Hypertrophic cardiomyopathy-related β-myosin mutations cause highly variable calcium sensitivity with functional imbalances among individual muscle cells

Sebastian E. Kirschner, Edgar Becker, Massimo Antognazzi, Hans-Peter Kubis, Antonio Francino, Francisco Navarro-López, Nana Bit-Avragim, Andreas Perrot, Mirsaid M. Mirrakhimov, Karl-Josef Osterziel, William J. McKenna, Bernhard Brenner, and Theresia Kraft. Hypertrophic cardiomyopathy-related β-myosin mutations cause highly variable calcium sensitivity with functional imbalances among individual muscle cells. Am J Physiol Heart Circ Physiol 288: H1242–H1251, 2005. First published November 18, 2004; doi:10.1152/ajpheart.00686.2004.—Disease-causing mutations in cardiac myosin heavy chain (β-MHC) are identified in about one-third of families with hypertrophic cardiomyopathy (HCM). The effect of myosin mutations on calcium sensitivity of the myofilaments, however, is largely unknown. Because normal and mutant cardiac MHC are also expressed in slow-twitch skeletal muscle, which is more easily accessible and less subject to the adaptive responses seen in myocardium, we compared the calcium sensitivity (pCa50) and the steepness of force-pCa relations (cooperativity) of single soleus muscle fibers from healthy individuals and from HCM patients of three families with selected myosin mutations. Fibers with the Arg723Gly and Arg719Trp mutations showed a decrease in mean pCa50, whereas those with the Ile736Thr mutation showed slightly increased mean pCa50 compared to fibers from healthy individuals and from HCM patients of three families.

Hypertrophic cardiomyopathy (HCM) is a relatively common (adult prevalence 1:500) genetically determined cardiac disorder that is defined clinically by the presence of unexplained left ventricular hypertrophy (LVH) with histological evidence of myocyte disarray (8). The hypertrophy may involve both ventricles, typically is segmental and maximal in the ventricular septum, and may be highly variable in severity (1.5–5 cm) (14, 36). Myocyte disarray, the pathological hallmark of HCM, involves 5–40% of the myocardium, and, within an individual patient, disarray is usually maximal in areas of more severe hypertrophy (35). Disease-causing mutations in genes encoding sarcomeric proteins are seen in 50–60% of families (30). The mechanisms for the development of hypertrophy and disarray, however, are poorly understood. Detailed knowledge of the primary functional consequences at the molecular level will help us to understand how mutations cause hypertrophy and disarray and what determines mild versus severe disease.

Current evidence indicates that most HCM-related mutant proteins are incorporated into the sarcomeres, leading to altered contractile function that can be hypo- or hypercontractile (poison polypeptide effect) (27, 30). Mutations in β-myosin heavy chain (β-MHC) appear to be the most common and have been the most studied. Studies of different point mutations in β-MHC reveal that myosin is incorporated into the sarcomeres of cardiac and slow-twitch skeletal muscle (6, 24) and does not cause alterations in the structure and assembly of thick filaments (15). β-MHC in slow-twitch muscle is the product of the same gene on chromosome 14, which is expressed in myocardium (6, 24, 39). In previous studies of slow-twitch skeletal muscle from HCM patients, we found an increased contractility for the β-MHC mutation Arg719Trp (15), whereas for most other myosin mutations decreased force generation was observed (19, 21).

Assessment of calcium sensitivity of active force generation is an important parameter for the understanding of myocardial dysfunction. The effects of myosin mutations on calcium sensitivity, however, have not yet been studied in tissue of HCM patients. Because binding of myosin heads to actin affects the activation level of the thin filament (12), it is not surprising that functional changes of the myosin heads due to a point mutation also influence the activation level of the thin filaments, as shown for a myosin mutation in a mouse model (25). In this study, we measured the calcium sensitivity (pCa0) as well as the steepness of the relationship between calcium concentration and active force generation (force-pCa relations)
of single, slow-twitch soleus muscle fibers of HCM patients carrying myosin mutations and of healthy controls. The advantage of using soleus muscle fibers instead of cardiac tissue with the mutation is both availability and the fact that in cardiac tissue the primary effects of the mutation are likely to be masked by cardiac-specific adaptational processes that are recognized in the failing heart such as, e.g., troponin I (TnI) phosphorylation (7, 33, 34, 37). Possible adaptation in soleus muscle through phosphorylation of slow myosin light chain 2 (31, 33) was readily excluded through treatment of the fibers with protein phosphatase-1α (PP1α).

