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*Am J Physiol Regul Integr Comp Physiol* 287:R685-R695, 2004. First published 18 March 2004; doi:10.1152/ajpregu.00620.2003

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## $\alpha$ -Tropomyosin mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly affect cardiac function in transgenic rats in different ways

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Submitted 24 October 2003; accepted in final form 8 March 2004

**Wernicke, Dirk, Corinna Thiel, Corina M. Duja-Isac, Kirill V. Essin, Matthias Spindler, Derek J. R. Nunez, Ralph Plehm, Niels Wessel, Annette Hammes, Robert-J. Edwards, Andrea Lippoldt, Ute Zacharias, Hinrik Strömer, Stefan Neubauer, Michael J. Davies, Ingo Morano, and Ludwig Thierfelder.**  $\alpha$ -Tropomyosin mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly affect cardiac function in transgenic rats in different ways. *Am J Physiol Regul Integr Comp Physiol* 287: R685–R695, 2004. First published March 18, 2004; 10.1152/ajpregu.00620.2003.—To study the mechanisms by which missense mutations in  $\alpha$ -tropomyosin cause familial hypertrophic cardiomyopathy, we generated transgenic rats overexpressing  $\alpha$ -tropomyosin with one of two disease-causing mutations, Asp<sup>175</sup>Asn or Glu<sup>180</sup>Gly, and analyzed phenotypic changes at molecular, morphological, and physiological levels. The transgenic proteins were stably integrated into the sarcomere, as shown by immunohistochemistry using a human-specific anti- $\alpha$ -tropomyosin antibody, ARG1. In transgenic rats with either  $\alpha$ -tropomyosin mutation, molecular markers of cardiac hypertrophy were induced. Ca<sup>2+</sup> sensitivity of cardiac skinned-fiber preparations from animals with mutation Asp<sup>175</sup>Asn, but not Glu<sup>180</sup>Gly, was decreased. Furthermore, elevated frequency and amplitude of spontaneous Ca<sup>2+</sup> waves were detected only in cardiomyocytes from animals with mutation Asp<sup>175</sup>Asn, suggesting an increase in intracellular Ca<sup>2+</sup> concentration compensating for the reduced Ca<sup>2+</sup> sensitivity of isometric force generation. Accordingly, in Langendorff-perfused heart preparations, myocardial contraction and relaxation were accelerated in animals with mutation Asp<sup>175</sup>Asn. The results allow us to propose a hypothesis of the pathogenetic changes caused by  $\alpha$ -tropomyosin mutation Asp<sup>175</sup>Asn in familial hypertrophic cardiomyopathy on the basis of changes in Ca<sup>2+</sup> handling as a sensitive mechanism to compensate for alterations in sarcomeric structure.

familial hypertrophic cardiomyopathy; animal model; calcium transient; cardiac skinned-fiber preparation

MORE THAN 150 mutations in 10 contractile proteins of the cardiac sarcomere cause familial hypertrophic cardiomyopathy (FHC), one of the most prevalent inherited disorders of the cardiovascular system (2, 8, 49). Among these sarcomeric proteins, mutations in  $\beta$ -myosin heavy chain (MHC), myosin-binding protein C, and troponin T (TnT) account for ~75% of FHC (2, 8, 49). The gene PRKAG2, encoding for the  $\gamma$ 2-subunit of AMP-activated protein kinase, represents the only

nonsarcomeric protein involved in the hypertrophic response in FHC (6). FHC is not only genetically divergent, but it is also clinically highly variable. Sudden cardiac death and unexplained cardiac hypertrophy are the most prominent features of the disease (49).

Accordingly, seven mutations in  $\alpha$ -tropomyosin (TPM1) have been associated with different clinical phenotypes in humans. Mutations Ala<sup>63</sup>Val, Lys<sup>70</sup>Thr (64), and Glu<sup>180</sup>Gly (55, 56) show mostly a benign course of FHC, mutations Val<sup>95</sup>Ala (23), Glu<sup>62</sup>Gln (21), and Leu<sup>185</sup>Arg (58) are associated with sudden cardiac death, and mutation Asp<sup>175</sup>Asn represents a “hot spot” mutation in TPM1, which was detected in five unrelated families (11, 19, 39, 59, 60).

In vitro, TPM1 mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly differently affect isometric force generation of single rat cardiac myocytes after adenoviral-mediated gene transfer (29). Mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly also have different effects on in vitro motility assay, a system using recombinant sarcomeric proteins to reconstitute the thin filament system (4, 5). However, when both TPM1 mutations were investigated in transgenic mouse models, differences were restricted to histopathological features: transgenic mice with TPM1 mutation Glu<sup>180</sup>Gly showed concentric left ventricular (LV) hypertrophy, interstitial fibrosis, and myocyte disarray (42), whereas animals with mutation Asp<sup>175</sup>Asn had normal heart weight-to-body weight ratios with only patchy areas of myocyte hypertrophy (38). In addition, transgenic hearts with either TPM1 mutation were characterized by reduced relaxation properties in work-performing heart preparations and by increased Ca<sup>2+</sup> sensitivity of isometric force generation of skinned LV fiber preparations.

On the basis of these clinical and experimental observations, it is still unclear how 1) specific mutations in TPM1 might lead to differential clinical features of FHC and 2) how to explain the slow progression of the disease over years and decades, as shown for most FHC-causing mutations, particularly Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly in TPM1. Little is known about 1) the influence of a particular mutation in TPM1 on cardiac structure and function and 2) the regulation of the stoichiometric relation of sarcomeric proteins in the myofilament system (45). TPM1 ablation in mice suggests a major role of transla-

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tional regulation in the control of TPM1 expression, but little detail is known about the relevant mechanisms (7, 43). In transgenic mice overexpressing mutation Asp<sup>175</sup>Asn or Glu<sup>180</sup>Gly in TPM1, phenotypic changes were seen if the expression level of the transgenic proteins was ~60% of the total tropomyosin (38, 42).

In this context, the present work was performed to study whether a low expression level of transgenic human TPM1, harboring mutation Asp<sup>175</sup>Asn or Glu<sup>180</sup>Gly, is associated with molecular, morphological, or physiological changes in rats. A 250-bp sequence of the rat cardiac myosin light chain (MLC)-2 gene promoter was used for cardiac-specific expression of the transgenes (16). Cardiac hypertrophy was not observed, but molecular markers of cardiac hypertrophy were induced in transgenic animals with either TPM1 mutation. In contrast, only rats carrying mutation Asp<sup>175</sup>Asn demonstrated reduced Ca<sup>2+</sup> sensitivity of cardiac skinned-fiber preparations. Frequency and amplitude of spontaneous Ca<sup>2+</sup> waves were elevated only in cardiomyocytes with mutation Asp<sup>175</sup>Asn, suggesting an increase in intracellular Ca<sup>2+</sup> concentration compensating for the reduced Ca<sup>2+</sup> sensitivity of isometric force generation. In addition, in Langendorff-perfused heart preparations, myocardial contraction and relaxation were accelerated only in animals with TPM1 mutation Asp<sup>175</sup>Asn. Thus this animal study may reflect the variable effects of these two FHC-causing TPM1 mutations on the human cardiac phenotype. The data show that changes in Ca<sup>2+</sup> handling represent a sensitive mechanism in response to changes in the sarcomeric architecture, particularly in TPM1.

