Diastolic Dysfunction and Altered Energetics in the $\alpha MHC^{403/+}$ Mouse Model of Familial Hypertrophic Cardiomyopathy

Matthias Spindler,* Kurt W. Saupe,* Michael E. Christe,§ H. Lee Sweeney,‖ Christine E. Seidman,§ J.G. Seidman,‡ and Joanne S. Ingwall*

**NMR Laboratory for Physiological Chemistry, Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115; ‡Howard Hughes Medical Institute, Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115; §Howard Hughes Medical Institute, Brigham and Women's Hospital, Boston, Massachusetts 02115; and ‖Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Abstract

An arginine to glutamine missense mutation at position 403 of the β-cardiac myosin heavy chain causes familial hypertrophic cardiomyopathy. Here we study mice which have this same missense mutation ($\alpha MHC^{403/+}$) using an isolated, isovolumic heart preparation where cardiac performance is measured simultaneously with cardiac energetics using ^{31}P nuclear magnetic resonance spectroscopy. We observed three major alterations in the physiology and bioenergetics of the $\alpha MHC^{403/+}$ mouse hearts. First, while there was no evidence of systolic dysfunction, diastolic function was impaired during inotropic stimulation. Diastolic dysfunction was manifest as both a decreased rate of left ventricular relaxation and an increase in end-diastolic pressure. Second, under baseline conditions $\alpha MHC^{403/+}$ hearts had lower phosphocreatine and increased inorganic phosphate contents resulting in a decrease in the calculated value for the free energy released from ATP hydrolysis. Third, hearts from $\alpha MHC^{403/+}$ hearts that were studied unpaced responded to increased perfusate calcium by decreasing heart rate approximately twice as much as wild types. We conclude that hearts from $\alpha MHC^{403/+}$ mice demonstrate work load–dependent diastolic dysfunction resembling the human form of familial hypertrophic cardiomyopathy. Changes in high-energy phosphate content suggest that an energy-requiring process may contribute to the observed diastolic dysfunction. (*J. Clin. Invest.* 1998. 101:1775–1783.) Key words: myosin • diastole • transgenic mice • bioenergetics • hypertrophic cardiomyopathy

Introduction

Hypertrophic cardiomyopathy is a disease characterized by a nondilated hypertrophied left ventricle in the absence of any overt cause, and an increased incidence of arrhythmias and sudden death. A prominent feature in nearly all patients with hypertrophic cardiomyopathy is abnormal diastolic function due to both impaired relaxation and reduced compliance of the left ventricle (1). These abnormalities lead to increased left ventricular end-diastolic pressure (EDP)[1] and result in pulmonary congestion and dyspnea, despite preserved or even hyperdynamic left ventricular systolic function (2).

In approximately half the patients with hypertrophic cardiomyopathy the disease is inherited as an autosomal-dominant familial hypertrophic cardiomyopathy (FHC). To date, over a dozen different mutations located on four different chromosomes have been associated with FHC (3). One of the most lethal forms discovered thus far is caused by a missense mutation in the β-myosin heavy chain gene on chromosome 14 that results in an amino acid exchange from arginine to glutamine at position 403 (Arg403Gln). Approximately half of the individuals with this mutation die by age 45 (3), primarily due to sudden death. This amino acid substitution occurs in myosin at the base of a loop which binds to actin. Although the site of the mutation has been identified, and the mutation is known to impair ATPase activity, the question of how this mutation causes the clinical syndrome of FHC remains unanswered.

Mice bearing the Arg403Gln point mutation in myosin were constructed so that the pathophysiology of this mutation could be studied (4). Mice that are homozygous for this mutation die within 7 d of birth while in contrast to human FHC, heterozygous mice appear to have a normal life span (4). Despite their normal life span, mice that are heterozygous for this mutation have some of the major characteristics of cardiomyopathy including myocyte disarray, interstitial fibrosis that progresses with age, relaxation abnormalities, electrocardiographic abnormalities, and left atrial dilation (4, 5).

Here we study this mouse model of FHC to determine which aspects of cardiac physiology and energetics are altered by this single amino acid substitution. Since diastolic dysfunction is a common feature of FHC and is known to be related to the energetic state of the heart (6), we measured cardiac energetics using the noninvasive tool of ^{31}P nuclear magnetic resonance (NMR) spectroscopy simultaneously with measurements

This study has been published in abstract form (1996. *Circulation.* 94: I-433 [Abstr.]).

Address correspondence to Joanne S. Ingwall, Ph.D., NMR Laboratory for Physiological Chemistry, Brigham and Women's Hospital, 221 Longwood Avenue, Room 247, Boston, MA 02115. Phone: 617-732-6994; FAX: 617-732-6990; E-mail: ingwall@bustoff.bwh.harvard.edu

Received for publication 6 October 1997 and accepted in revised form 13 February 1998.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/98/04/1775/09 $2.00
Volume 101, Number 8, April 1998, 1775–1783
http://www.jci.org

1. *Abbreviations used in this paper:* $\alpha MHC^{403/+}$, heterozygous mutation in α-myosin at amino acid 403; Arg403Gln, arginine to glutamine substitution at amino acid 403; DevP, developed pressure; dP/dt, first derivative of left ventricular pressure; EDP, end-diastolic pressure; FHC, familial hypertrophic cardiomyopathy; $|\Delta G_{\sim p}|$, free energy of ATP hydrolysis; HR, heart rate; NMR, nuclear magnetic resonance; PCr, phosphocreatine; Pi, inorganic phosphate; RPP, rate–pressure product; SERCA, sarcoendoplasmic reticulum calcium ATPase; SP, systolic pressure.

of systolic and diastolic performance in isolated isovolumic hearts. In this way we determined whether biochemical candidates known to contribute to diastolic dysfunction in other settings contribute to dysfunction in hearts bearing the Arg403Gln mutation. By measuring systolic and diastolic function at different work loads in the $\alpha MHC^{403/+}$ and wild-type hearts, we have identified several types of contractile dysfunction at the whole heart level that are caused by the Arg403Gln mutation.

Methods

Animals

Heterozygous $\alpha MHC^{403/+}$ mice (strain 129/BS) were generated using the "hit and run" technique as described (7). It should be noted that αMHC is the dominant isoform in mouse hearts whereas βMHC is the dominant isoform in human hearts but that these two isoforms are 100% homologous for 30 amino acids in each direction of amino acid 403. 30 adult (20–24 wk of age) male (for exception, see below) $\alpha MHC^{+/+}$ (wild-type) and 31 age-matched littermate mutant $\alpha MHC^{+/-}$ mice were studied. Each animal was genotyped by PCR-amplified tail DNA and restriction enzyme digestion to confirm the presence or absence of the mutation (4). The experimental protocol for this study was approved by the Standing Committee on Animals of Harvard Medical Area and followed the recommendations of current NIH and American Physiological Society guidelines for the use and care of laboratory animals.