Muscle fibers from three patients carrying different point mutations were studied: Arg723Gly [Barcelona mutation (9)], Arg719Trp (1), and Ile736Thr (3). With the use of our specifically developed method for long-term preservation of fully functional skinned fibers, it was possible to study an adequate number of muscle fibers from each individual patient. Therefore, interindividual variability was excluded that otherwise could have masked functional variability among fibers of the individual patients. Our data reveal a slight increase in calcium sensitivity and higher active force generation particularly at the lower end of the force-pCa relation for Ile736Thr with residual active force generation (vs. controls) and a decrease in calcium sensitivity for the other two mutations (Arg723Gly and Arg719Trp). No overall common pattern of change in calcium sensitivity was found. In addition, there was a much larger variability (vs. controls) in the force-pCa relations between individual fibers carrying the same mutation ranging from almost normal response to highly significant differences. This suggests that factors, e.g., the content of mutated protein, might vary substantially among individual fibers and cause functional variability. Resulting imbalances in contractile function from myocyte to myocyte might therefore represent a primary cause for the development of LVH, myocyte disarray, and myocardial dysfunction.

PATIENTS AND METHODS

Biopsies, long-term storage of the muscle, and fiber preparation. Soleus muscle biopsies were obtained from three patients who were previously clinically and genetically characterized as HCM patients caused by mutations in the converter domain of β-MHC. One patient is the member of a HCM family from Barcelona who carries the β-MHC mutation Arg723Gly (9). Clinical details of this patient, patient II-2, from family 26 in Enjuto et al. (9) have been published. He was successfully transplanted at the age of 59 yr. His exercise capacity at the time of biopsy (pretransplant) was restricted [New York Heart Association (NYHA) functional class IV]. The brother of this patient received an implantable cardioverter-defibrillator, and two other members of the same family died suddenly (9). The patient who carries the β-MHC mutation Arg719Trp is a female member of a British family. She presented with an out-of-hospital cardiac arrest and subsequently received an implantable cardioverter-defibrillator. She has moderate LVH (22 mm) with reduced exercise capacity (peak oxygen consumption 60% predicted, NYHA class I). The HCM patient with the Ile736Thr β-MHC mutation is a female of Kyrgyz (central asian) origin. She was diagnosed with HCM at the age of 32 yr. Currently, she is 55 yr old and in functional NYHA class II with moderate septal hypertrophy (18 mm). The Ile736Thr mutation cosegregated in her family with the HCM phenotype. No sudden cardiac death was reported in the family.

In addition, biopsies of the soleus muscles of three healthy control individuals (all men, age 29, 30, and 52 yr) were obtained. These individuals were volunteers without any known impairment of cardiac function. Informed consent was obtained according to approved Ethics Committee protocols of all involved centers. The investigation conforms with the principles outlined in the Declaration of Helsinki. The soleus muscle biopsies of HCM patients and control individuals were excised under local anesthesia. All biopsies were ~10 × 10 × 15 mm. Immediately after surgery, the biopsies were cooled down while a few pieces of muscle tissue were frozen directly in liquid nitrogen for later mRNA analysis. The rest of the biopsies was carefully dissected into small muscle fiber bundles, which were pinned down in a chamber with a layer of sylgard (Dow Corning; Midland, MI) and permeabilized in skinnning solution with Triton X-100 as previously described (16). For long-term storage, permeabilized fiber bundles were equilibrated with skinnning solutions containing increasing sucrose concentrations (up to 2.0 M), rapidly frozen in liquid propane, and stored in liquid nitrogen, as previously described (15). Note that biopsies from patients and from control individuals were treated in the same way during each step of the procedures. To avoid any effects of improper treatment or shipment on the quality of the muscle tissue, the processing of all biopsies was carried out by the same experimentalists. The biopsy with mutation Arg723Gly was dissected and frozen in Barcelona and shipped to Hannover in liquid nitrogen. The biopsy with mutation Arg719Trp was obtained and dissected in London and taken to Hannover while the separated bundles were equilibrating in the different sucrose concentrations. After arrival in Hannover and completion of the equilibration protocol, the bundles were frozen in liquid propane as described above. The biopsies from the Kyrgyz patient and from all control individuals were obtained and processed in Han- nover. All experiments were carried out in our mechanical setup in Hannover.