## MATERIALS AND METHODS

**Generation of transgenic rat lines.** The transgenic expression cassettes are shown in Fig. 1. A PCR-amplified human striated muscle TPM1 cDNA fragment (57–913, accession no. m19713) was modified with an oligonucleotide-mediated site-directed mutagenesis approach to introduce a G-to-A transition at position 579 and an A-to-G transition at position 595 to generate mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly, respectively. Three transgenic expression cassettes with human wild-type (WT) or mutated TPM1 were produced, each containing at the 5' end a 250-bp sequence of the rat cardiac MLC-2 gene promoter (16) and at the 3' end a 2,159-bp genomic fragment of the human growth hormone (hGH) gene (498–2,657, accession no. m13438), consisting of five exons varying in length from 71 to 302 bp, and a simian virus 40 polyadenylation sequence. The expression

cassettes were excised from pBluescript II KS(+/-) (Stratagene, Heidelberg, Germany) as *Hind*III/*Not*I fragments, gel purified, and microinjected into pronuclei of fertilized oocytes from Sprague-Dawley rats to produce transgenic rats according to the procedure described by Mullins and Ganten (35). Integration of the transgenes was assessed by PCR and Southern blot analysis of genomic DNA from rat tail biopsies. *Probe 1* [forward primer: 5'-ACGCTCA-GAACTGAAGTACA-3', TPM1-(838–858); reverse primer: 5'-TGCCTGCATTTTCGCGGTGCG-3', hGH-(1,090–1,109)] and *probe 2* [forward primer: 5'-GACCCAGAGCACAGAGCA-3', MLC-2 promoter(-250 to -233); reverse primer: 5'-CATTGCAGCTAGGT-GAGCTGT-3', hGH-(561–541)] were used for PCR screening procedures for transgene integration (Fig. 1). For Southern blot analysis, 20  $\mu$ g of genomic rat tail DNA were digested overnight with *Bam*HI and hybridized with [<sup>32</sup>P]dATP-labeled *probe 3* (forward primer: 5'-GACGTCCCTGCTCCTGGCTT-3'; reverse primer: 5'-ACAA-ACTCCTGGTAGGTGTC-3') corresponding to exon II of the hGH fragment (Fig. 1). All experiments were performed on homozygous animals. Transgenic animals with either TPM1 mutation did not demonstrate reduced viability or any gross phenotypic alterations.

**Analysis of mRNA expression.** Total RNA was isolated from tissue preparations with the TRIzol reagent according to the manufacturer's instructions (Life Technologies, Paisley, UK). For Northern blot analysis, 20  $\mu$ g of total RNA were separated on 1.2% formaldehyde-agarose gels and transferred to Hybond N<sup>+</sup> membranes (Amersham, Buckinghamshire, UK) by the electrotransfer procedure described for the semidry transfer unit (model TE 70, Hoefer Pharmacia Biotech, San Francisco, CA). *Probe 3* was used to detect transgenic mRNA, and another probe, *probe 4* [forward primer: 5'-AAGCCGACG-TAGCTTCTCTGAACAG-3', TPM1-(301–325); reverse primer: 5'-TGAGCCTCCAGTGACTTCAA-3', TPM1-(666–685)], was used to quantify transgenic human TPM1 mRNA relative to endogenous rat TPM1 mRNA expression. For detection of the rat atrial natriuretic factor (ANF) mRNA, a 268-bp cDNA fragment (forward primer: 5'-TGGAGCAAATCCCGTATACA-3'; reverse primer: 5'-TT-TCAAGAGGGCAGATCTAT-3') was used as a radioactively labeled probe (63). Rat  $\alpha$ -skeletal actin was detected using a 577-bp DNA probe (forward primer: 5'-TTGAATCCTCTGCCTCAGAC-3'; reverse primer: 5'-GAGCAACCAGAGTCAGACTA-3') (66). The entire cDNA of rat GAPDH was used for Northern blot quantification (15). The radioactivity on these blots was analyzed with FUJIX Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film).

In situ hybridization of cardiac tissue sections was performed as described by Lippoldt et al. (24). RNA probes were generated by in vitro transcription of *probe 3*, with T3 RNA polymerase used at the 3' end to generate a sense probe and T7 RNA polymerase at the 5' end

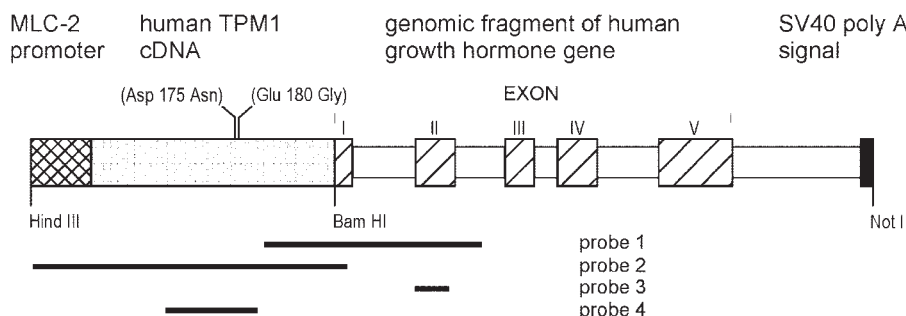


Fig. 1. Structure of transgenic constructs. Transgenic cassettes contained the entire coding sequence of human striated muscle TPM1 or 1 of 2 mutated TPM1 cDNAs with an Asp<sup>175</sup>Asn or a Glu<sup>180</sup>Gly transition. A 250-bp fragment of rat myosin light chain (MLC)-2 promoter was placed 5' to the TPM1 cDNA; 3' to the TPM1 cDNA, a 2.1-kb human growth hormone (hGH) gene fragment consisting of 5 exons and a simian virus 40 (SV40) polyadenylation (poly A) sequence was inserted. *Probes 1* and *2* were designed to analyze genomic integration of the transgenes. *Probe 3* was used for Southern and Northern blot analysis and in situ hybridization. *Probe 4* was produced to quantify transgenic mRNA expression relative to endogenous rat TPM1 mRNA expression.

to synthesize an antisense probe. Radiolabeling of the RNA probes was performed using [ $^{35}$ S]UTP.

**Protein isolation and Western blot analysis.** Cardiac myofibrillar proteins were prepared by homogenizing myocardium in 10 volumes (wt/vol) of ice-cold homogenization buffer containing 50 mmol/l Tris·HCl (pH 7.4), 50 mmol/l KCl, 1% Triton X-100, and 0.5  $\mu$ g/l each of the protease inhibitors E-64, chymostatin, and pepstatin A. The suspension was then centrifuged at 12,000  $g$  for 5 min at 4°C. The myofibrillar protein pellet was resuspended in homogenization buffer and centrifuged as described above. This procedure was repeated six times to obtain a pale yellow-white pellet, which was finally resuspended in water, and aliquots were stored at -80°C until used for Western analysis. Protein concentrations were determined by a protein assay (Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein homogenates (5  $\mu$ g/lane) were separated by SDS-PAGE on two 12.5% gels. One gel was stained with Coomassie blue for 3 h and destained overnight to confirm equal protein loading. The second gel was used for protein transfer onto 0.45- $\mu$ m nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories). The protein transfer was performed in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 1.5 h. For immunodetection of transgenic human TPM1 and its mutants, an antibody, ARG1, was used. ARG1 recognizes human TPM1, but not rat TPM1, allowing the detection of transgenic human TPM1 in the presence of endogenous rat TPM1. Human and rat TPM1 vary by only two amino acid residues. To produce the antibody ARG1, a peptide was synthesized comprising residues 214–226 (Tyr-Ser-Gln-Lys-Glu-Asp-Arg-Tyr-Glu-Glu-Ile-Lys) of human TPM1, in which rat TPM1 differs from human TPM1 at position 220 (Lys for Arg). The peptide was coupled to keyhole limpet hemocyanin, and antisera were raised in rabbits using the procedures described previously (12). Nitrocellulose membranes were incubated with blocking buffer [5% horse serum and 0.5% Tween 20 in 1 $\times$  Tris-buffered saline (TBS)] at 4°C overnight. After the blocking procedure, membranes were briefly rinsed with 1 $\times$  TBS containing 0.5% Tween 20. The membranes were then incubated with a 1:1,000 dilution of ARG1 in 1 $\times$  TBS containing 4% BSA and 0.5% Tween 20 for 4 h at room temperature. The membranes were washed four times for 5 min in 1 $\times$  TBS supplemented with 0.5% Tween 20. Subsequently, membranes were exposed to a 1:4,000 dilution of alkaline phosphatase-conjugated Affinipure goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA) in 1 $\times$  TBS containing 4% BSA and 0.5% Tween 20 for 2 h at room temperature. Membranes were washed three times in 1 $\times$  TBS supplemented with 0.5% Tween 20. Bands were visualized using the chromogenic substrate nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics, Mannheim, Germany) after 45–60 min of exposure.

**Preparation of adult rat cardiomyocytes and immunostaining.** Adult rat cardiomyocytes were prepared as described elsewhere (46). The cells were plated onto chamber slides (Nunc, Wiesbaden, Germany) and fixed for 20 min with 4% paraformaldehyde. The antibody ARG1, used for the immunodetection of transgenic human proteins in Western blot analysis, was applied for immunostaining of adult cardiomyocytes. The cells were incubated with blocking buffer (2.5% horse serum, 1% nonfat milk, and 0.1% Triton X-100 in 1 $\times$  PBS) at room temperature for 4 h. After the blocking procedure, the cells were incubated with a 1:25 dilution of ARG1 in blocking buffer at 4°C overnight. Subsequently, the cells were washed four times for 5 min in 1 $\times$  PBS supplemented with 0.1% Triton X-100 and exposed to a 1:500 dilution of Cy3-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany). The procedure was similar for the non-species-selective anti-TPM1 antibody TM311 (Sigma, Deisenhofen, Germany), except for use of 4% BSA as a blocking reagent. The transgenic and endogenous TPM1 were visualized using a confocal microscope (model MRC 1000, Bio-Rad Laboratories). Cardiomyocytes were obtained from three 5- to 6-mo-old female animals per line.