Isolated perfused heart preparation

Hearts of wild-type and $\alpha MHC^{403/+}$ mice were isolated and perfused in the Langendorff mode in a 10-mm glass NMR tube as described previously for mouse hearts (8). In brief, the chest was opened, and the heart was rapidly excised and arrested in ice-cold buffer. Retrograde perfusion via the aorta was carried out at a constant coronary perfusion pressure of 75 mmHg at 37°C. Right ventricular drainage was accomplished by incision of the pulmonary artery. The flow of Thebesian veins was drained by a thin polyethylene tube (PE-10) pierced through the apex of the left ventricle. Coronary flow was measured by collecting coronary sinus effluent through the suction tube. Phosphate-free Krebs-Henseleit buffer containing (mM): NaCl (118), KCl (5.3), $CaCl_2$ (2.0), $MgSO_4$ (1.2), EDTA (0.5), $NaHCO_3$ (25), pyruvate (0.5), and glucose (10) was prepared at the time of the experiment and equilibrated with 95% O_2 + 5% CO_2 yielding a pH of 7.4. For the calcium dose–response experiments, the perfusion was switched to a recirculating system containing a total volume of 660 ml and the calcium concentration was increased in steps of either 0.5 or 1.0 mM. The free calcium concentration in the baseline buffer (total calcium of 2.0 mM with 0.5 mM EDTA) was 1.1 mM. Except where noted, hearts were paced at 7 Hz using monophasic square-wave pulses delivered from a stimulator (model S 88; Grass Instrument Co., Quincy, MA) through salt bridge pacing wires consisting of PE-160 tubing filled with 4 M KCl in 2% agarose.

Measurement of isovolumic contractile performance

A water-filled balloon custom-made of polyvinylchloride film was inserted through the mitral valve into the left ventricle via an incision in the left atrium. The balloon was connected to a pressure transducer (Statham P23Db; Gould, Oxnard, CA) for continuous recording of left ventricular pressure and heart rate (HR). The size of the balloon was carefully matched to the ventricle size. The balloon was inflated to set left ventricular EDP between 6 and 8 mmHg for all hearts and the balloon volume was then held constant. Contractile performance data were collected on-line at a sampling rate of 200 Hz using a commercially available data acquisition system (MacLab ADInstruments, Milford, MA). Left ventricular developed pressure (DevP) (the difference between systolic pressure [SP] and EDP), the minimum and maximum values within a beat of the first derivative of left ventricular pressure (+dP/dt and −dP/dt), and rate–pressure product (RPP) (product of DevP and HR) were calculated off-line.

Experimental groups and protocols

Four protocols were used. In the first protocol (eight wild-type and nine $\alpha MHC^{403/+}$ hearts), isovolumic contractile performance and ^{31}P NMR spectroscopy were measured simultaneously under baseline perfusion conditions (perfusate $[Ca^{2+}]$ equal to 2.0 mM). The perfusion was then switched to a recirculating system containing low calcium buffer (perfusate $[Ca^{2+}]$ of 1.0 mM) and the relationship between perfusate $[Ca^{2+}]$ and isovolumic performance was measured by incrementally increasing the $[Ca^{2+}]$ (in 0.5 or 1.0 mM steps) to 5.0 mM. Each Ca^{2+} dose was administered for 3 min and functional measurements were made when a new steady state was achieved, typically after 2 min.

To compare changes in ATP, phosphocreatine (PCr), and their hydrolysis products in wild-type and $\alpha MHC^{403/+}$ mouse hearts due to increased cardiac work, a second group of animals was studied. In this group (eight wild-type and eight $\alpha MHC^{403/+}$ hearts), after baseline ^{31}P NMR and functional measurements had been made, the perfusion was switched to a buffer containing a total $[Ca^{2+}]$ of 4.0 mM. This high work load condition was maintained for 12 min during which time high-energy phosphate content using ^{31}P NMR spectroscopy and cardiac function were measured.

To test whether pacing at a fixed HR influences the systolic and diastolic performance (of the $\alpha MHC^{403/+}$ hearts), a third group of hearts (eight wild-type and eight $\alpha MHC^{403/+}$) underwent the same protocol as described for the second group except that these hearts were studied without pacing. This cohort of animals included three wild-type and two $\alpha MHC^{403/+}$ hearts from female mice. Because the HR data for male and female hearts were not different in this protocol, the data are pooled. At the end of each of these experiments for these three protocols, hearts were blotted, weighed, and stored at −80°C for subsequent biochemical assays.

In the fourth protocol, hearts from six wild-type and six $\alpha MHC^{403/+}$ mice were perfused for 16 min and then rapidly frozen for biochemical measurements.

^{31}P NMR spectroscopy

^{31}P NMR spectra were obtained at 161.94 MHz using a GE-400 wide-bore spectrometer (Omega, Fremont, CA). Hearts were placed in a 10-mm glass NMR tube and inserted into a custom-made $^{1}H/^{31}P$ double-tuned probe situated in a 89-mm bore, 9.4 T superconducting magnet. To improve homogeneity of the NMR-sensitive volume, the perfusate level was adjusted so that the heart was submerged in buffer. Spectra were collected without proton decoupling at a pulse width of 15 μs, pulse angle of 60°, recycle time of 2.14 s, and sweep width of 6,000 Hz. Single spectra were collected during 8-min periods and consisted of data averaged from 208 free induction decays. Spectra were analyzed using 20-Hz exponential multiplication and zero and first-order phase corrections. The resonance areas corresponding to ATP, PCr, and inorganic phosphate (Pi) were fitted to Lorentzian functions and calculated using a commercially available program (NMR1, Syracuse, NY). By comparing the areas under the peaks from fully relaxed (recycle time 15 s) and those of partially saturated (recycle time 2.14 s) spectra, correction factors for saturation were calculated for ATP (1.0), PCr (1.2), and Pi (1.15).

Biochemical measurements

Between 5 and 10 mg of ventricular tissue was homogenized for 10 s at 4°C in potassium phosphate buffer containing 1 mM EDTA and 1 mM β-mercaptoethanol, pH 7.4 (final concentration of 5 mg wet wt/ml). Aliquots were removed for measurement of protein according to the method of Lowry et al. (9) with BSA as the standard and measurement of total creatine content according to the fluorometric assay of Kammermeier (10). Triton X-100 was then added to the homogenate at a final concentration of 0.1% for analysis of creatine kinase activity (11).

Data analysis

Metabolite concentrations. To determine the cytosolic concentration of ATP, the absolute resonance areas corresponding to the γ- and β-phosphates of ATP in the ^{31}P NMR spectra were normalized by heart weight. Since the Lowry protein content (which minimizes detection of extracellular matrix protein and thereby approximates myocyte protein) was the same in both groups (0.153±0.004 mg/mg wet wt, see Table I), we make the assumption that the fractional volumes of intracellular water in the myocytes of wild-type and αMHC$^{403/+}$ hearts are similar and equal to values typical of well-perfused rodent hearts (0.48 μl/mg wet wt). In this case, area units/mg wet weight is directly proportional to the absolute intracellular concentrations. The value of 10 mM for [ATP] for wild-type mouse myocardium was used to calibrate the [γ-P] and [β-P]ATP peak areas of the ^{31}P NMR spectrum obtained during baseline perfusion period. In this study, the mean of the areas of the [γ-P] and [β-P]ATP resonances was used for this calculation. The possibility that free ADP could contribute to the [γ-P]ATP area and thereby lead to an overestimation of the ATP concentration can be excluded since the ADP concentrations in all protocols reported here were at least one order of magnitude lower than the NMR-detectable threshold. Changes in ATP, PCr, and Pi concentrations during the protocols were calculated by multiplying the ratio of their resonance peak areas to the mean area of the [β-P] and the [γ-P]ATP peaks from the initial baseline spectrum by 10 mM.

Intracellular pH was determined by comparing the chemical shift of Pi and PCr in each spectrum to values from a standard curve.

Cytosolic free ADP was calculated using the equilibrium constant of the creatine kinase reaction and values obtained by NMR spectroscopy and biochemical assays: $[ADP] = ([ATP][free\ creatine])/([PCr][H^+]K_{eq})$; where the equilibrium constant (K_{eq}) is $1.66 \cdot 10^9$ M^{-1} for a [Mg^{2+}] of 1.0 mM (12, 13).