For the experiments, the bundles of soleus muscle fibers were thawed in skinnning solution containing 2.0 M sucrose and equilibrated with skinnning solution of decreasing sucrose concentrations. Finally, the bundles were transferred into skinnning solution without sucrose for subsequent dissection of individual muscle fibers according to a previously published method (38). Several fibers were isolated from each bundle and kept in skinnning solution, and all experiments were carried out within the following 4 days. Previously, we found that storage of isolated single fibers in contrast to fiber bundles for a couple of days resulted in complete preservation of the regulatory properties and of the structural stability of the fibers (16, 17). It was particularly important for the present study to prevent any kind of rundown or loss of regulatory properties of the fibers. Table 1 reveals that the calcium sensitivity of fibers isolated from one bundle and measured on 4 subsequent days did not show any systematic shift or change to suggest altered regulatory properties. Note that in addition all skinnning solutions contained a cocktail of six different protease inhibitors (details described in Ref. 16) as well as 5 mM 2,3-butanediene monoxide (BDM) (15). Special care was taken to keep the muscle, the dissected bundles before and after freezing, and the isolated fibers during all procedures at low temperature (2–5°C).
that each of the bundles contained fibers with pCa50-values over the whole range of pCa50-variability. There were no signs of systematic degradation of the fibers out of one bundle during the 2–4 days of experiments. The same was observed with the other two mutants and control fibers.

**Mechanical experiments.** For functional studies, single soleus muscle fibers were mounted into the setup as previously described (16). Sarcomere length, which was monitored by laser light diffraction, was adjusted to 2.4 μm for all fibers. The force-pCa curves were obtained by measuring each data point individually in random order, and fibers were relaxed between each calcium concentration. A quick release-restretch protocol was used to stabilize the actively contracting fibers during all measurements (4). Structural integrity of the fibers was controlled at every stage of the measurements using an inverted microscope integrated into the setup. Furthermore, mutant and control fibers were treated in the same way during each step of the preparation, and experiments were carried out with mutant and control fibers by switching between mutant and controls frequently.

At the beginning of each experiment, the fiber type was identified by determining the rate constant of force redevelopment (k<sub>redev</sub>) after a period of unloaded shortening (where force drops to zero) at maximum calcium activation (pCa 4.4) and 10°C (5). To further ensure that only fibers with exclusively the slow myosin isofrom (β-MHC) were included in the summarized data, the fibers were transferred into SDS buffer and frozen for later SDS-PAGE analysis (18) immediately after the measurements of force-pCa relations had been finished. In all cases, the fibers identified as slow fibers (type I) using k<sub>redev</sub> contained solely β-MHC, whereas fibers identified as fast soleus muscle fibers contained a fast myosin isofrom.

Two-dimensional gel electrophoresis of fibers treated with PP1α. Because all fibers were treated with BDM during the skinning procedure, they were already partly dephosphorylated after isolation from the bundle (Ref. 32; see RESULTS). Nevertheless, to ensure that the fibers were essentially in the same state of phosphorylation, all fibers were treated with PP1α at the beginning of each experiment as described above. Two-dimensional gel electrophoresis was performed on several fibers as a control for the phosphorylation state of slow myosin light chain 2 (LC2S). As the first dimension, a native gel electrophoresis containing 8 M urea was carried out as described by Perrie et al. (26) to separate the phosphorylated and dephosphorylated myosin light chains. Run strips of the slab gels containing myosin light chains were then cut out and equilibrated with sample buffer containing SDS. In the second dimension, the strips were loaded onto a horizontal slab gel (SDS-PAGE (15%)) to separate the myosin light chains from tropomyosins. Gels were silver stained by the method of Heukeshoven and Dernick (13).

**Data analysis.** To best reveal the possible changes in calcium sensitivity (pCa50) and steepness (cooperativity) of the force-pCa relationships, all active forces measured at the different calcium concentrations are shown normalized to the force generated at maximum calcium activation of the respective fiber. To determine the pCa50 values and the steepness of the curve, force-pCa relations of the individual fibers were fitted according to the following modified Hill equation (29, 33) where curves with residual active forces at very low calcium concentrations could be fitted correctly using an offset parameter (k):

\[
F(Ca^{2+})/F_0 = (1 - k)[Ca^{2+}]^{nH}/[EC_{50}^{nH} + [Ca^{2+}]^{nH}) + k
\]

where \(F_0\) is the force at saturating calcium concentration, \(F(Ca^{2+})\) is the force at the actual calcium concentration, \(EC_{50}\) is the concentration of calcium at pCa50.
is the calcium concentration giving 50% of maximum force (pCa50), and \( n_H \) is the Hill coefficient, which represents a measure of the steepness of the curve. The fitting curves were calculated using Origin software (Microcal Software; Northampton, MA) including the \( \chi^2 \)-minimization, which gives an estimate of how much the data points deviate from the model curve. The averages of the individual pCa50 values and of individual \( n_H \) from \( n \) muscle fibers of each patient and of the controls are indicated as pCa50 and \( n_H \). These values are given in the text together with their SEs. Analysis of statistical significance was carried out using Student’s \( t \)-test.