**Morphological analysis.** Transverse sections through both ventricles at midseptal level were taken for histological evaluation and stained with hematoxylin and eosin or phosphotungstic acid-hematoxylin. Ventricular free wall and septal thickness were measured. The degree of myocyte disarray in the myocardium was quantified by placing a grid containing 100 squares in the eyepiece of a microscope and dividing the septum into 10 fields. The number of squares exhibiting myocyte disarray was counted. The histological sections were examined by one observer blinded to whether the myocardial sections were derived from mutant transgenic rats or WT transgenic and nontransgenic (NTG) controls.

**Investigation of skinned heart muscle fiber preparations.** Force measurements on detergent-extracted LV muscle fibers were performed as described previously (34). The pCa-force relation data were fitted to the Hill equation to determine pCa<sub>50</sub> and the Hill coefficient (17). Mean values were calculated from eight fiber preparations per animal, five female animals per line, aged 12–14 wk.

**Ca<sup>2+</sup> imaging.** For Ca<sup>2+</sup> imaging, the cells were incubated with the Ca<sup>2+</sup> indicator fluo 3-AM (1  $\mu$ M) and pluronic acid (0.04%) for 45 min at room temperature in M199 cell culture medium supplemented with Earle's salts, 0.2% BSA, insulin (15  $\mu$ g/ml), 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, penicillin (100 IU/ml), and streptomycin (100  $\mu$ g/ml). After the cells were loaded with fluo 3-AM, they were washed with the HEPES-buffered physiological saline solution: 134 mM NaCl, 6 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose, with pH adjusted to 7.4 with NaOH. The recording chamber contained physiological solution of the same composition, except for the experiments with the higher external K<sup>+</sup> concentration. All experiments were performed at room temperature.

The confocal microscope consisted of a Bio-Rad imaging system connected to a Nikon Diaphot microscope. Images were obtained by illumination with a krypton-argon laser at 488 nm and recording of all emitted light >500 nm. Bio-Rad software was used for data analysis. Changes in fluorescence intensity reflected as changes in intracellular Ca<sup>2+</sup> concentration were calculated as follows

$$\Delta F/F_0 = (F - F_{\text{base}})/(F_{\text{base}} - F_0)$$

where  $F$  is the measured fluorescence intensity of the Ca<sup>2+</sup> indicator,  $F_{\text{base}}$  is the fluorescence intensity of the Ca<sup>2+</sup> indicator before stimulation, and  $F_0$  is the background signal determined from the average of areas adjacent to the cell.

**Functional analysis of Langendorff-perfused heart preparations.** Animals aged 5–7 mo were anesthetized with pentobarbital sodium, and hearts were isolated and analyzed as described previously (48). LV end-diastolic pressure, LV developed pressure (LVDP), time to peak pressure, time constant of exponential pressure decay, and maximal rates of pressure rise and decay normalized by LVDP [(+dp/dt)/LVDP and (-dp/dt)/LVDP] were derived from the pressure trace. After determination of LV volume at which peak LVDP was obtained, LV volume was set at half-maximal for the remainder of the experiments (50).

**Exercise protocol.** The animals were trained in “passive” running wheels, which were driven by an electric motor to standardize the exercise procedure. Six animals of each transgenic line and six NTG controls underwent the training procedure. The exercise program was commenced with animals aged 6–7 mo. During the first 4 wk of training, rotation speed of the wheels and duration of exercise were progressively increased to a maximum of 9–10 rotations/min and 45 min/day, respectively. This exercise protocol was maintained for 4 mo; then the animals were killed, and the hearts were removed for molecular and histopathological analysis.

**Statistical analysis.** Nonparametric Kruskal-Wallis test was used to detect intergroup differences, whereas differences between two groups were analyzed using Mann-Whitney's  $U$  test. In all tests, the criterion for statistical significance was Bonferroni-Holm-corrected  $P < 0.05$ .

All animal studies have been approved and were performed in accordance with international as well as federal guidelines of animal care.

## RESULTS

*Transgenes are moderately expressed in cardiomyocytes.* A total of 172 offspring were generated by microinjection of one of three transgenic constructs into fertilized rat oocytes containing the human TPM1 cDNA with mutation Asp<sup>175</sup>Asn or Glu<sup>180</sup>Gly or, as control, the nonmutated human TPM1 cDNA (Fig. 1). For 17 transgenic rat lines, 6 with nonmutated human TPM1, 4 with TPM1 mutation Asp<sup>175</sup>Asn, and 7 with TPM1 mutation Glu<sup>180</sup>Gly, single integration sites of the transgenic constructs were confirmed by Southern blot analysis (data not shown). Among these 17 transgenic lines, 8 showed stable and cardiac-specific mRNA expression of the transgenes, as shown in Fig. 2A for line C(Glu<sup>180</sup>Gly). Transgenic mRNA expression levels were comparable in mutant lines B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly) as well as in transgenic control line A(WT), amounting to ~5% of endogenous rat TPM1 mRNA as shown by Northern blot analysis (Fig. 2B). In situ hybridization revealed that all transgenes were equally expressed in atrial and ventricular cardiomyocytes of male and female animals (data not shown).

The expression of the transgenes at the protein level was confirmed by Western blot analysis using an antibody, ARG1, targeted toward residues 214–226 (Tyr-Ser-Gln-Lys-Glu-Asp-Arg-Tyr-Glu-Glu-Glu-Ile-Lys) of human TPM1. The amino acid sequence of human TPM1 differs from that of rat TPM1 by an Arg-to-Lys amino acid exchange in position 220. As shown in Fig. 2C, the antibody ARG1 specifically detected transgenic human TPM1 but not endogenous rat TPM1. In Fig. 2C, transgenic protein expression levels were similar in lines A(WT), B(Asp<sup>175</sup>Asn), and C(Glu<sup>180</sup>Gly). A similar signal is shown for human heart tissue, but in this case, four times less protein homogenate was loaded than for the transgenic rat experiments. Because of the comparable mRNA and protein expression levels of the transgenes in lines A(WT), B(Asp<sup>175</sup>Asn), and C(Glu<sup>180</sup>Gly), these three transgenic lines were subjected to further molecular, histopathological, and physiological analyses.

*Stable integration of the transgenic proteins in the sarcomere.* The integration of the transgenic proteins in the sarcomere was analyzed by immunostaining of adult cardiomyocytes. The species-unspecific anti-TPM1 antibody TM311 was used to detect a regular sarcomeric structure in cardiomyocytes from NTG controls (Fig. 3A) and transgenic animals of line B(Asp<sup>175</sup>Asn) (Fig. 3B). In contrast, the human-specific anti-TPM1 antibody ARG1 revealed sarcomeric staining in cardiomyocytes from line B(Asp<sup>175</sup>Asn) (Fig. 3D), but not from NTG controls (Fig. 3C). The results illustrated for line B(Asp<sup>175</sup>Asn) were similar to those for lines A(WT) and C(Glu<sup>180</sup>Gly) (data not shown).

