain. With increasing calcium, an ever-increasing proportion of this change in passive force, together with the active force [B and C], is borne by the thin filaments.)

The magnitude of component A (ie, the value of parameter \( A \)) reflects the extent to which passive structural elements contribute to the dynamic stiffness of the fiber. At low calcium concentrations (pCa 7 to 8, at which changes in passive viscoelasticity are expected to be most apparent), parameter \( A \) of a MHC403/1 strips was 24% to 44% larger than that of wild-type strips. Part of the passive stiffness at the 2.2-μm sarcomere length is that of interstitial collagen, the accumulation of which (fibrosis) would contribute to \( A \), both by increasing the passive stiffness of the strip and by simply occupying a greater fraction of the cross-sectional area (parameters \( A \), \( B \), and \( C \) are normalized to strip cross-sectional area).

The papillary muscles from the a MHC403/1 hearts showed significant but variable fibrosis, as assessed by collagen stain. In 1 mutant animal, the papillary muscle showed more evidence of fibrosis (4% to 5%) than the free wall (1% to 2%). In the second mutant animal, the papillary muscle showed less evidence of fibrosis (<1%) than the free wall (2% to 3%). In the third mutant animal, the papillary muscle and free wall had no evident fibrosis. The wild-type control animals showed little or no evidence of fibrosis. In those mutant hearts that showed fibrosis, the extent varied considerably, even between adjacent regions within the same papillary muscle or free wall. The within-group variation is reminiscent of the pronounced variability of myofibrillar

**Figure 5.** Viscoelastic model parameters as functions of calcium concentration. Shown are amplitudes of process B (panel A), process C (panel B), apparent rate constant 2 \( p_b \) (panel C), and apparent rate constant 2 \( p_c \) (panel D) at 27°C. ●, control strips; □, aMHC403/1 strips. Significance levels, mutant versus wild type, are as follows: * \( P < 0.05 \), ** \( P < 0.001 \), \( P = 0.05 \) to 0.075. Data shown are mean ± SEM.

**Figure 6.** Three-state crossbridge model. Processes B and C represent strain-induced transitions between postulated pre-force state \( X_1 \) and postforce states \( X_2 \) and \( X_3 \), respectively. The apparent rate constants of these transitions are 2 \( p_b \) and 2 \( p_c \), where 2 \( p_b = k_{12} + k_{13} \) and 2 \( p_c = k_{31} + k_{32} \). The \( k_s \) are the unidirectional forward and backward rate constants, respectively. The rate-limiting step is between \( X_2 \) and \( X_3 \). The thin filament is assumed to be cooperatively activated by attachment of the myosin head in the \( X_1 \) (open) state. Our results indicate that 2 \( p_b \) and 2 \( p_c \) are significantly depressed in aMHC403/1 strips (2 \( p_b \) more so than 2 \( p_c \)). We speculate that the depression is due primarily to reductions of \( k_{12} \) and \( k_{32} \), attributable to destabilization of the actomyosin interaction via substitution of the α-myosin heavy chain residue glu403 for arg403.
disarray (Figure 2). On the basis of this limited study, we provisionally conclude that both the increase in coefficient $A$ and its variability can be at least partly attributed to the variable increase in collagen content.

The magnitude of processes $B$ and $C$ (ie, the $B$ and $C$ values) reflect the number of force-producing crossbridges as well as stiffness per crossbridge (time-averaged unitary stiffness). Because $B$ and $C$ are normalized to strip cross-sectional area, the marginal reduction and variability of $B$ and $C$ at maximal activation (Figure 5A and 5B) may reflect a fibrosis-related variable reduction in cross-sectional area occupied by the myofibrils. Alternatively, reduced $B$ and $C$ may indicate a fundamental change in crossbridge stiffness or redistribution of crossbridge states. (Since the imposed length perturbations was small [<0.25% of the sarcomere length, or <4 nm per half sarcomere], the viscoelastic properties represented by $B$ and $C$ are those of a steady-state population of crossbridges undergoing relatively small fluctuations in strain about some mean position.)