To quantify the spread of the individual pCa50 values within each group, i.e., for each patient, for one control individual alone, and for all three controls together, we calculated within each group the SD and the associated confidence limits of the pCa50 values and defined this as SDpCa50. These calculations were carried out using the statistical routine in Mathematica (Wolfram Research; Champaign, IL). In Fig. 2, the SDpCa50 and the corresponding 95% confidence ranges of each group are shown. To establish whether there is a statistically significant difference in the SD of the pCa50 values of controls versus individual patients, a variance ratio test (\( F \)-test) was carried out.

RESULTS

Effects of phosphorylation on the force-calcium relationship. Phosphorylation of the regulatory light chain of myosin (LC2S or MLC-2 fast) affects the calcium sensitivity of active force generation in ventricular myocytes as well as in skeletal muscle fibers (31, 33). Because for the present study it is very important to exclude variations in calcium sensitivity due to effects of variable regulatory light chain phosphorylation, the muscle fibers were treated with PP1\( \alpha \) to bring all fibers into a similar dephosphorylated state and to reduce possible variabilities among the different biopsies and individual muscle fibers. Dephosphorylation due to PP1\( \alpha \) incubation caused a rightward shift of the curve to higher calcium concentrations \( [\text{pCa50}] = 6.20 \pm 0.03 \) (\( n = 16 \)) before and \( 6.12 \pm 0.02 \) (\( n = 19 \)) after PP1\( \alpha \)-treatment \( (P < 0.05) \) and no change in steepness of the force-pCa relationship \( (\bar{n}_H = 2.94 \pm 0.18 \text{ before and } 2.92 \pm 0.15 \text{ after PP1\( \alpha \) treatment}). Dephosphorylation caused a slight reduction of the variability of calcium sensitivity among the individual fibers, as illustrated by the smaller error bars in Fig. 1A. The effect of PP1\( \alpha \) treatment was small, indicating that the skinning procedure of the fibers in the presence of BDM [which acts as a chemical phosphatase (32)] may already have dephosphorylated the fibers to a large extent.

Figure 1B shows the individual force-pCa relationships of fibers after treatment with PP1\( \alpha \) from three control individuals and illustrates the distribution of the pCa50 values and the steepness of the curves. For comparison, in Fig. 1C, the force-pCa relations of only one control individual are presented and demonstrate that the pCa50 values of one individual vary less than those of three individuals (Fig. 1B). This observation is further supported by the SDpCa50 in Fig. 2, which is clearly smaller for single control individuals (e.g., SDpCa50 = 0.043, \( n = 10 \) fibers) compared with three control individuals added together (SDpCa50 = 0.069, \( n = 19 \) fibers). Note that the SDpCa50 value of all control individuals taken together is larger than that of single control individuals. This is because of the interindividual variability of the pCa50 values among the control individuals.

To examine whether the observed effects of PP1\( \alpha \) treatment were due to time-dependent alterations during the incubation period, five fibers were incubated under the same conditions but with an inactive batch of PP1\( \alpha \). After 1 h of incubation with inactive phosphatase at 20°C, the pCa50 values and the steepness of the force-pCa relationships showed no significant change in either SDpCa50 or \( n_H \), and the force-pCa relationship \( (\text{pCa50} = 6.20 \pm 0.03 \text{ before and } 6.12 \pm 0.02 \text{ after PP1\( \alpha \) treatment})\) are shown. To establish whether there is a statistically significant difference in the SD of the pCa50 values of controls versus individual patients, a variance ratio test (\( F \)-test) was carried out using Student’s \( t \)-test.
changes ($pC_{50} = 6.14 \pm 0.02$ vs. $6.14 \pm 0.01$; $n_H = 3.31 \pm 0.54$ vs. $3.69 \pm 0.34$ before and after incubation, respectively). This indicates that the effects of PP1α treatment (Fig. 1A) are not due to the incubation itself.

As a control, using two-dimensional gel electrophoresis, we also analyzed the phosphorylation state of LC2S in several fibers that were treated with the active batch of PP1α. In Fig. 1D, a gel of a single slow soleus fiber that was not dephosphorylated is shown. This fiber contains both phosphorylated and unphosphorylated LC2S. Figure 1E shows, as an example, a two-dimensional gel of a fiber that was treated with PP1α for 1 h. Only dephosphorylated LC2S appears on the gel (the asterisk marks the approximate position where phosphorylated LC2S is expected). This reveals that the incubation with PP1α is sufficient for complete dephosphorylation of regulatory light chains. It should, however, be noted that most of the untreated fibers, when incubated with BDM, already showed low phosphorylation levels of LC2S on two-dimensional gels. Nevertheless, to ensure a comparable state of low phosphorylation, we treated all fibers from control individuals and from patients carrying the β-MHC mutations with PP1α for 1 h under the same conditions used for the experiments shown in Fig. 1 except where stated otherwise.