The free energy stored in the high-energy phosphate bonds of ATP ($\Delta G_{\sim P}$) is released by ATP hydrolysis. Although $\Delta G_{\sim P}$ is a negative value, the change in free energy state due to release of $\Delta G_{\sim P}$ by ATP hydrolysis is a positive value and is calculated here as: $|\Delta G_{\sim P}|(kJ/mol) = |\Delta G^\circ + RT\ ln\ ([ADP][P_i]/[ATP])|$; where ΔG° (−30.5 kJ/mol) is the value of $\Delta G_{\sim P}$ under standard conditions of molarity, temperature, pH, and [Mg^{2+}] (14), R is the gas constant (8.3 J/mol K), and T is the temperature in Kelvin.

Statistical analysis. All results are expressed as means±SEM. Paired and unpaired Student's *t* tests as appropriate were used to compare αMHC$^{403/+}$ and wild-type hearts at baseline and high calcium perfusion. Statistical analyses were performed with the use of Statview (Brainpower, Calabasas, CA) and a value of $P \leq 0.05$ was considered significant.

Results

General characteristics of wild-type and αMHC$^{403/+}$ mice. To determine if gross cardiac hypertrophy was present in hearts from αMHC$^{403/+}$ mice, body weight, ventricular weight, and ventricular to body weight ratios were determined (Table I). In agreement with previous reports, body weight, ventricular weight, and ventricular to body weight ratio were the same in both groups (4). Left atrial enlargement of various degrees was observed in approximately one-third of the αMHC$^{403/+}$ hearts. Quantification was not possible since part of the left atria had to be removed as part of the isovolumic heart preparation. Lowry protein content, which approximates the myocyte protein content, and coronary flow were also unchanged in the αMHC$^{403/+}$ compared with wild-type hearts.

Contractile performance during baseline perfusion and Ca^{2+} dose–response relationship. Left ventricular isovolumic contractile performance was measured over a range of perfusate calcium concentrations to determine if the αMHC$^{403/+}$ mutations cause changes in the calcium sensitivity of myocardial tension development. Systolic function was assessed as SP and +dP/dt while diastolic function was assessed by measuring EDP and −dP/dt. Representative tracings of isovolumic contractile performance during baseline perfusion ([Ca^{2+}] set to 2.0 mM) for a wild-type and an αMHC$^{403/+}$ heart are displayed in Fig. 1. When paced at 420 bpm and perfused under identical perfusion conditions with an EDP of 8 mmHg, SP and DevP as

Table I. Characteristics of Wild-Type and αMHC$^{403/+}$ Mice

	Wild-Type	αMHC$^{403/+}$
n	24	25
Body wt (g)	36±1	39±1
Ventricular wt (mg)	144±5	145±5
Ventricular/body wt ratio (mg/g)	4.0±0.1	3.8±0.1
Protein content (mg/mg wet wt)	0.153±0.004	0.153±0.004

Data are means±SEM. For the protein measurements: $n = 14$ for wild-type and $n = 15$ for αMHC$^{403/+}$ hearts.

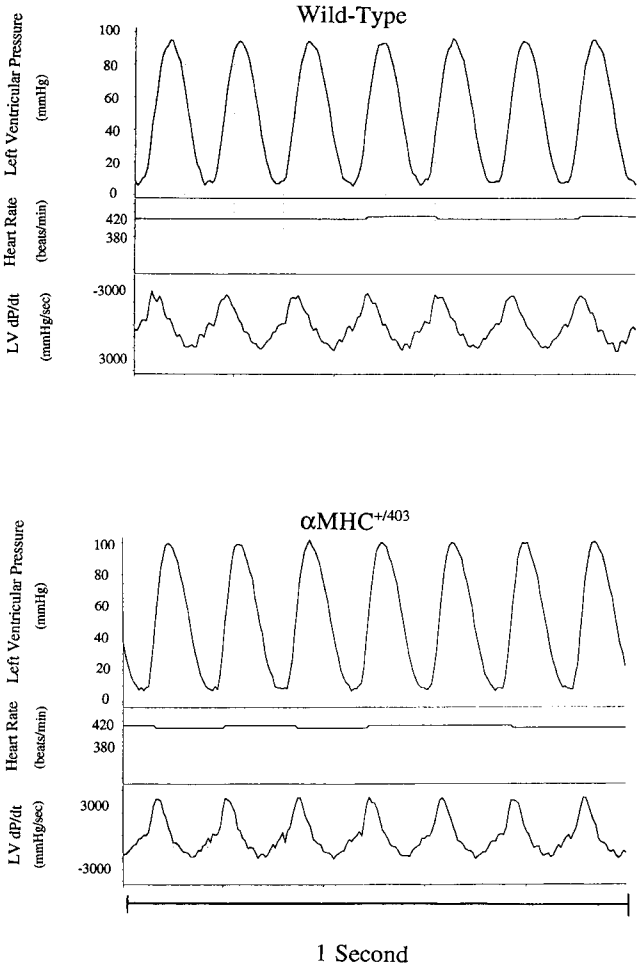

Figure 1. Representative tracings of isovolumic contractile performance in a wild-type and an αMHC$^{403/+}$ heart under baseline perfusion conditions. When paced at 420 bpm there were no differences in baseline contractile performance between the wild-type and αMHC$^{403/+}$ hearts.

Table II. Baseline Isovolumic Contractile Performance and Coronary Flow in Wild-Type and αMHC$^{403/+}$ Mouse Hearts

	Wild-Type	αMHC$^{403/+}$
n	8	9
EDP (mmHg)	8±1	8±1
SP (mmHg)	94±4	95±4
DevP (mmHg)	85±4	87±4
RPP (mmHg/min)	35900±1900	36500±1700
+dP/dt (mmHg/s)	2800±150	3100±140
−dP/dt (mmHg/s)	2200±130	2000±80
Coronary flow (ml/min)	2.5±0.1	2.7±0.1

Data are means±SEM; All hearts were paced at 420 bpm.

well as +dP/dt and −dP/dt were the same in wild-type and αMHC$^{403/+}$ hearts (Table II).

Changes in cardiac performance in response to changes in perfusate [Ca^{2+}] are shown in Fig. 2. When the perfusate was switched to 1.0 mM [Ca^{2+}], EDP increased by 6–7 mmHg for both wild-type and αMHC$^{403/+}$ hearts. DevP decreased from 85±4 to 21±2 mmHg in wild-type hearts, while DevP decreased much less, from 87±4 to 42±4 mmHg in αMHC$^{403/+}$ hearts. Increasing the perfusate [Ca^{2+}] toward baseline levels normalized EDP in both groups. SP and consequently DevP were again indistinguishable between wild-type and αMHC$^{403/+}$ hearts. At higher [Ca^{2+}], SP increased in both groups. However, whereas EDP remained essentially constant in wild-type hearts at high [Ca^{2+}], EDP for αMHC$^{403/+}$ hearts showed a progressive increase from 9 mmHg at 2.0 mM [Ca^{2+}] to 14 and 20 mmHg at 4.0 and 5.0 mM [Ca^{2+}], respectively. When [Ca^{2+}] was returned to 2.0 mM at the end of the protocol, EDP returned to baseline levels in both groups (data not shown). Since EDP progressively increased in the αMHC$^{403/+}$ hearts while SP was comparable to that in the wild types, DevP was lower for αMHC$^{403/+}$ than for wild types at perfusate [Ca^{2+}] > 3.0 mM. When the [Ca^{2+}]–DevP relationship is expressed normalized to the maximal achieved DevP, a leftward shift in the dose–response relation for the αMHC$^{403/+}$ hearts was observed (Fig. 2).