We assumed that changes in the rate constants of the exponential responses $B$ and $C$ (Figure 5C and 5D) reflected changes in crossbridge kinetics due to the R403Q mutation. Our method does not allow us to assess the degree to which fibrosis (in the form of additional parallel elasticity) directly affects, if it affects at all, the elementary crossbridge rate constants. However, our model does take into account an effect of fibrosis on strip kinetics through the passive viscoelastic A term of $y(f)$, such that an increased passive viscoelasticity will reduce the frequency ($f_{max}$) at which power output is maximal. This prediction is consistent with the tendency of $f_{max}$ to be less in R402Q strips than in wild-type strips (see Results section).

In the context of a simple 3-state crossbridge scheme (Figure 6), process $B$ represents transitions between a preforce state $X_1$ and a postforce state $X_2$, the apparent rate constant of which is $2\pi b$. For a stepwise change in average crossbridge strain of <$4$ nm [$y(f)$], $2\pi b= k_{12}+k_{21}$, where $k_{12}$ and $k_{21}$ are the apparent forward and backward unidirectional rate constants, respectively. Process $C$ represents transitions between another postforce state $X_3$ (Figure 6, asterisk) and the preforce state $X_1$. The apparent rate constant of this transition is $2\pi c=k_{31}+k_{13}$, where the $k$s are the unidirectional rate constants. Assuming that bridges in the preforce state $X_1$ are not force bearing over the range of frequencies used (0.1 to 100 Hz), our mechanical measurements cannot distinguish myosin in the preforce state from detached (or weakly attached; see below) myosin; thus, $X_1$ constitutes a lumped state.

Rat $\alpha$-myosin that contains the R403Q mutation has an elevated $K_m$ for actin-activated MgATPase activity, which implies a reduced actin-binding affinity. Reductions of $2\pi b$ and $2\pi c$ (Figure 5C and 5D), or, more specifically, reductions of $k_{12}$ and $k_{13}$, are consistent with a reduced actin-binding affinity. The elevation of tension and dynamic stiffness (ie, increased $B$) of $\alpha$MHC403Q strips at submaximal activation (pCa 5.5, Table 1), tension is increased because of enhanced thin filament activation; above $50\%$ activation (pCa more than $=5.8$), tension is depressed. Fibrosis is likely to reduce $\alpha$MHC403Q strip tension by a variable extent, depending on the progression of the disease. Near-maximal or at-maximal activation oscillatory power is reduced, for reasons explained above. It is tempting to speculate that both peak tension and power reserve are compromised during heavy exercise, when systolic calcium may approach maximal levels (pCa$=5.5$).

The major findings of the present study are (1) the elevated passive dynamic stiffness of relaxed myocardium, (2) the enhanced tension at submaximal calcium activation, (3) the reduced oscillatory power at maximal calcium activation, and (4) the depressed kinetics of MHC403Q strips compared with wild-type strips. Because the mutant animal was heterozygous, the observed differences from wild-type animals are probably less than would be observed for homozygous mutants, if they lived to a comparable age. In any case, the observed differences in strip mechanics are at least partly responsible for altered cardiac function. An elevated passive dynamic stiffness and increased calcium sensitivity of active tension in MHC403Q strips, for example, would be expected to increase tone during diastole, a prediction that is consistent with evidence that diastolic function is impaired in MHC403Q mouse hearts. Several human clinical studies also report impaired relaxation of the left ventricle in FHC, with improved performance by calcium-channel blockers. Although the results of these clinical studies have been used to argue for diastolic calcium overload in FHC, impaired relaxation could just as well reflect greater tone due to increased calcium sensitivity.

Near maximal or at maximal calcium activation (pCa$=5.5$), the reduced force production and oscillatory power output due to depressed crossbridge kinetics and reduced actomyosin content (fibrosis), may impair systolic reserve during strenuous exercise. Thus, the calcium-dependent effect of the mutation (whether facilitating or debilitating), together with a variable degree of fibrosis and myofibrillar disorder, may contribute to the diversity of clinical symptoms observed in murine FHC.