**Force-pCa relations of fibers with the Ile736Thr mutation.** Figure 3A shows individual force-pCa relations of fibers with the mutation Ile736Thr compared with summarized pCa relations from fibers of control individuals (thick solid line; cf. data in Fig. 1A). All fibers had been treated with PP1α. For some fibers with the mutation Ile736Thr, the force-pCa relations were similar to the control fibers or even appear shifted to the right (higher calcium concentrations), whereas other curves were shifted to lower calcium concentrations (higher pCa values) compared with the average of the control fibers. Most importantly, however, some fibers showed much shallower force-pCa relations with high residual active forces at very low calcium concentrations (pCa 8), and several fibers generated three to four times more force at low calcium concentrations (pCa 6.54 and 6.89) compared with other fibers. Overall, there was a large variability in the behavior of individual fibers with the Ile736Thr mutation. The larger scale of Fig. 3A presents individual curves to demonstrate that there was no large scatter of the data points around the respective fitted curves. The
\( \chi^2 \)-minimization of the fitting routine yielded small values ranging from \( 1.7 \times 10^{-4} \) to \( 4.2 \times 10^{-3} \) for the curves shown in Fig. 3A. Importantly, \( \chi^2 \)-values for the force-pCa relations of the control fibers shown in Fig. 1B were between \( 5 \times 10^{-5} \) to \( 4.6 \times 10^{-3} \), i.e., in a similar range as for the curves of mutation Ile736Thr. This shows that the quality of the data was as good for mutant fibers as for controls, and the data points of control individuals and of the patient follow their model curves in a similar fashion. This is shown here as an example for one mutation but it can also be seen for the other mutants.

It is therefore clear that the large variability among the individual curves (pCa50 values) from fibers with the Ile736Thr mutation (Fig. 3A), which is greater than that seen in controls (Fig. 1, B and C), must originate from a larger variability of intrinsic fiber properties. The F-test applied to the data in Fig. 2 showed that the variability in the pCa50 values of the Ile736Thr mutation (SDpCa50 = 0.14) was significantly larger (\( P < 0.05 \)) than the SDpCa50 of the three control individuals (cf. Fig. 1B: SDpCa50 = 0.069). The difference in variability became even more evident when the mutant fibers were compared with fibers from only one control individual (cf. Fig. 1C; SDpCa50 = 0.043; \( P < 0.01 \), mutation vs. single control). The latter comparison excluded interindividual variabilities among the controls, e.g., due to different genetic background of the control individuals.

In Fig. 3B, the summarized data from all mutated muscle fibers in Fig. 3A are shown compared with the data from fibers of controls (cf. Fig. 1A). The force-pCa relation of all fibers with the mutation was slightly less steep compared with control fibers (\( n_{HI} = 2.59 \pm 0.19 \) vs. \( 2.92 \pm 0.15 \) for control fibers, \( P > 0.05 \)) and showed incomplete relaxation at very low calcium concentrations. The relative force generated by fibers with mutation Ile736Thr at pCa 8, pCa 6.89, and pCa 6.54 was significantly different from controls (\( P < 0.01 \)). The curves in Fig. 3B demonstrate that if one only looks at the summarized data, the wide distribution in calcium sensitivity may escape detection [Ile736Thr: pCa50 = 6.15 \pm 0.04 (\( n = 10 \)); controls: 6.12 \pm 0.02 (\( P > 0.05 \))] and the extent of incomplete relaxation of some fibers would be greatly underestimated.

To determine whether phosphatase treatment affects the variability of force-pCa relations, we also examined untreated fibers from the patient with the mutation Ile736Thr (Fig. 3C). The data were very similar to what was found after PP1α treatment (Fig. 3A). The group of untreated fibers (Fig. 3C) represents a whole continuum of fibers ranging from fibers producing quite high forces at lower calcium concentrations with less steep force-pCa curves to fibers that are similar to the controls (thick solid line; cf. Fig. 1A). This indicates that the wide range of calcium sensitivities of individual fibers shown for the mutation Ile736Thr (Fig. 3A) could neither be reduced by phosphatase treatment nor was it the result of such treatment.