*Molecular markers of cardiac hypertrophy are induced in transgenic animals with TPM1 mutation Asp<sup>175</sup>Asn or Glu<sup>180</sup>Gly.* Heart weight-to-body weight ratio, as well as septal and ventricular wall thickness, remained unaltered in transgenic mutant and control lines at all ages, even after strenuous physical exercise (data not shown). However, the mRNA expression of fetal cardiac genes, known to be an early event in myocardial hypertrophy (9), was induced in both mutant trans-

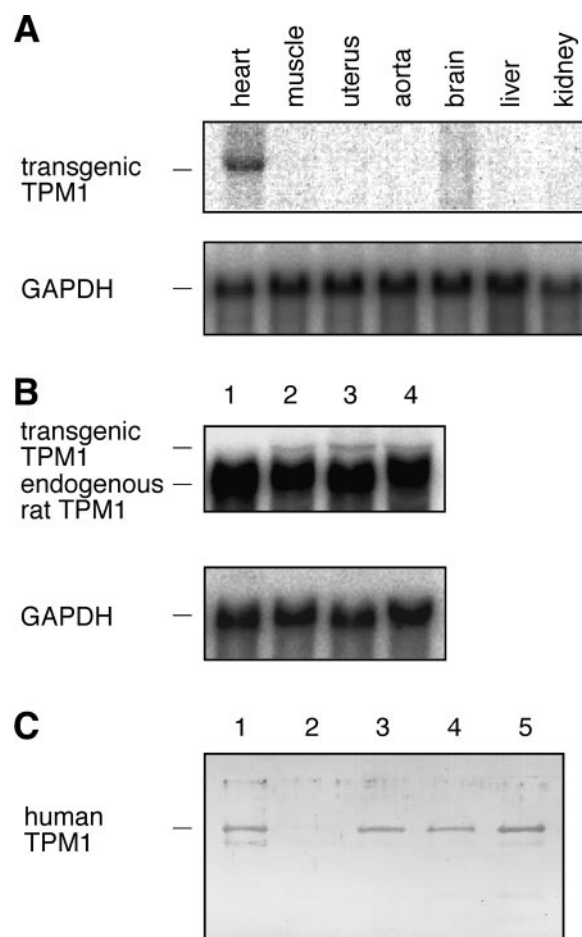


Fig. 2. Transgenic mRNA and protein expression. A: Northern blot showing cardiac-specific mRNA expression of transgene in line C(Glu<sup>180</sup>Gly). A single 1.7-kb transgenic transcript was detected using radioactively labeled probe 3. An identical mRNA expression pattern was observed for line A(WT) and B(Asp<sup>175</sup>Asn) (data not shown). B: 2 bands of ~1.7 and 1.0 kb representing transgenic and endogenous TPM1 mRNA, respectively, in Northern blot analysis from heart tissue. The larger transgenic transcript could not be detected in heart preparations from nontransgenic (NTG) animals (1). Northern blot illustrates similar mRNA expression level of the transgenes in line A(WT) (2), B(Asp<sup>175</sup>Asn) (3), and C(Glu<sup>180</sup>Gly) (4), which amounts to ~5% of total TPM1 mRNA content. Data were confirmed by analyzing  $\geq 10$  animals per line. Radioactively labeled probe 4 was used for TPM1 mRNA detection. GAPDH was used to check loading of RNA. C: Western blot analysis performed with an antibody discriminating between human and rat TPM1 (see MATERIALS AND METHODS). A 33-kDa TPM1 band was detected in protein homogenates from human heart tissue (1), but not from NTG rat (2). Western blot indicates similar levels of transgenic protein expression in line A(WT) (3), B(Asp<sup>175</sup>Asn) (4), and C(Glu<sup>180</sup>Gly) (5). Data were confirmed by analyzing 6 animals per line.

genic lines. As shown in Fig. 4, ANF mRNA expression was elevated about fourfold in transgenic animals older than 9 mo from lines B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly) compared with age-matched transgenic controls. Physical exercise did not further stimulate ANF expression. Although less pronounced, the mRNA level of  $\alpha$ -skeletal actin was also increased in hearts of transgenic rats carrying either mutation, but not in transgenic controls (data not shown).

No histopathological abnormalities were detected in sedentary animals. However, after completion of the physical training program at 12 mo of age, four of six rats of each mutant

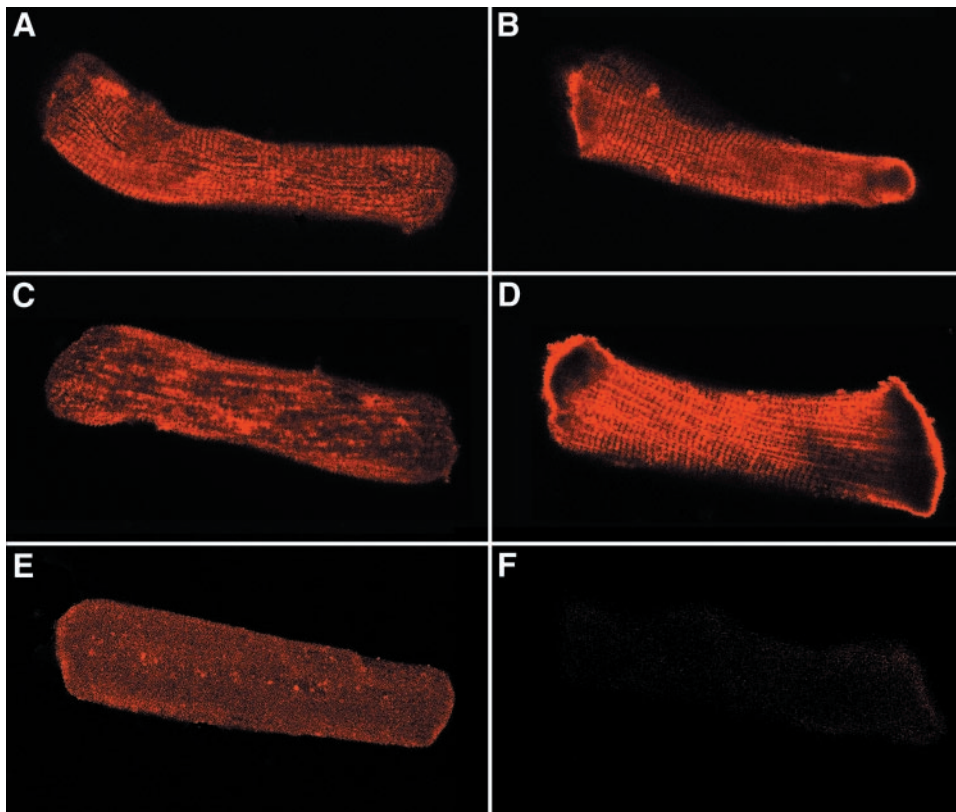


Fig. 3. Immunostaining of cardiomyocytes. Immunostaining of cardiomyocytes was performed with species-unspecific anti-TPM1 antibody TM311 (A and B) and with the human-specific anti-TPM1 antibody ARG1 (C and D). A regular sarcomeric structure was detected in cardiomyocytes from NTG controls (A) and transgenic animals of line B(Asp<sup>175</sup>Asn) (B) using species-unspecific anti-TPM1 antibody TM311. In contrast, human-specific anti-TPM1 antibody ARG1 revealed sarcomeric staining in cardiomyocytes from the transgenic line B(Asp<sup>175</sup>Asn) (D), but not from NTG controls (C). Some unspecific immunostaining was observed with ARG1 preimmune serum (E), whereas Cy3-conjugated goat anti-rabbit IgG showed no background signals. Similar data were observed for lines A(WT) and C(Glu<sup>180</sup>Gly).

line showed signs of myocyte disarray, which was not seen in transgenic animals with the WT human TPM1 cDNA (Fig. 5). Myocyte disarray was predominantly located in the anterior and posterior septum, affecting ~4% of the cross-sectional area in both mutant lines. Other histopathological changes typical for FHC, such as interstitial fibrosis or thickening of the media of intramural coronary arteries, were not seen.

**Ventricular muscle fibers with TPM1 mutation Asp<sup>175</sup>Asn are less sensitive to Ca<sup>2+</sup>.** Physiological analyses were performed to compare functional effects caused by TPM1 missense mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly in vitro. The pCa-force relation of demembranated LV muscle fiber preparations was measured in 3-mo-old animals to determine whether TPM1 mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly would affect the Ca<sup>2+</sup> sensitivity of force generation. The mean values of force measurements at various Ca<sup>2+</sup> concentrations are plotted in Fig. 6. pCa<sub>50</sub> (i.e.,  $-\log$  of the free Ca<sup>2+</sup> concentration required for half-maximal tension development) was  $5.31 \pm 0.03$  for line B(Asp<sup>175</sup>Asn) compared with  $5.51 \pm 0.01$  for line C(Glu<sup>180</sup>Gly) and  $5.55 \pm 0.04$  for transgenic controls from line A(WT). Therefore, force development is less sensitive to Ca<sup>2+</sup> with skinned-fiber preparations from animals with mutation Asp<sup>175</sup>Asn than with skinned fibers from mutation Glu<sup>180</sup>Gly hearts. Furthermore, cooperativity of the pCa-force curves was lower in skinned-fiber preparations of line B(Asp<sup>175</sup>Asn) than line C(Glu<sup>180</sup>Gly) or transgenic controls, as shown by a decline of the Hill coefficient (Table 1). At the same time, maximal shortening velocity and maximal force remained unchanged among all transgenic lines. The results of the pCa-force relation shown for lines B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly) (Fig. 6) were confirmed by analysis of skinned-fiber preparations of two other transgenic lines, D(Asp<sup>175</sup>Asn) and E(Glu<sup>180</sup>Gly)

(data not shown). There were no significant differences in the pCa-force relation between the transgenic line A(WT) and NTG controls (data not shown).