Peak +dP/dt plotted against perfusate [Ca^{2+}] follows the pattern of SP in each group. Maximal rate of relaxation (−dP/dt) in the αMHC$^{403/+}$ hearts, however, was lower than in wild-type hearts for [Ca^{2+}] greater than 1.5 mM (Fig. 3).

Taken together, these results indicate that αMHC$^{403/+}$ hearts demonstrate increased systolic performance at low [Ca^{2+}] and comparable systolic performance for [Ca^{2+}] > 2.0 mM. The reversible increase in EDP and the decreased rate of relaxation at high [Ca^{2+}] observed for the αMHC$^{403/+}$ hearts demonstrate two aspects of diastolic dysfunction.

^{31}P NMR measurements and biochemical characteristics at baseline. Biochemical markers of myocardial energy status were measured with ^{31}P NMR spectroscopy to determine if the αMHC$^{403/+}$ mutation causes changes in energetics similar to those observed during some forms of heart failure. Fig. 4 shows representative ^{31}P NMR spectra from a wild-type and an αMHC$^{403/+}$ heart acquired during baseline perfusion. The spectra show that the PCr resonance area and thus the amount

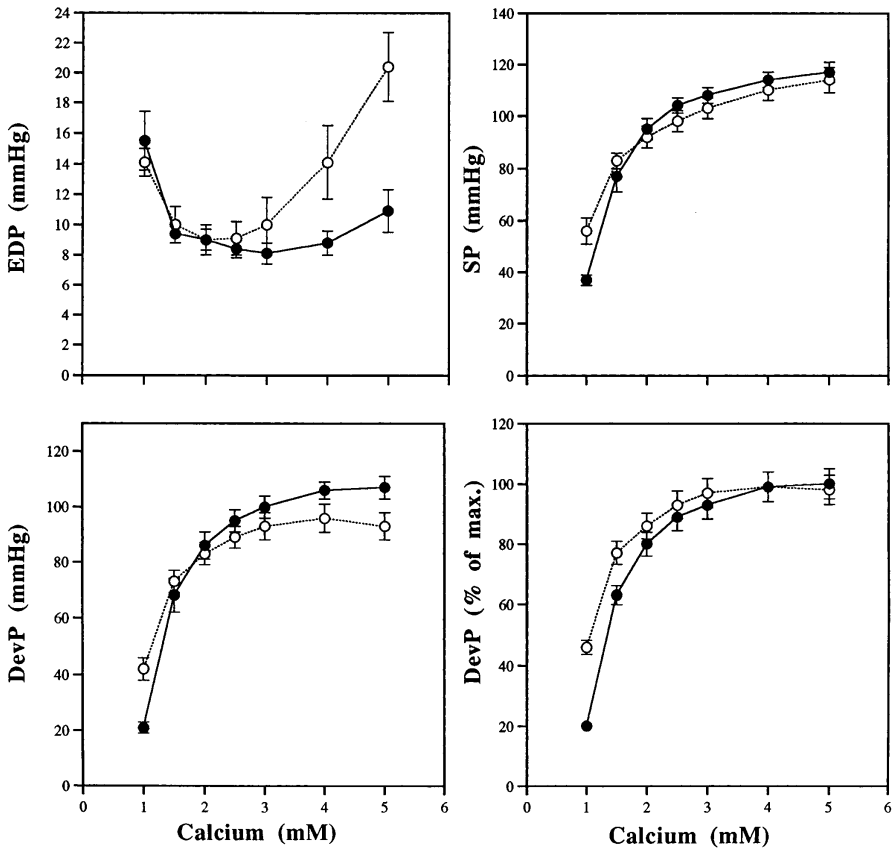

Figure 2. Relationships between perfusate calcium concentration and parameters of left ventricular function in wild-type (*filled circles*) and αMHC$^{403/+}$ (*open circles*) hearts. αMHC$^{403/+}$ hearts generated more left ventricular pressure than wild types at 1 mM Ca^{2+}, but had a lower maximal DevP due to an increased EDP. When DevP was normalized as a percentage of maximal DevP, there is a leftward shift in the calcium dose–response relationship in αMHC$^{403/+}$ hearts, suggesting increased calcium sensitivity at low Ca^{2+} concentrations.

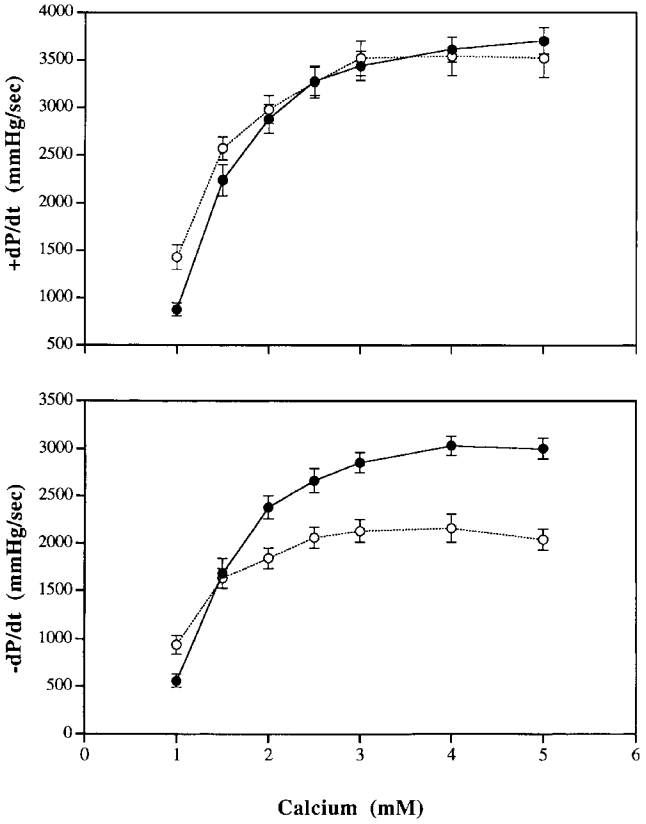

Figure 3. Relationships between perfusate Ca^{2+} concentration and peak positive and negative dP/dt. Note that −dP/dt (rate of left ventricular relaxation) was less in $\alpha MHC^{403/+}$ at Ca^{2+} concentrations above 2.0 mM, despite similar values for +dP/dt and SP. Wild type (*filled circles*), $\alpha MHC^{403/+}$ (*open circles*).

of PCr in the $\alpha MHC^{403/+}$ heart was less than in wild-type hearts. Similarly, the resonance area for Pi was larger in $\alpha MHC^{403/+}$ than wild-type hearts. The ATP resonance area for the $\alpha MHC^{403/+}$ hearts was indistinguishable from wild-type hearts (232±11 vs. 228±7 area units/mg of wet weight).

The mean values of [ATP], [ADP], [PCr], [Pi], and total creatine pool measured or calculated from ^{31}P NMR spectroscopy and chemical assays are summarized in Table III. $\alpha MHC^{403/+}$ hearts, performing the same amount of work estimated as RPP and the same contractility as estimated by +dP/dt, had a lower [PCr] (−22%) and higher [Pi] (+77%) compared with wild-type hearts. The total amount of NMR visible phosphate-containing metabolites was the same in both groups of hearts, 51.7 and 51.2 mM for wild-type and $\alpha MHC^{403/+}$ hearts, respectively. Intracellular pH was similar in all groups and averaged 7.14. The total creatine pool measured chemically was 16% lower in $\alpha MHC^{403/+}$ hearts. In combination with the 22% decreased [PCr] and the unchanged [ATP], calculated [ADP] was 39% higher in $\alpha MHC^{403/+}$ hearts than in wild-type hearts. These changes were not due to changes in either total creatine kinase activity or isoenzyme distribution which were the same in wild-type and $\alpha MHC^{403/+}$ hearts (data not shown).