For force-pCa relations of fibers with the Arg719Trp mutation and the Arg723Gly mutation. Figures 4A and 5A show the force-pCa relationships of individual fibers with the mutation Arg719Trp and with the Barcelona mutation Arg723Gly, respectively, together with the summarized curve for the control individuals (thick solid line, cf. Fig. 1A). All fibers had been incubated with PP1α. Most force-pCa relationships of the fibers with mutation Arg719Trp and also with mutation Arg723Gly were shifted to the right, i.e., to higher calcium concentrations, although some were very close to the control curve. For both mutations, a few force-pCa curves appeared to be slightly less steep than the curve of the control fibers. With mutation Arg719Trp, the force-pCa relation on average (Fig. 4B) was shifted to higher calcium concentrations by 0.21 pCa units [Arg719Trp: pCa50 = 5.90 \pm 0.03 (\( n = 16 \)) (\( P < 0.001 \)); see Table 2], and steepness was significantly reduced [\( n_{HI} = 2.45 \pm 0.12 \) for mutant fibers vs. 2.92 \pm 0.15 for controls (\( P < 0.05 \))]. Mutation Arg723Gly on average (Fig. 5B) also caused a significant shift to higher calcium concentrations [pCa50 = 5.98 \pm 0.03 (\( n = 13 \)) (\( P < 0.001 \)); see Table 2], whereas the steepness of the force-pCa relationship was only very slightly reduced (\( n_{HI} = 2.81 \pm 0.21 \)). At low calcium concentrations (pCa \( \approx 7 \)), all fibers with mutation Arg723Gly fully relaxed. Yet, not all fibers with mutation Arg719Trp were fully relaxed at low calcium concentration; the force of mutated fibers at pCa 8.0 was significantly higher than that for controls (\( P < 0.01 \)) and indicates incomplete relaxation of mutated fibers.

Comparison of the individual force-pCa relationships and of the SDpCa50 values (Fig. 2) of mutation Arg719Trp (Fig. 4A) and of mutation Arg723Gly (Fig. 5A) with the control curves (Fig. 1) revealed that the spread of pCa50 values was significantly wider for mutation Arg719Trp (SDpCa50 = 0.125) and for mutation Arg723Gly (SDpCa50 = 0.105) compared with three controls (SDpCa50 = 0.069 (\( P < 0.01 \) and \( P < 0.05 \), respectively)) and particularly compared with only one control individual (SDpCa50 = 0.043 (\( P < 0.05 \) mutation vs. single control)). The larger variability among the mutated fibers is not due to wider scattering of the individual data points around their respective fitted curves, as can be seen from the \( \chi^2 \)-minimization values of the fitting [Arg719Trp: 3 \times 10^{-5} to 2.2 \times 10^{-3} (Fig. 4A); Arg723Gly: 8 \times 10^{-5} to 4.6 \times 10^{-3} (Fig. 5A)], which is in both cases similar to the values of the

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**Fig. 4. A:** individual force-pCa relations of muscle fibers from a patient with the Arg719Trp mutation. The different symbols represent data from individual fibers. For individual pCa50 values of these fibers, see Table 1. The heavy solid line shows the force-pCa relation of the control fibers (cf. Fig. 1A). **B:** summary of all individual force-pCa relations of the patient with the Arg719Trp mutation (n = 16 fibers) compared with the control data (n = 19 fibers). **\( * \)P < 0.001 and **\( * \)P < 0.01 for force of controls vs. mutated fibers. All data shown here were recorded after treatment of the fibers with PP1α.
control curves. Therefore, it can be concluded that mutations Arg719Trp and Arg723Gly both cause an overall reduction in calcium sensitivity of the soleus muscle fibers but with a large variability among individual fibers, which seems to be due to large differences in intrinsic properties of the fibers.

**DISCUSSION**

The three converter domain myosin mutations that were studied altered calcium sensitivity and the slope of the force-pCa relations in different ways. There were significant changes to lower calcium sensitivity (mutations Arg719Trp and Arg723Gly) as well as a small shift to higher calcium sensitivity with higher active forces, particularly at lower calcium concentrations (mutation Ile736Thr). For mutation Ile736Thr and Arg719Trp, some fibers showed abnormal relaxation. For all three mutations studied, the calcium sensitivity (pCa50) of fibers with the mutation varied much more than for control fibers, with some fibers from patients being essentially identical to controls.