**Spontaneous Ca<sup>2+</sup> waves in cardiomyocytes of line B(Asp<sup>175</sup>Asn).** To investigate whether the decreased Ca<sup>2+</sup> sensitivity of the myofilament system in line B(Asp<sup>175</sup>Asn) is associated with changes in Ca<sup>2+</sup> handling, the fluorescent Ca<sup>2+</sup> indicator fluo 3-AM was used to measure intracellular spontaneous Ca<sup>2+</sup> waves in cardiomyocytes. Cardiomyocytes were obtained from 6 animals of line A(WT), 6 animals of line B(Asp<sup>175</sup>Asn), and 5 animals of line C(Glu<sup>180</sup>Gly); 10–15 cardiomyocytes were analyzed per animal. Spontaneous Ca<sup>2+</sup> waves, known to be the consequence of Ca<sup>2+</sup> overload in cardiomyocytes (25), were detected in 14 of 69 cardiomyocytes of line B(Asp<sup>175</sup>Asn) (20%), but in only 9 of 88 cardiomyocytes of line A(WT) (9%) and 4 of 49 cardiomyocytes of line C(Glu<sup>180</sup>Gly) (8%; Fig. 7A). Accordingly, after the extracellular Ca<sup>2+</sup> concentration was elevated from 2 to 10 mM, 91% of cardiomyocytes of line B(Asp<sup>175</sup>Asn) showed spontaneous Ca<sup>2+</sup> waves, in contrast to 28% and 34% of cardiomyocytes of lines A(WT) and C(Glu<sup>180</sup>Gly), respectively. Additionally, under these “Ca<sup>2+</sup>-overload” conditions, the frequency of spontaneous Ca<sup>2+</sup> waves increased fourfold in cardiomyocytes of line B(Asp<sup>175</sup>Asn) but only twofold in cardiomyocytes of lines A(WT) and C(Glu<sup>180</sup>Gly), as shown in Table 2. At the same time, in cardiomyocytes isolated from line B(Asp<sup>175</sup>Asn), the increase in fluorescence intensity ( $\Delta F/F_0$ ) evoked by Ca<sup>2+</sup> overload was twofold higher than in cardiomyocytes of lines A(WT) and C(Glu<sup>180</sup>Gly). These data are illustrated in Fig. 7, B and C, showing the time course of spontaneous Ca<sup>2+</sup> waves in cardiomyocytes representative of lines B(Asp<sup>175</sup>Asn) and A(WT). As further demonstrated, in

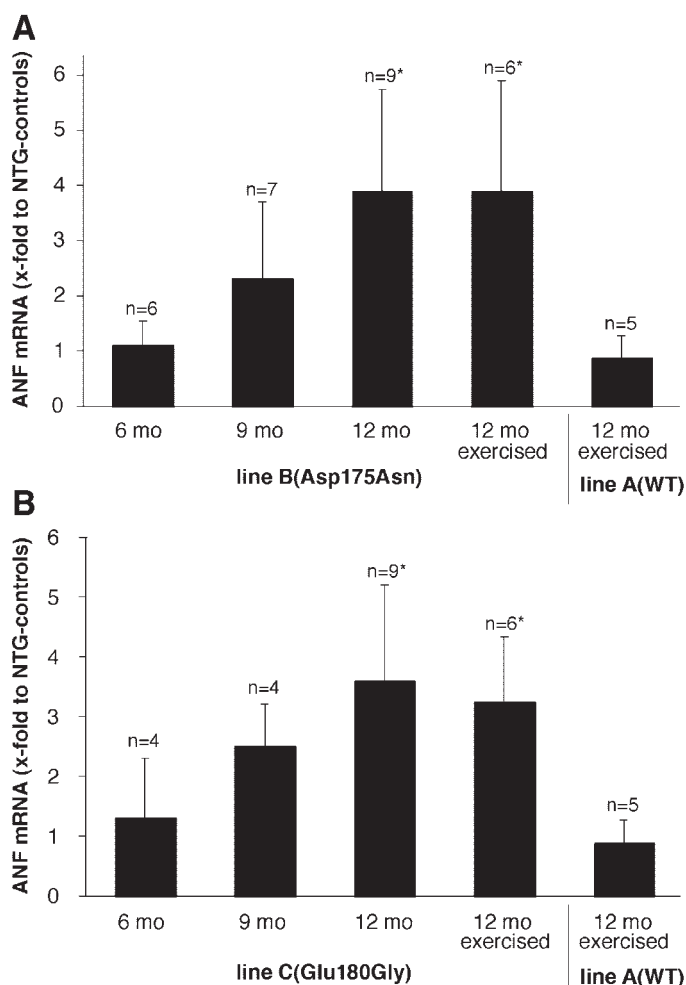


Fig. 4. Stimulation of ventricular atrial natriuretic factor (ANF) mRNA expression. ANF mRNA expression is elevated 4-fold in animals, aged 12 mo, from mutant transgenic lines B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly), but not from transgenic controls of line A(WT). ANF mRNA level was not further stimulated by exercise. Increase in ANF mRNA expression was calculated compared with age-matched NTG controls. \*Corrected  $P < 0.05$ .

cardiomyocytes of all three transgenic lines, spontaneous  $\text{Ca}^{2+}$  waves were completely abolished by the inhibition of the ryanodine receptors with 10  $\mu\text{M}$  ryanodine, suggesting that spontaneous  $\text{Ca}^{2+}$  waves are evoked by  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum.

To analyze whether abnormal  $\text{Ca}^{2+}$  homeostasis in cardiomyocytes of line B(Asp<sup>175</sup>Asn) is associated with changes of the expression of  $\text{Ca}^{2+}$ -handling proteins, the mRNA expression of the  $\text{Ca}^{2+}$ -ATPase, its regulating subunit phospholamban, and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger were studied. The mRNA expression levels of these two  $\text{Ca}^{2+}$  transport systems were not different between cardiomyocytes of lines A(WT), B(Asp<sup>175</sup>Asn), and C(Glu<sup>180</sup>Gly), as revealed by RT-PCR and Northern blot analyses (data not shown).

**Accelerated contraction and relaxation of hearts with TPM1 mutation Asp<sup>175</sup>Asn.** Functional parameters of Langendorff-perfused heart preparations of animals 5–7 mo of age were analyzed to determine whether the reduced  $\text{Ca}^{2+}$  sensitivity of skinned-fiber preparations and the increased cytosolic  $\text{Ca}^{2+}$  concentration in cardiomyocytes of line B(Asp<sup>175</sup>Asn) are

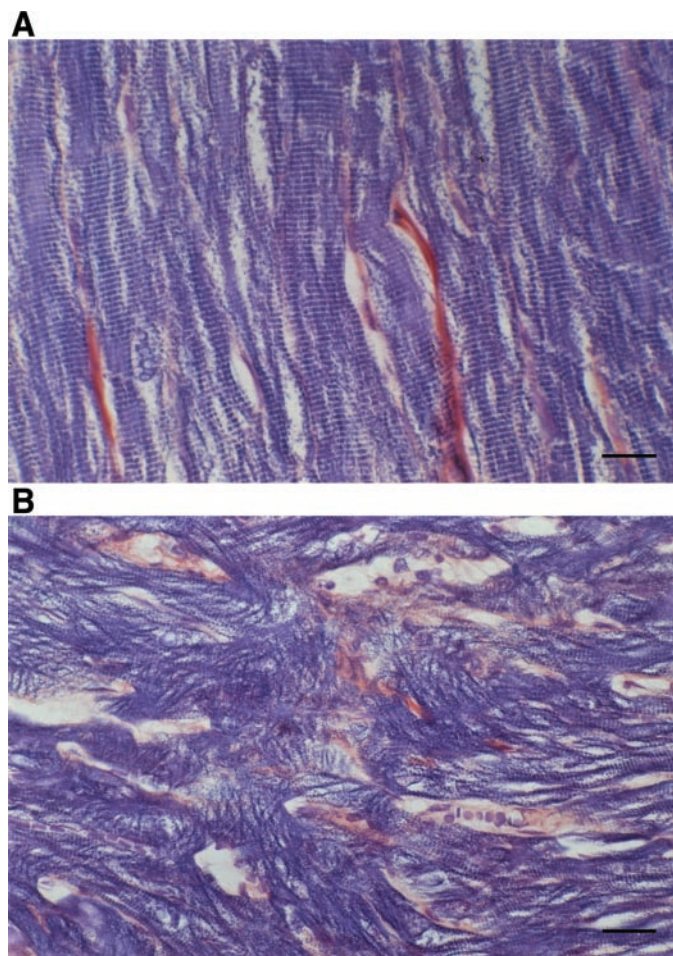


Fig. 5. Histological evidence for myocyte disarray. Transverse sections through the heart of an exercised animal from line B(Asp<sup>175</sup>Asn) are shown. Preparations were stained with phosphotungstic acid-hematoxylin. A: area of normal histological structure of left ventricular free wall. B: area with myocyte disarray of ventricular septum. Similar results were observed for line C(Glu<sup>180</sup>Gly), but not for line A(WT) or NTG controls. Scale bar, 20  $\mu\text{m}$ .