Table III. Concentrations of High and Low Energy Phosphate Metabolites and Creatine in Wild-Type and $\alpha MHC^{403/+}$ Mouse Hearts during Baseline Conditions

	Wild-Type	$\alpha MHC^{403/+}$
n	16	17
[ATP] (mM)	10.0±0.1	9.9±0.3
[PCr] (mM)	17.0±0.4	13.2±0.7*
[Pi] (mM)	4.7±0.4	8.3±0.7*
[ADP] (μM)	33±3	46±6*
Total [creatine] (mM)	26.3±1.3	22.2±1.0*

Data are means±SEM; *n*, number of hearts. For creatine measurements: $n = 6$ for wild-type and $\alpha MHC^{403/+}$ hearts; $^*P \leq 0.05$ vs. wild-type.

Thus, under conditions of comparable contractile performance, $\alpha MHC^{403/+}$ hearts had a markedly decreased [PCr] and [total creatine] and an increased [Pi] and [ADP], while [ATP] was unchanged.

Diastolic performance and energetic responses to increased perfusate [Ca^{2+}]. To determine if the differences in myocardial energetics seen at baseline are greater during increased

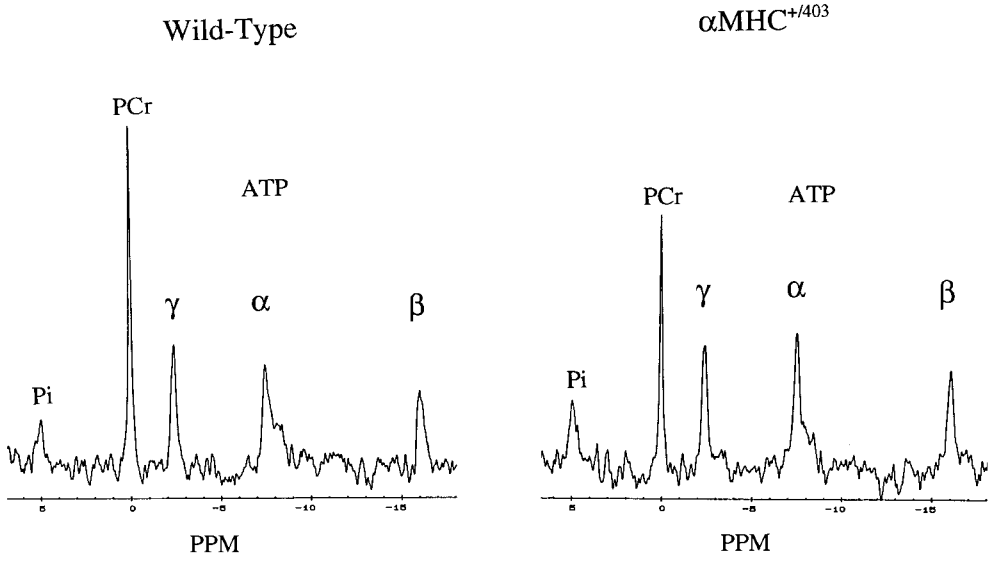

Figure 4. Representative ^{31}P NMR spectra from a wild-type heart (*left*) and an $\alpha MHC^{403/+}$ heart (*right*). Each spectrum is the average of 208 consecutive scans collected over 8 min. The major resonances are assigned (from left to right) as Pi, PCr, and γ-, α-, and β-phosphates of ATP. The area under each peak is proportional to the number of molecules of that substance in the heart. $\alpha MHC^{403/+}$ hearts have similar ATP areas as wild-type hearts but smaller PCr and larger Pi areas.

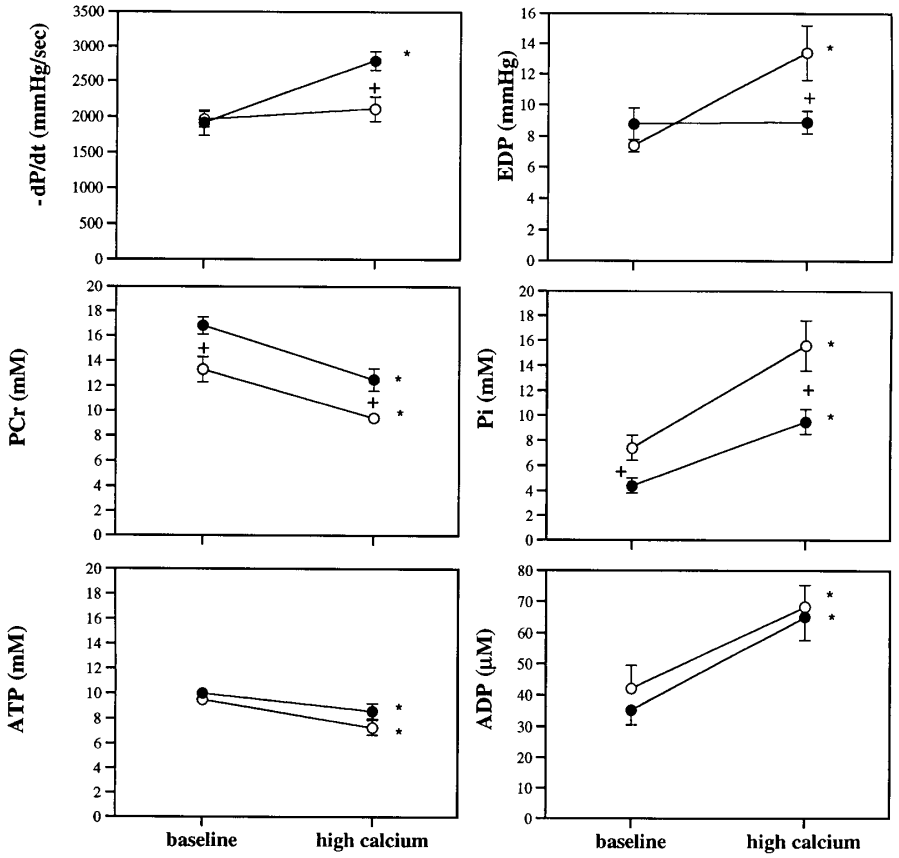

Figure 5. Effect of increased cardiac work on energetics and two measures of diastolic function. In these hearts (eight in each group) cardiac function and energetics were measured at baseline and after a step change to 4.0 mM perfusate $[Ca^{2+}]$. The energetic response to increased ventricular performance was very similar in wild-type (*filled circles*) and $\alpha MHC^{403/+}$ (*open circles*) hearts despite differences in baseline [PCr] and [Pi]. These hearts from $\alpha MHC^{403/+}$ mice demonstrated the same defect of diastolic function during this step change in $[Ca^{2+}]$ as observed during the dose–response protocol in Fig. 2, no increase in $-dP/dt$ at high $[Ca^{2+}]$ but an increase in EDP. *Significantly different from baseline; $^{+}$wild-type and $\alpha MHC^{403/+}$ hearts significantly different.

contractile performance, thereby explaining the observed diastolic dysfunction, we measured high-energy phosphate content at baseline and at a perfusate $[Ca^{2+}]$ of 4.0 mM. This protocol also tests whether a step increase in $[Ca^{2+}]$ from 2.0 to 4.0 mM reproduces the diastolic abnormalities observed in the Ca^{2+} dose–response protocol. Fig. 5 shows that in wild-type hearts $-dP/dt$ increased in parallel with increased work load while EDP remained constant. In $\alpha MHC^{403/+}$ hearts, however, increasing perfusate $[Ca^{2+}]$ from 2.0 to 4.0 mM caused a 6-mmHg increase in EDP with no increase in $-dP/dt$. Therefore, a step increase in perfusate $[Ca^{2+}]$ caused changes in contractile performance that are very similar to the changes observed during the dose–response protocol, confirming these results.