**Myosin mutations affect calcium sensitivity and relaxation at low calcium concentrations.** The data shown here for three HCM-causing mutations in β-MHC demonstrate for the first time that myosin mutations can affect the force-pCa relationship of human slow skeletal muscle fibers. The calcium-sensitizing and -desensitizing effects of the mutations studied here as well as of other mutations affecting calcium sensitivity (for a review, see Ref. 20) might represent one factor that contributes to the variable response to pharmacological treatment observed with drugs that affect the calcium homeostasis of the myocardium (8, 22). Furthermore, if the significant residual force at low calcium concentrations observed with fibers from the patient with the mutation Ile736Thr also holds true for cardiomyocytes, this will result in incomplete relaxation of the myocardium in diastole. Such residual force at low calcium concentration could well be one factor contributing to the diastolic dysfunction often observed in HCM patients (8, 22).

Mutations in β-MHC may also cause dilated cardiomyopathy (DCM), a condition characterized by impaired left ventricular contractile performance that is clinically and histologically distinct from HCM. It was speculated that HCM results from mutations causing an increased calcium sensitivity, whereas DCM results from mutations causing reduced calcium sensitivity (28). Our data are inconsistent with such a concept, because for the three mutations studied, which all cause HCM, two mutations showed decreased calcium sensitivity. In addition, HCM-causing mutations in other sarcomeric proteins such as tropomyosin or TnT can also induce either lower or higher calcium sensitivity (20). This indicates that from the shift in calcium sensitivity caused by the mutations, similar to several other parameters of contractility (27, 30), no common mode of pathogenesis can be derived and no definite distinction between sarcomeric mutations causing HCM or DCM is apparent.

**Force-pCa relations show large variabilities among individual muscle fibers.** The slow-twitch fibers from all three HCM patients exhibit a larger variability of their force-pCa relations than control fibers (Fig. 2). With our newly developed conservation method and careful preservation of the isolated fibers during each series of experiments (16), sufficient fibers from each patient could be characterized with high reproducibility so that the large variability did not escape detection. The variability, which is most prominent for mutations Ile736Thr and Arg719Trp, is such that a substantial number of fibers from patients are even in the range of control fibers. Considering that for each mutation the examined fibers were from one individual only, the large variability is quite surprising.

For the three controls (Fig. 1B), variability arises from 1) intrinsic variations among different fibers of each individual and 2) from different pCa50 values of the three individuals. The remaining variability among fibers from just one control individual, i.e., when interindividual variations of pCa50 are excluded (Figs. 1C and 2) most likely is due to intrinsic differences among individual fibers and to the normal divergence between individual measurements, e.g., due to slight inaccuracies in pH, temperature, etc., during the experiments.

To demonstrate that the much larger variability in calcium sensitivity for fibers from the patients is not due to a systematic change in experimental conditions but rather represents the variability among fibers with the mutation, the dates when the experiments of mutation Arg719Trp were carried out and their

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**Table 2. pCa50 and nH values of mutated and control fibers after treatment with PPLα**

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<th>pCa50</th>
<th>nH</th>
<th>n</th>
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<td>Control individuals</td>
<td>6.12 ± 0.02</td>
<td>2.92 ± 0.15</td>
<td>19</td>
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<tr>
<td>Mutations</td>
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<tr>
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<td>10</td>
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<tr>
<td>Arg719Trp</td>
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<td>16</td>
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<tr>
<td>Arg723Gly</td>
<td>5.98 ± 0.03 †</td>
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Values are means ± SE; n, no. of soleus muscle fibers. pCa50, mean calcium sensitivity; nH, mean Hill coefficient; PPLα, protein phosphatase-1α. * P < 0.05 and † P < 0.001, fibers with mutations vs. control individuals.
pCa50 values are presented in Table 1. In the sets of pCa curves recorded within a few days using the same experimental conditions and from fibers that were isolated from one fiber bundle of the biopsy, calcium sensitivity varied over the whole range of pCa50 values observed in the fibers with this mutation (Fig. 4A). No systematic shift in one direction, e.g., arising from a gradual change in pH during the day, was detectable. Furthermore, the data indicate that the variability in pCa50 values does not arise from different handling or different properties of individual fiber bundles of a biopsy or from aging of the fibers. The same was found for the other mutations and control fibers. In addition, the data shown for individual fibers (Figs. 1, B and C, 2, A and C, 3A, and 4A) together with the χ2-minimization analysis reveal that the scatter of data points of individual fibers around the respective fitted force-pCa relation is much smaller than the total scatter of all data points. Therefore, pCa50 values and nH are very sensitive parameters and are characteristic for each individual fiber.