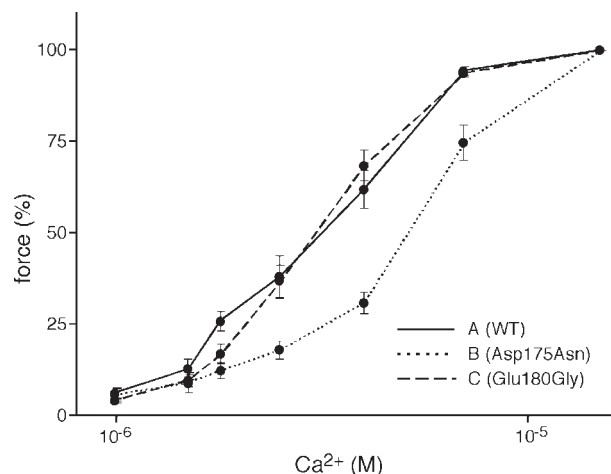


Fig. 6. pCa-force relation of skinned-fiber preparations. Sarcomere length was set at 1.9–2.0  $\mu\text{m}$ , and force was normalized to the corresponding maximum force at pCa 4.50. Curves show decreased  $\text{Ca}^{2+}$  sensitivity for line B(Asp<sup>175</sup>Arg) compared with line C(Glu<sup>180</sup>Gly) and transgenic human wild-type (WT) controls. Hearts from 5 animals per line, 8 fiber preparations per animal, were analyzed.

Table 1. Analysis of heart skinned-fiber preparations of the transgenic lines

| Parameter                             | A(WT)      | B(Asp <sup>175</sup> Asn) | C(Glu <sup>180</sup> Gly) |
|---------------------------------------|------------|---------------------------|---------------------------|
| pCa <sub>50</sub>                     | 5.55±0.04  | 5.31±0.03*                | 5.51±0.01                 |
| V <sub>max</sub> , ML/s               | 1.54±0.10  | 1.49±0.09                 | 1.52±0.10                 |
| F <sub>max</sub> , mN/mm <sup>2</sup> | 47.92±5.35 | 51.06±5.30                | 45.93±2.90                |
| Hill coeff                            | 3.20±0.16  | 2.13±0.30*                | 3.21±0.09                 |

Values are means ± SD. Experiments were performed with heart skinned-fiber preparations of female animals, age 12–14 wk. Hearts from 5 animals per line, 8 fiber preparations per animal, were analyzed. pCa<sub>50</sub>, negative logarithm of free Ca<sup>2+</sup> concentration required for half-maximal force development; V<sub>max</sub>, maximal shortening velocity; ML/s, muscle lengths/s; F<sub>max</sub>, maximal force. Differences revealed by Kruskal-Wallis test: \*B(Asp<sup>175</sup>Asn) vs. A(WT) and C(Glu<sup>180</sup>Gly) at corrected *P* < 0.05.

associated with functional changes in cardiac performance (Table 3). Systolic contraction of Langendorff-perfused heart preparations was accelerated in line B(Asp<sup>175</sup>Asn) compared with line C(Glu<sup>180</sup>Gly) and transgenic WT and NTG controls, as shown by a decreased time to LV peak pressure. Furthermore, diastolic relaxation was accelerated, but only in hearts with mutation Asp<sup>175</sup>Asn, as indicated by a reduced time constant of exponential pressure decay and a decreased time to 90% relaxation accompanied by an increased maximal rate of pressure decay normalized for LVDP [(-dp/dt)/LVDP]. Pressure-volume and Ca<sup>2+</sup> dose-response curves were superimposable for isolated hearts of all transgenic lines as well as NTG controls (data not shown).

## DISCUSSION

Mutations in TPM1 have been associated with variable clinical phenotypes of FHC in humans (11, 21, 23, 56, 58). This high degree of clinical heterogeneity has been observed not only between different missense mutations in TPM1, but

Table 2. Analysis of spontaneous Ca<sup>2+</sup> waves in cardiomyocytes

| Parameter                            | A(WT)      | B(Asp <sup>175</sup> Asn) | C(Glu <sup>180</sup> Gly) |
|--------------------------------------|------------|---------------------------|---------------------------|
| 2 mM extracellular Ca <sup>2+</sup>  |            |                           |                           |
| ΔF/F <sub>0</sub>                    | 18±5       | 28±6                      | 19±6                      |
| f, Hz                                | 0.04±0.01  | 0.06±0.02                 | 0.05±0.02                 |
| n                                    | 21         | 19                        | 17                        |
| 10 mM extracellular Ca <sup>2+</sup> |            |                           |                           |
| ΔF/F <sub>0</sub>                    | 12±4       | 24±7*                     | 11±4                      |
| f, Hz                                | 0.08±0.03† | 0.25±0.07†                | 0.09±0.03†                |
| n                                    | 18         | 22                        | 19                        |

Values are means ± SD. Spontaneous Ca<sup>2+</sup> waves were analyzed in cardiomyocytes using the fluorescent Ca<sup>2+</sup> indicator fluo 3-AM. ΔF/F<sub>0</sub>, fluorescence intensity; f, frequency of spontaneous Ca<sup>2+</sup> waves; n, number of cardiomyocytes derived from 4–6 animals, 3–5 cardiomyocytes per animal. Differences revealed by Kruskal-Wallis test: \*B(Asp<sup>175</sup>Asn) vs. A(WT) and C(Glu<sup>180</sup>Gly); †10 mM vs. 2 mM extracellular Ca<sup>2+</sup> at corrected *P* < 0.05.

also between affected members of families. It is unclear whether these differences are dependent on the nature of individual mutations or, alternatively, are due to genetic, epigenetic, or environmental factors. In addition, the slow progression of FHC suggests that pathophysiological changes affect sarcomeric structure and function in a rather subtle manner and/or can be functionally well compensated. For this reason, we were interested to know how low-level overexpression of human TPM1, harboring mutation Asp<sup>175</sup>Asn or Glu<sup>180</sup>Gly, affects cardiac physiology in transgenic rats. In the present study, the expression of the transgenes was driven by a 250-bp sequence of the rat MLC-2 promoter, which has been shown to confer cardiomyocyte-specific expression but does not contain regulatory elements known to have function in muscle-specific transcription (16).