Our results suggest a possible mechanism related to papillary muscle dysfunction to explain mitral regurgitation in FHC. During diastole, the mitral valve is open and the
chordae tendineae that attach the papillary muscles to the valve leaflets are slack. With the onset of systole, the mitral valve closes and the chordae are passively stretched. At the same time, the papillary muscles actively contract, which helps prevent the valve leaflets from prolapsing into the atrium. The papillary muscles attached to the chordae will be stretch-activated, thereby developing additional tension and doing work (proportional to parameter B, Figure 5A) that helps maintain the valve in the closed position. Precise timing of contraction and the additional benefit of prolonged activation that is independent of electrical activity may be critical in maintaining mitral competence, especially at very rapid heart rates. If so, papillary muscle function will benefit from an enhanced oscillatory power output, especially at heart rates near \( f_{\text{max}} \) (mutant heart, 13±1 Hz; wild-type heart, 15±1 Hz at 37°C), ie, heart rates during strenuous exercise. Clearly, further work is required to establish whether \( \alpha \text{MHC}^{403+/+} \) mouse hearts in fact exhibit mitral regurgitation.

Appendix

1. Response to a Stepwise Stretch

In the response to a stepwise stretch,\(^{31,32}\) force increases concurrently with stretch, then falls abruptly after the stretch is complete (rapidly at first, then more slowly, with an exponential rate constant \( p \)). An even slower rise in force develops with an exponential rate constant \( 2mb \) (the stretch activation response), which finally gives way to another slow fall in force. The initial and final declines in force together resemble conventional stress-relaxation, which can be approximated (for \( t>1 \text{ ms} \)) by the term \( At^{-s} \), where \( A \) is a coefficient (in mN/mm\(^2\), if expressed as a tension or stiffness) and \( k \) is a unitless exponent. For very small amplitudes (<0.125% strip length), the response to a step decrease in length (ie, a release) mirrors that of a step increase (stretch); that is, the response is "linear."\(^{33} \) The amplitude coefficients are positive with stretch and negative with step increase (stretch); that is, the response is "linear."\(^{33} \)

2. \( A(2\pi f/\alpha)^k \)

In the presence of 30 mmol/L BDM, the number of attached force-generating crossbridges is sharply reduced.\(^{33} \) When 30 mmol/L BDM was added to activating solution in our experiments, coefficients B and C became very small, and the Nyquist plot approximated a straight line with a slope (\( k \) value) that was similar to that seen in the rigor state (data not given). This observation supports our use of the term \( A(2\pi f/\alpha)^k \) in the Nyquist plot to characterize process A, which appears to differ in magnitude (coefficient A) but not in form (straight line, constant \( k \)) among relaxed, active, and rigor states.

3. Depressed Kinetic Response of the \( \alpha \text{MHC}^{403+/+} \) Strips

Additional tests suggest that the depressed kinetic response of the \( \alpha \text{MHC}^{403+/+} \) strips is not due to substrate limitation. Kinetic parameters \( b \) and \( c \), which are sensitive to [MgATP],\(^{34} \) were significantly reduced by lowering the [PCr] to 20 mmol/L, but a reduction from 40 to 33 mmol/L produced only a marginal reduction of \( b \) and \( c \) in both mutant and wild-type strips (<5% at 27°C and <10% at 37°C). Doubling the concentration of CK to 580 U/mL had no effect on \( b \) and \( c \), confirming saturation at 240 U/mL. These results provide additional support that 40 mmol/L PCr and 240 U/mL CK are adequate (with slightly less regenerating capacity at 37°C). This conclusion is supported by the absence of any correlation between any of the kinetic constants of \( y(f) \) and strip diameter (range, both groups, 77 to 163 \( \mu \text{m} \)).

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