To minimize possible variabilities in phosphorylation levels of the regulatory myosin light chain, all but one set of fibers (Fig. 3C) were treated with PP1β before force-pCa relationships were recorded. The soleus muscle also contains the slow isoforms of regulatory proteins, but whether phosphorylation of, e.g., slow skeletal TnT (and TnI) occurs and whether it affects calcium sensitivity of the thin filaments are currently not clear (for a review, see Ref. 10). On the other hand, treatment of the muscle fibers with the chemical phosphatase BDM during the skinning procedure, at least in heart muscle, has been reported to dephosphorylate myofibrillar proteins to a large extent (32). Thus an effect of variable phosphorylation of myofibrillar proteins on the force-pCa relations cannot be ruled out completely but appears unlikely. However, even if there is still some variability in phosphorylation of myofibrillar proteins, it is expected to be seen in muscle fibers from healthy controls as well as from HCM patients and should cause a similarly wide distribution of the force-pCa relationships for mutant fibers and for controls, unless the larger variability is a distinct feature of the mutant tissue, i.e., that phosphorylation of myofibrillar proteins varies more in fibers with the mutations, e.g., to compensate for functional effects of the mutation. It is quite unlikely that the low physical activity of the HCM patients could have caused the larger variability of the force-pCa relationships compared with controls because impairment of the patients was quite variable (cf. NYHA classes) while the variability among individual fibers was similar for all patients.

Another possible explanation for the large functional variability among individual fibers with the same mutation may relate to variable amounts of mutated protein from fiber to fiber. Preliminary results from analysis of mutant and wild-type protein by mass spectrometry in single β-MHC-expressing soleus fibers from the patient with the Ile736Thr mutation showed some fibers that contained both mutated and wild-type myosin, whereas in other fibers only wild-type myosin could be detected (unpublished observations). However, quantification of mutated and wild-type protein at the individual fiber level is currently not feasible. Interestingly, in larger tissue pieces from HCM patients with myosin mutations, we found that the content of mutated myosin varies over a large range for the different mutations. For example, the biopsy from the patient with the mutation Ile736Thr was found to contain on average 33% mutated myosin, the muscle sample with the mutation Arg719Trp had on average 54% mutated myosin, and the soleus biopsy with another disease-causing mutation, Val606Met, was found to contain only 12% mutated protein [details published elsewhere (2, 24)]. Furthermore, we also found similar proportions of mutant β-MHC mRNA in the muscle biopsies of these patients (2). Taken together, this indicates that not every fiber of these biopsies contains wild-type and mutant protein in a 1:1 ratio. Therefore, different amounts of mutated protein from fiber to fiber appear as a realistic possibility to explain the functional variability among individual fibers.

Implications of the functional variability among individual muscle fibers. Irrespective of whether the variability in the extent of functional changes (calcium sensitivity) among individual fibers is caused by different amounts of mutated myosin or some other unidentified mechanism, some important consequences of such variability need to be considered. In muscle tissue, the highly variable force generation at each calcium concentration ranging from fibers with almost normal active force to fibers with a very pronounced decrease or increase in force means that fibers parallel to each other will generate very different amounts of force at the same calcium concentration. With mutation Ile736Thr, both the varying levels of force generation at low activation levels and the incomplete relaxation of some fibers under normally relaxing conditions could also cause large variability in force generation from fiber to fiber.

Provided that the observed large variability of functional parameters also holds true for cardiomyocytes, then the imbalance in force generation and in relaxation especially of myocytes arranged in series is expected to lead to local imbalances among individual myocytes including overload, extension, and possibly disruption of weaker cells or sarcomeres. Although the myocardium might be able to compensate for some of the imbalances, e.g., by adjusting the phosphorylation level of some sarcomeric proteins (7, 33, 34, 37), it appears quite reasonable that in the long run the imbalanced force generation would result in the development of myocyte disarray, the histological hallmark of HCM, which may be found even in the absence of hypertrophy (23).

If such functional imbalances from myocyte to myocyte, e.g., in force generation, occur also with mutations in other sarcomeric proteins, functional imbalance might represent a unifying mechanism for the development into a common pathological pathway for different mutations despite very different primary functional effects, leading to many of the typical features of HCM (30). In addition, large functional variabilities of cardiomyocytes would present a new perspective on pharmacological interventions. Because treatment with β-blockers or calcium antagonists presumably affects all cardiomyocytes in a similar manner, the variability in function would remain and no improvement of the differences among cells and no improvement of instabilities would be achieved. This could contribute to the variable success of pharmacological treatments for the majority of patients who do not have obstruction (8). To understand the mechanisms that lead to the development of HCM, it will be important to determine whether the variability in function of individual muscle fibers with the same mutation seen here for slow-twitch skeletal muscle is also found in myocardium of HCM patients and whether it is due to...
variable amounts of mutated protein in the individual fibers or results from other factors.

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Increased Ca\textsuperscript{2+}-sensitivity of the contractile apparatus in end-stage human heart failure results from altered phosphorylation of contractile proteins. 