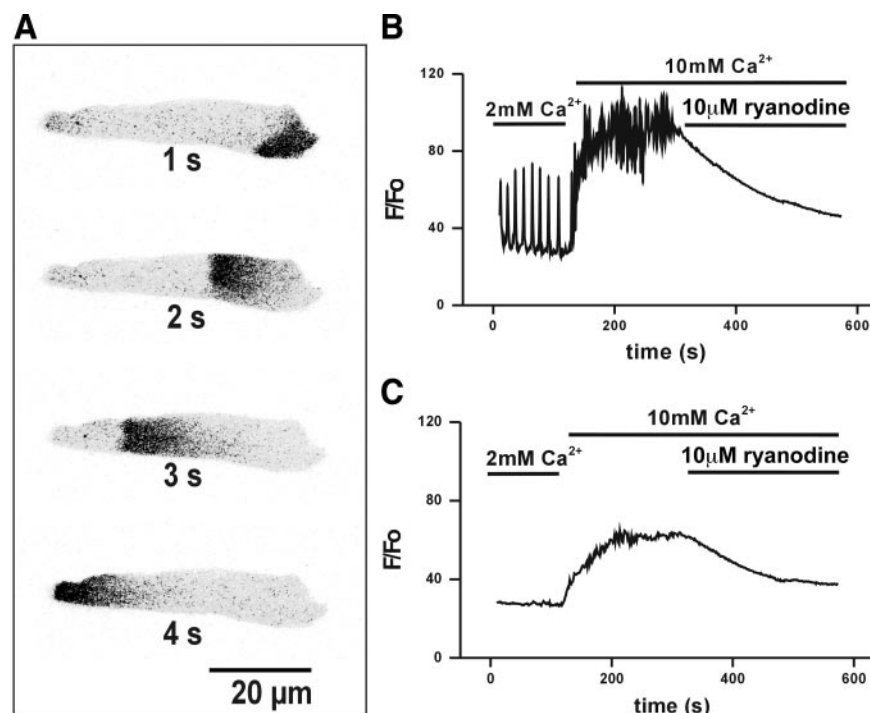


Fig. 7. Increased frequency of spontaneous Ca<sup>2+</sup> waves in cardiomyocytes of line B(Asp<sup>175</sup>Asn). A: spontaneous Ca<sup>2+</sup> wave in a cardiomyocyte of a rat of line B(Asp<sup>175</sup>Asn) in the presence of 10 mM extracellular Ca<sup>2+</sup>. Four consecutive full-field images (1, 2, 3, and 4) were taken 1 s apart. B and C: time course of spontaneous Ca<sup>2+</sup> waves in cardiomyocytes representative for line B(Asp<sup>175</sup>Asn) and A(WT), respectively. B: fluorescence intensity (ΔF/F<sub>0</sub>) for a cardiomyocyte isolated from an animal of line B(Asp<sup>175</sup>Asn). A 4-fold increase in the frequency of spontaneous Ca<sup>2+</sup> waves was detected after elevation of extracellular Ca<sup>2+</sup> concentration from 2 to 10 mM. Under this condition, ΔF/F<sub>0</sub> increased 2-fold. Spontaneous Ca<sup>2+</sup> waves were completely abolished by addition of ryanodine. C: spontaneous Ca<sup>2+</sup> waves were not detected in a cardiomyocyte of a control animal of line A(WT), even after increase in extracellular Ca<sup>2+</sup> concentration from 2 to 10 mM.

Table 3. Isovolumic contractile performance of Langendorff-perfused heart preparations

| Parameter                                                         | Controls<br>(n = 19) | B(Asp <sup>175</sup> Asn)<br>(n = 15) | C(Glu <sup>180</sup> Gly)<br>(n = 10) |
|-------------------------------------------------------------------|----------------------|---------------------------------------|---------------------------------------|
| Heart wt/body wt, mg/g                                            | 0.43±0.01            | 0.43±0.01                             | 0.44±0.01                             |
| End-diastolic pressure, mmHg                                      | 6.6±0.7              | 5.3±0.7                               | 6.8±0.7                               |
| LVDP, mmHg                                                        | 93.0±2.9             | 87.6±2.9                              | 85.4±2.8                              |
| +dP/dt/LVDP, s <sup>-1</sup>                                      | 28.6±0.5             | 29.6±0.7                              | 30.3±0.2                              |
| -dP/dt/LVDP, s <sup>-1</sup>                                      | 21.4±0.4             | 22.2±0.2*†                            | 20.7±0.3                              |
| TPP, ms                                                           | 63.0±0.8             | 60.0±0.7*†                            | 64.6±0.5                              |
| $\tau$ , ms                                                       | 24.5±0.7             | 22.5±0.5*†                            | 24.8±0.6                              |
| T <sub>90</sub> , ms                                              | 65.2±1.3             | 61.8±1.0*                             | 64.1±1.0                              |
| Coronary flow/heart wt,<br>ml·min <sup>-1</sup> ·mg <sup>-1</sup> | 12.0±0.1             | 12.0±0.1                              | 12.0±0.1                              |

Values are means  $\pm$  SE. All hearts were paced at 300 beats/min. Experiments were performed with heart preparations of female animals aged 5–7 mo. In the control group, 8 animals of the transgenic control line A(WT) and 11 nontransgenic (NTG) animals were pooled, because separate analysis of both control groups revealed no difference in measured parameters.  $\pm$ dP/dt, maximal rate of pressure rise or decay; LVDP, left ventricular developed pressure; TPP, time to peak pressure;  $\tau$ , relaxation coefficient; T<sub>90</sub>, time to 90% relaxation; n, number of animals. Differences revealed by Kruskal-Wallis test: \*B(Asp<sup>175</sup>Asn) vs. controls; †B(Asp<sup>175</sup>Asn) vs. C(Glu<sup>180</sup>Gly) at corrected  $P < 0.05$ .

Histopathological analysis revealed discrete and similar changes for mutant transgenic lines B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly). Myocardial hypertrophy did not develop, even when a chronic exercise workload was imposed. However, in both mutant lines, B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly), mRNA expression of ANF and  $\alpha$ -skeletal actin was increased in animals older than 9 mo. It is known that the induction of genes normally expressed in fetal myocardium is an early event of the hypertrophic cardiac response (9). The induction of ANF and  $\alpha$ -skeletal actin mRNA in our transgenic animals was less pronounced by nearly one order of magnitude than in hearts showing marked myocardial hypertrophy, as seen, for example, in stroke-prone spontaneously hypertensive rats (data not shown).

Myocyte disarray was detected in the ventricular septum of mutant transgenic lines B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly), whereas in other animal models of FHC, myocyte disarray is equally distributed within the myocardium (14, 27, 40, 53). The ventricular septum is the most common site of histopathological changes in hearts of FHC patients (28). In the transgenic animals studied here, the degree of myocyte disarray was small compared with the human disease and developed only after exercise. A low degree of histopathological changes, including myocardial hypertrophy, myocyte disarray, and interstitial fibrosis, is consistently observed in most animal models of FHC, particularly when mutations of thin filament proteins are studied (38, 40, 53, 65). These experimental observations are in agreement with clinical data correlating the genetic course of FHC with histopathological changes (2, 8, 26).

Although the induction of molecular markers of myocardial hypertrophy is similar between lines B(Asp<sup>175</sup>Asn) and C(Glu<sup>180</sup>Gly), these mutant transgenic lines differed with respect to cardiac physiology in vitro. Investigation of LV skinned-fiber preparations revealed decreased Ca<sup>2+</sup> sensitivity of isometric force generation in line B(Asp<sup>175</sup>Asn), but not in line C(Glu<sup>180</sup>Gly). This observation is surprising, because both

mutations are located in the COOH-terminal region near Cys<sup>190</sup>, the putative TnT-binding region (57). It is possible that mutations in this already destabilized region of the TPM1 molecule further disturb the local structure of the protein, therefore altering the cascade of Ca<sup>2+</sup>-induced protein-protein interactions in the thin filament system. The formation of force-generating cross bridges, initiated by Ca<sup>2+</sup> binding to troponin C, is highly cooperative and characterized by the Hill coefficient (17). Although cardiac troponin C has only one essential Ca<sup>2+</sup>-binding site (57), skinned cardiac fiber preparations of transgenic control line A(WT) demonstrate a more than threefold elevated Hill coefficient (Table 1). This high cooperativity cannot be explained solely by cooperative interactions between thin filament regulatory proteins. Rather, the formation of force-generating cross bridges changes the conformation of thin filament regulatory proteins, increasing the Ca<sup>2+</sup> affinity of troponin C and, therefore, facilitating the formation of additional force-generating cross bridges (67). This reciprocal coupling may be altered in line B(Asp<sup>175</sup>Asn), producing reduced Ca<sup>2+</sup> sensitivity and a decreased Hill coefficient compared with line C(Glu<sup>180</sup>Gly) and transgenic controls. Decreased Ca<sup>2+</sup> sensitivity of isometric force generation observed for line B(Asp<sup>175</sup>Asn), but not for line C(Glu<sup>180</sup>Gly), does not seem to be related to nonspecific effects, such as the influence of the genomic integration site, because similar results were observed with two other transgenic rat lines with either TPM1 mutation.