The changes in high-energy phosphate content of these hearts measured simultaneously with the changes in contractile performance are also summarized in Fig. 5. When cardiac work was increased by changing $[Ca^{2+}]$ from 2.0 to 4.0 mM, [PCr] and [ATP] decreased while [Pi] and [ADP] increased both in the wild-type and in the $\alpha MHC^{403/+}$ hearts. Thus, although starting from different phosphate metabolite levels during baseline perfusion, the work-induced changes were very similar in wild-type and in $\alpha MHC^{403/+}$ hearts.

During baseline perfusion, the free energy release from ATP hydrolysis ($|\Delta G_{\sim P}|$), was 59.7±0.3 kJ/mol for wild-type hearts and 57.6±0.7 kJ/mol for $\alpha MHC^{403/+}$ hearts. For the same increase in RPP, $|\Delta G_{\sim P}|$ decreased to 55.2±0.6 kJ/mol in wild-type and to 53.4±0.5 kJ/mol in $\alpha MHC^{403/+}$ hearts.

Response of unpaced hearts to increased $[Ca^{2+}]$. Although pacing hearts at a constant HR allowed us to more easily interpret changes in left ventricular pressures and rate of pressure change (dP/dt), it could mask any differences in HR behavior (and rhythm) in the two groups of hearts. To address this issue, we studied a separate group of unpaced hearts when perfusate $[Ca^{2+}]$ was increased from 2.0 to 4.0 mM. Although there was a trend toward a lower intrinsic HR and higher DevP in $\alpha MHC^{403/+}$ compared with wild-type hearts during baseline perfusion, there were no significant differences in HR, DevP, or RPP between groups (Fig. 6). However, when the perfusate $[Ca^{2+}]$ was increased to 4.0 mM, $\alpha MHC^{403/+}$ and wild-type hearts responded differently. HR in wild-type hearts decreased 18%, DevP increased 66% and RPP increased 33%. In the $\alpha MHC^{403/+}$ hearts, HR decreased 40% while DevP increased only 23%; as a consequence, RPP decreased by 29%. EDP remained constant during the protocol in both groups (data not shown).

In summary, increasing the perfusate Ca^{2+} concentration in unpaced $\alpha MHC^{403/+}$ hearts led to a profound decrease in HR such that RPP decreased instead of increased as observed in wild-type hearts.

Discussion

Here we report studies on mice heterozygous for the Arg403Gln point mutation in cardiac α-MHC ($\alpha MHC^{403/+}$). In humans, this mutation causes FHC with a 50% mortality by age 45 (3). We studied hearts from these mice with regard to major factors thought to contribute to the complex clinical syndrome of FHC, specifically, systolic and diastolic function, response to inotropic stimulation, and myocardial high-energy phosphate content. Our goal was to determine which aspects

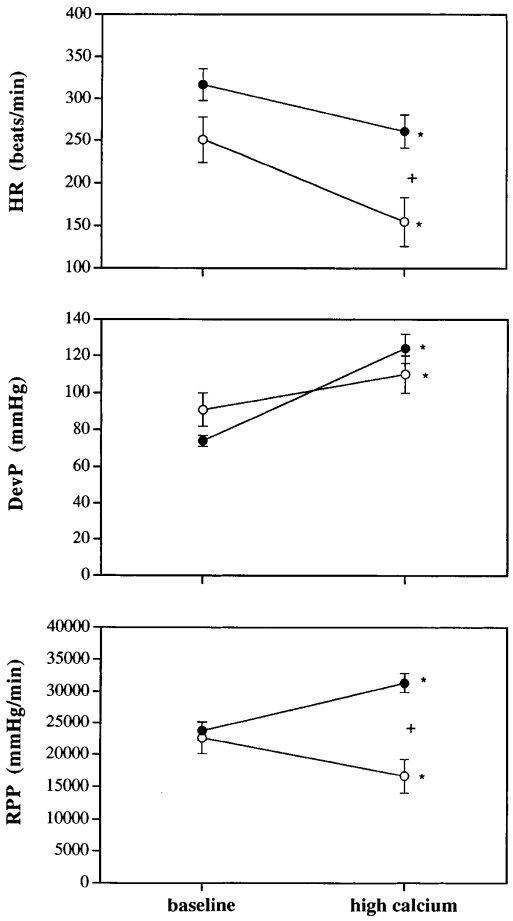

Figure 6. Unpaced αMHC$^{403/+}$ (*open circles*) and wild-type (*filled circles*) hearts at baseline (2 mM [Ca^{2+}]) and during increased cardiac work (4.0 mM [Ca^{2+}]). Note the large (40%) HR decrease in αMHC$^{403/+}$ hearts in response to increased [Ca^{2+}] as compared with only a 18% decrease in wild-type hearts. RPP fell in response to increased perfusate [Ca^{2+}] in the αMHC$^{403/+}$ hearts due to this large decrease in HR, whereas it increased in the wild-type hearts. *Significantly different from baseline; $^{+}$wild-type and αMHC$^{403/+}$ hearts significantly different.

of cardiac physiology and energetics are altered by this single amino acid substitution which in human FHC is associated with left ventricular diastolic dysfunction and premature death.

Mice bearing this mutation demonstrated the following characteristics. First, while hearts isolated from these mice had normal systolic function, diastolic dysfunction was manifest during inotropic challenge both as impaired relaxation (−dP/dt) and elevated EDP. Second, myocardial energetics were altered under baseline conditions in αMHC$^{403/+}$ mice such that [PCr] was decreased and [Pi] increased, while [ATP] was unchanged. These changes resulted in a decrease in calculated free energy release from ATP hydrolysis. While αMHC$^{403/+}$ and wild-type hearts responded similarly with regard to changes in phosphate-containing metabolite concentrations during increased cardiac work, in αMHC$^{403/+}$ hearts $|\Delta G_{\sim P}|$ fell to a lower absolute level. This may be one mechanism leading to diastolic dysfunction. Our third major finding was that spontaneously beating (unpaced) hearts from αMHC$^{403/+}$ responded to the inotropic challenge of increased perfusate [Ca^{2+}] by decreasing HR to such an extent that cardiac work (as estimated by RPP) decreased.

Systolic function. Systolic dysfunction, manifest as decreased ejection fraction or left ventricular pressure generation, is not commonly observed in patients with the Arg403Gln mutation (15). Consistent with this clinical observation in humans, we found no evidence of systolic dysfunction in hearts from αMHC$^{403/+}$ mice. The only condition where there was a difference in systolic performance in αMHC$^{403/+}$ compared with wild-type mouse hearts was during perfusion with low (1.0 mM) calcium. Under this condition hearts bearing the mutation demonstrated increased SP and +dP/dt compared with wild-type hearts. This augmented SP generation in mutants at low perfusate calcium could have been caused by either more intracellular calcium during systole (larger calcium transient) or more force generated for a given amount of calcium (increased calcium sensitivity). Increased calcium sensitivity at low [Ca^{2+}] in the αMHC$^{403/+}$ hearts could be caused by cooperative activation of the thin filament as a result of the altered actin-myosin binding kinetics of the Arg403Gln cross-bridges. Such cooperative activation has been demonstrated by increasing the number of noncycling cross-bridges in cardiac and skeletal muscle preparations (16). In skeletal muscle, Arg403Gln cross-bridges have been shown to cycle more slowly and to remain bound longer to the actin filament than wild-type cross-bridges (17, 18). A clear prediction of these altered kinetics for the intact hearts studied here is that by increasing the number of cross-bridges in the bound state, the Arg403Gln mutation results in increased cooperative activation of the thin filament, and thus greater pressure generation at low levels of calcium activation. This effect should saturate at high levels of calcium activation, where the thin filament approaches the state of maximal activation. This prediction was verified by the data of Fig. 2 showing that pressure development was increased at low calcium in αMHC$^{403/+}$ hearts but converged with wild-type hearts at high concentrations of extracellular calcium.

HR. The primary cause of death in patients with the Arg403Gln mutation is sudden cardiac death thought to be secondary to cardiac arrhythmias (19). While the focus of our study was not on cardiac arrhythmias, we did make a surprising observation consistent with an abnormality in controlling heart rhythm in mutant hearts. In spontaneously beating (unpaced) mice hearts, increasing perfusate calcium concentration from 2.0 to 4.0 mM led to a decrease in HR by 18% in wild-type hearts while mutant hearts decreased HR by 40%. The magnitude of this decrease was sufficient to reduce overall cardiac work (as assessed by RPP) in the mutant hearts by 29%. In contrast, under the same conditions, wild-type hearts increased cardiac work by 33%. The 18% decrease in HR in wild-type hearts was likely caused by a hyperpolarization of the pacemaker cells secondary to increased extracellular calcium. Whether the larger HR decrease in mutants was due to an augmented hyperpolarization of the pacemaker or some other mechanism such as induction of a conduction disturbance is not known. In an electrophysiological study of αMHC$^{403/+}$ mice, Berul et al. reported that αMHC$^{403/+}$ mice demonstrate a normal increase in HR in response to isoproterenol in vivo (5). This suggests that the large decrease in HR in the isolated αMHC$^{403/+}$ hearts in response to 4 mM calcium

observed here was not a general response to inotropic stimulation.

Diastolic function. Diastolic function in patients with the Arg403Gln mutation is often impaired. Fananapazir et al. report that eight out of nine members of one family with the Arg403Gln mutation had an EDP of > 15 mmHg (15). The isometric Langendorff preparation used in the present study is particularly well suited to the study of diastolic dysfunction since changes in left ventricular relaxation during diastole are seen as changes in diastolic pressure. In $\alpha MHC^{403/+}$ mice we observed two forms of diastolic dysfunction during inotropic stimulation, an increase in EDP, and a slower maximal rate of relaxation (−dP/dt). The increase in EDP in the mutants during inotropic stimulation could be caused by mechanical and/or biochemical abnormalities. A mechanical cause could be an increase in turgor. One would expect turgor (the garden hose effect) to increase EDP when perfusion pressure increased or when SP decreased below perfusion pressure causing vascular engorgement in the Langendorff preparation. This is the likely explanation for the increase in EDP seen in both groups of hearts when calcium was decreased to 1 mM, resulting in a SP of less than the perfusion pressure of 75 mmHg. It is unlikely that increased turgor is responsible for the increased EDP at high $[Ca^{2+}]$, since SP is well above perfusion pressure in all hearts.

There are three possible biochemical mechanisms which could explain an increase in EDP. First, [ATP] could have decreased to the point where rigor tension developed. Second, ADP could have accumulated leading to slowed cross-bridge cycling (6). Third, an increased or prolonged Ca^{2+} availability to the myofibril due to a thermodynamic limitation (decreased $|\Delta G_{\sim P}|$) of the sarcoendoplasmic reticulum calcium ATPase (SERCA). The first possibility is unlikely since the changes in [ATP], [ADP], and [Pi] with increased contractile performance are similar in the $\alpha MHC^{403/+}$ and wild-type hearts. In assessing the second possibility, while it is true that the ADP levels are similar in both wild-type and $\alpha MHC^{403/+}$ hearts, it is not known if the threshold concentration for ADP-induced slowing of myosin cross-bridge cycling is affected by the Arg403Gln mutation, or indeed whether the mutant myosin is affected in some unanticipated manner by Pi accumulation or pH decrease. The third possible explanation for the increase in EDP is an increase in diastolic calcium concentration secondary to a fall in $|\Delta G_{\sim P}|$ below that which is necessary to maintain a normal calcium gradient between the cytoplasm and sarcoplasmic reticulum.

The second type of diastolic dysfunction present in the $\alpha MHC^{403/+}$ hearts was a failure to increase the rate of ventricular relaxation during inotropic stimulation. This was observed even though SP and +dP/dt increased similarly in wild-type and $\alpha MHC^{403/+}$ hearts. Impaired relaxation could be caused by a change in ventricular compliance, decreased rate of calcium resequestration into the sarcoplasmic reticulum, or prolonged thin-filament activation caused by a decreased rate of actin-myosin cross-bridge detachment. It is unlikely that changes in left ventricular compliance due to either interstitial fibrosis or intracellular disarray could have caused impaired isometric relaxation because the hearts studied here were contracting isometrically (no muscle shortening or relengthening). However, we cannot rule out the possibility that impaired relaxation is caused by some secondary effect of increased fibrosis on the contractile proteins. Evidence against slowed calcium removal as the cause of the impaired relaxation rate comes from the observation that when cardiac work was increased by a calcium-independent method (increasing preload) impaired relaxation still occurred (Christe, M., unpublished observations). The most direct explanation for impaired relaxation in the $\alpha MHC^{403/+}$ hearts is that the arginine to glutamine amino acid switch has slowed the kinetics of actin-myosin dissociation and led to prolonged activation of the thin filament (17).

Using the working (ejecting) heart preparation, it has been reported previously that the $\alpha MHC^{403/+}$ hearts have a discontinuity in the left ventricular pressure tracing during left ventricular relaxation (4). In the isovolumic (isometric) preparation described here, this upward deflection was not observed. In pilot studies we found that this altered pressure waveform disappears in $\alpha MHC^{403/+}$ hearts when they are switched from the ejecting preparation to isovolumic, and reappears when switched back to ejecting. Since this discontinuity was present in the ejecting heart which shortens and relengthens during each beat, but not the isovolumic heart, it appears that the discontinuity phenomenon is caused by some effect of the Arg403Gln mutation on muscle relengthening during left ventricular relaxation. Taken together these observations indicate that the relaxation dysfunction is a primary consequence of altered myosin binding kinetics and not a secondary consequence of hypertrophy.

Cardiac energetics. In this study we observed that under baseline conditions [ATP] was not different in the two groups but that [PCr] was lower and [Pi] higher in the $\alpha MHC^{403/+}$ hearts compared with wild-type hearts. Although quantitatively small, this pattern of decreased [PCr] and creatine pool is similar to that observed during heart failure in humans (20).

What could cause [PCr] to be decreased in the $\alpha MHC^{403/+}$ mice? The concentration of PCr is determined by the size of the total creatine pool and the degree of its phosphorylation. The observation that the total creatine pool was decreased by 16% likely is sufficient to explain most of the 22% decrease in [PCr]. Since heart weight and myocyte mass were the same, and we could not detect any overt changes in left ventricular geometry, it is unlikely that increased wall stress in the $\alpha MHC^{403/+}$ mice accounts for the decreased [PCr]. Finally, the decreased concentration of PCr cannot be explained by lack of creatine kinase activity or shift in creatine kinase isoenzyme distribution (data not shown) as they were the same in $\alpha MHC^{403/+}$ and wild-type hearts.