To investigate the mechanisms compensating for the reduced Ca<sup>2+</sup> sensitivity of isometric force generation observed with skinned cardiac fibers from hearts with mutation Asp<sup>175</sup>Asn, Ca<sup>2+</sup> handling in adult cardiomyocytes was analyzed. In cardiomyocytes of line B(Asp<sup>175</sup>Asn), increased frequency and amplitude of spontaneous Ca<sup>2+</sup> waves suggest an increase in intracellular Ca<sup>2+</sup> concentration, as demonstrated for isolated cardiomyocytes (10, 25, 62), cardiac trabeculae (61), and the intact heart (22). We presume that the reduced number of Ca<sup>2+</sup>-binding sites in the myofilament system of line B(Asp<sup>175</sup>Asn), shown in experiments on demembranated LV muscle fiber preparations, shifts the equilibrium between bound to the myofilament system and unbound Ca<sup>2+</sup> toward free intracellular Ca<sup>2+</sup>. As a result, the probability of the regeneration of spontaneous Ca<sup>2+</sup> waves is increased in cardiomyocytes of line B(Asp<sup>175</sup>Asn). At the same time, the elevated intracellular Ca<sup>2+</sup> concentration in cardiomyocytes of line B(Asp<sup>175</sup>Asn) is not associated with changes in the mRNA expression of Ca<sup>2+</sup>-ATPase and the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, known to be altered in several pathological conditions (1, 47). The regulation and function of Ca<sup>2+</sup>-handling proteins in transgenic rats harboring mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly in TPM1 should be analyzed in further research.

Langendorff-perfused heart preparations revealed an accelerated contraction and relaxation with hearts from animals of line B(Asp<sup>175</sup>Asn), but not line C(Glu<sup>180</sup>Gly). Interestingly, transgenic mice overexpressing Arg<sup>92</sup>Gln-mutated TnT show accelerated myocardial contractility in vitro and reduced sarcomere length of isolated cardiomyocytes (54). The location of TnT mutation Arg<sup>92</sup>Gln within the TPM1 binding domain of the TnT molecule suggests that altered protein-protein interactions between TPM1 and TnT in the thin filament system could be functionally compensated by increased intracellular Ca<sup>2+</sup>, which leads to a "hypercontractile" state of the myofilament

system. Accordingly,  $\text{Ca}^{2+}$  load is one of the main determinants of the velocity of propagation of spontaneous  $\text{Ca}^{2+}$  waves in cardiac muscle (32), correlating with cell shortening and membrane depolarization (31, 33). Furthermore, a spontaneous increase in intracellular  $\text{Ca}^{2+}$  concentration could activate transient inward currents (3, 51), generally assumed to be arrhythmogenic (13) and one of the proposed mechanisms of sudden cardiac death in FHC (8, 49).

It is difficult to explain how changes in the  $\text{Ca}^{2+}$  sensitivity of the myofilament system might lead to myocardial hypertrophy as postulated for FHC (49). In the present study, changes in the  $\text{Ca}^{2+}$  sensitivity of the myofilament system preceded the induction of molecular markers of myocardial hypertrophy. We speculate that myocardial hypertrophy is compensatory and the result of a decreased functional efficacy of the contractile apparatus associated with alterations in cardiac myocyte metabolism, in particular in energy metabolism, as has been suggested for MHC mutation Arg<sup>403</sup>Gln (48).

A large number of FHC-causing mutations in different sarcomeric proteins have been described (2, 26, 49), and several animal models were designed to study the pathogenetic mechanisms for selected mutations (27, 38, 40, 42, 53, 65). However, none of these animal models was suitable to reproduce the entire cardiac phenotype of the human disease; moreover, phenotypical changes that are not features of the human disease were observed in these animal models (14, 30, 40, 53, 54). Data of animal studies show that the design of the genetic manipulation can have a strong influence on the cardiac phenotype of the transgenic animals. This was well demonstrated for TnT mutation Arg<sup>92</sup>Gln, which was studied in two transgenic mouse models with use of different promoter sequences and transgenes from distinct species. Both models were associated with significant differences in cardiac histopathology (40, 54).

In this context, it is interesting to note that human TPM1 mutation Asp<sup>175</sup>Asn expressed in the rat, which in line B(Asp<sup>175</sup>Asn) causes decreased  $\text{Ca}^{2+}$  sensitivity of isometric force generation as well as accelerated contraction and relaxation in Langendorff-perfused heart preparations, produces opposite functional effects when it is present within a mouse TPM1 transgene and overexpressed in mice (38). These opposing observations on  $\text{Ca}^{2+}$  sensitivity of isometric force generation and functional parameters of work-performing heart preparations between transgenic rats and mice with mutation Asp<sup>175</sup>Asn might be related to species-specific differences and/or the distinct transgenic design. Although the 250-bp fragment of the rat MLC-2 promoter leads to weak transgenic expression in rats (Fig. 2), the 5.5-kb promoter sequence of the murine  $\alpha$ -MHC gene has been shown to be a strong promoter for transgenic overexpression in mice (37, 38, 42, 65). With the use of this 5.5-kb promoter sequence of the murine  $\alpha$ -MHC gene for transgenic overexpression in mice, increased  $\text{Ca}^{2+}$  sensitivity of isometric force generation of LV skinned-fiber preparations and slower relaxation properties in work-performing heart preparations were observed not only in animals with mutation Asp<sup>175</sup>Asn in the transgenic mouse TPM1, but also in animals with mutation Glu<sup>180</sup>Gly (30, 42) and in mice overexpressing mouse  $\beta$ -tropomyosin (36, 37, 41). Interestingly, in these three animal models, the expression level of the transgenic proteins driven by this promoter fragment of the  $\alpha$ -MHC gene was ~60% of the total tropomyosin that was associated

with a concomitant decrease in endogenous TPM1. It should be noted that the 5'-untranslated region of the murine  $\alpha$ -MHC gene contains a number of regulatory elements known to play a role in muscle-specific transcription (37, 44, 52), which could interfere with phenotypic changes caused by transgenic TPM1 mutation Asp<sup>175</sup>Asn or Glu<sup>180</sup>Gly. High transgene expression of reporter genes and normal proteins in transgenic mice can in itself induce phenotypical changes in the myocardium (18, 20).

The phenotype of transgenic animals is not only determined by regulatory elements of the promoter and the expression level of the transgenes but also is influenced by unexpected events, which are often much less understood. For instance, mice overexpressing mutation Glu<sup>180</sup>Gly in TPM1 did not survive beyond 6 mo of age, exhibiting extreme lethargy, and, most likely, succumb to starvation (42). In contrast, transgenic mice overexpressing mutation Asp<sup>175</sup>Asn in TPM1 (38), as well as transgenic rats with either TPM1 mutation characterized here, did not demonstrate reduced viability or any gross phenotypic alterations.

In conclusion, the data of the present study indicate that TPM1 mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly cause phenotypic changes via distinct pathogenetic mechanisms. Changes in  $\text{Ca}^{2+}$  handling represent a sensitive mechanism in response to alterations in the sarcomeric structure, particularly in the thin filament system, as shown here for mutation Asp<sup>175</sup>Asn in TPM1. On the basis of data of this study, we propose that mutation Asp<sup>175</sup>Asn leads to decreased  $\text{Ca}^{2+}$  sensitivity of isometric force generation in the myofilament system, which is compensated by increased intracellular  $\text{Ca}^{2+}$  concentration, leading to accelerated myocardial contraction and relaxation. The results show that changes in  $\text{Ca}^{2+}$  handling precede the induction of molecular markers of myocardial hypertrophy in the absence of histopathological abnormalities known to be typical for FHC, such as myocyte disarray and interstitial fibrosis. Therefore, the precise mechanisms whereby mutations Asp<sup>175</sup>Asn and Glu<sup>180</sup>Gly cause myocardial hypertrophy and other histopathological changes in FHC remain to be further elucidated. Pathogenetic mechanisms in FHC have to be addressed separately for each particular disease-causing mutation by summarizing data from distinct experimental approaches in vitro, the characterization of animal models, and clinical investigations.

#### ACKNOWLEDGMENTS

We thank Ursula Ganten and Andrea Leupold for support of the experimental work and helpful discussion of the manuscript. We appreciate the technical help of Ilona Trippmacher, Annegret Dahlke, Marion Somnitz, Kerstin Voigt, and Helmut Mattausch.

Present address of K. V. Essin: Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences, St. Petersburg, Russia.

#### GRANTS

This work was supported by Deutsche Forschungsgemeinschaft Grant Th476/2 (to L. Thierfelder). D. Wernicke's work was supported by European Social Funds for the Berlin-Brandenburg area (nos. 20010019 and 20030005).

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