A further consequence of the Arg403Gln mutation that may affect cardiac energetics is that the mutant myosin puts a drag on the wild-type myosin during shortening (17). When the $\alpha MHC^{403/+}$ heart is shortening against a load, the cost of doing this work will be higher than for a wild-type heart. It seems likely that this chronic increase in the cost of maintaining cardiac function could result in the depressed energetic state that we observed in the form of decreased [PCr] and creatine pools.

To determine if the differences in myocardial energetics seen at baseline are maintained or even greater during increased cardiac energy consumption, possibly explaining the observed diastolic dysfunction, we measured myocardial energetics after an $\sim$ 40% increase in cardiac RPP. Although starting from different metabolite levels during baseline perfusion, hearts from $\alpha MHC^{403/+}$ mice responded very similarly to wild-type hearts in terms of decreased [PCr] and increased [Pi].

Calculating the free energy release from ATP hydrolysis

integrates the changes of phosphate-containing metabolites and defines the amount of energy available to the ATPases in the myocytes. The free energy available to the $\alpha MHC^{403/+}$ hearts at high rates of energy consumption was in the range of the minimal energy requirement of the SERCA, the ATPase with the highest minimal energy requirement in muscle cells (21). We hypothesize that at high work loads the $\alpha MHC^{403/+}$ hearts reach an energetic state where the SERCA is unable to maintain the cytoplasm-sarcoplasmic reticulum Ca^{2+} gradient, resulting in diastolic Ca^{2+} overload. The possibility that the SERCA becomes thermodynamically limited has been explored for the normal heart (6) but this hypothesis remains to be directly tested for the $\alpha MHC^{403/+}$ hearts.

Conclusions. In conclusion, the $\alpha MHC^{403/+}$ hearts were able to maintain systolic function, even though they contain slowly cycling Arg403Gln cross-bridges. However, the $\alpha MHC^{403/+}$ cross-bridges caused impaired relaxation that apparently cannot be compensated for by the heart. In addition, the cost of developing pressure may be higher in the mutant hearts. Importantly, this study demonstrates that both the diastolic dysfunction and the energetic abnormalities are likely to be primary effects of the myosin mutation, and are unlikely to be secondary consequences of hypertrophy. Extrapolation from this experimental model to the clinical setting can only be done with caution, particularly since these mice do not reproduce two important signs of FHC, namely gross cardiac hypertrophy and sudden death. Nevertheless, our data suggest that an increased energetic cost of cardiac function may be an important common feature of many of the FHC mutations that have been unappreciated in earlier investigations and may contribute to the sudden death that is characteristic of FHC.

Acknowledgments

We would like to thank Dr. Nancy Sweitzer for her thoughtful contributions to this research.

This work was supported by SCOR grant HL-52350. Dr. Spindler was supported by a Deutsche Forschungsgemeinschaft research fellowship, and Dr. Saupe was supported by National Research Service Award grant HL-09259.

References

1. Maron, B.J., P. Spirito, K.J. Green, Y.E. Wesley, R.O. Bonow, and J. Arce. 1987. Noninvasive assessment of left ventricular diastolic function by pulsed Doppler echocardiography in patients with hypertrophic cardiomyopathy. *J. Am. Coll. Cardiol.* 10:733–742.

2. Wynne, J., and E. Braunwald. 1997. The cardiomyopathies and myocarditides. *In* Heart Disease. 5th edition. E. Braunwald, editor. W.B. Saunders, Philadelphia. 1414–1426.

3. Seidman, C.E., and J.G. Seidman. 1995. Gene mutations that cause familial hypertrophic cardiomyopathy. *In* Molecular Cardiovascular Medicine. E. Haber, editor. Scientific American Press, New York. 193–210.

4. Geisterfer-Lowrance, A.A.T., M. Christe, D.A. Conner, J.S. Ingwall, F.J. Schoen, C.E. Seidman, and J.G. Seidman. 1996. A mouse model of familial hypertrophic cardiomyopathy. *Science.* 272:731–734.

5. Berul, C.I., M.E. Christe, M.J. Aronovitz, C.E. Seidman, J.G. Seidman, and M.E. Mendelsohn. 1997. Electrophysiological abnormalities and arrhythmias in αMHC mutant familial hypertrophic cardiomyopathy mice. *J. Clin. Invest.* 99:570–576.

6. Tian, R., M.E. Christe, M. Spindler, J.C.A. Hopkins, J.M. Halow, S.A. Camacho, and J.S. Ingwall. 1997. Role of MgADP in the development of diastolic dysfunction in the intact beating rat heart. *J. Clin. Invest.* 99:745–751.

7. Hasty, P., R. Ramirez-Solis, R. Krumlauf, and A. Bradley. 1991. Introduction of a subtle mutation in the Hox-2.6 locus in embryonic stem cells. *Nature.* 350:243–246.

8. Chu, G., W. Luo, J.P. Slack, C. Tilgmann, W.E. Sweet, M. Spindler, K.W. Saupe, G. Boivin, C.S. Moranec, M.A. Matlib, et al. 1996. Compensatory mechanisms associated with the hyperdynamic function of phospholamban-deficient mouse hearts. *Circ. Res.* 78:1064–1076.

9. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

10. Kammermeier, H. 1973. Microassay of free and total creatine from tissue extracts by combination of chromatographic and fluorometric methods. *Anal. Biochem.* 56:341–345.

11. Rosalki, S.B. 1967. An improved procedure for serum creatine phosphokinase determination. *J. Lab. Clin. Med.* 69:696–705.

12. Lawson, J.W.R., and R.L. Veech. 1979. Effect of pH and free Mg^{2+} on the K_{eq} of the creatine kinase reaction and other phosphate hydrolysis and phosphate transfer reactions. *J. Biol. Chem.* 254:6528–6537.

13. Veech, R.L., J.W.R. Lawson, N.W. Cornell, and H.A. Krebs. 1979. Cytosolic phosphorylation potential. *J. Biol. Chem.* 254:6538–6547.

14. Gibbs, C. 1985. The cytoplasmic phosphorylation potential. Its possible role in the control of myocardial respiration and cardiac contractility. *J. Mol. Cell. Cardiol.* 17:727–731.

15. Fananapazir, L., and N.D. Epstein. 1994. Genotype-phenotype correlations in hypertrophic cardiomyopathy. *Circulation.* 89:22–32.

16. Buck, S.H., P.J. Konyn, and R.L. Moss. 1997. Cooperative activation of contraction of cardiac muscle by bound cross-bridges. *Biophys. J.* 72:A202. (Abstr.)

17. Sweeney, H.L., A.J. Straceski, L.A. Leinwand, B.A. Tikunov, and L. Faust. 1994. Heterologous expression of cardiomyopathic myosin that is defective in its actin interaction. *J. Biol. Chem.* 269:1603–1605.

18. Lankford, E.B., N.D. Epstein, L. Fananapazir, and H.L. Sweeney. 1995. Abnormal contractile properties of muscle fibers expressing β-myosin heavy chain gene mutations in patients with hypertrophic cardiomyopathy. *J. Clin. Invest.* 95:1409–1414.

19. Marion, B.J., W.C. Roberts, and S.E. Epstein. 1982. Sudden death in hypertrophic cardiomyopathy: a profile of 78 patients. *Circulation.* 65:1388–1394.

20. Ingwall, J. 1993. Is cardiac failure a consequence of decreased energy reserve? *Circulation.* 87(Suppl.):VII58–VII62.

21. Kammermeier, H. 1987. High energy phosphate of the myocardium: concentration versus free energy change. *Basic Res. Cardiol.* 82(Suppl. 2):31–36.