An abnormal Ca^{2+} response in mutant sarcomere protein–mediated familial hypertrophic cardiomyopathy

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Dominant-negative sarcomere protein gene mutations cause familial hypertrophic cardiomyopathy (FHC), a disease characterized by left-ventricular hypertrophy, angina, and dyspnea that can result in sudden death. We report here that a murine model of FHC bearing a cardiac myosin heavy-chain gene missense mutation ($\alpha MHC^{403/+}$), when treated with calcineurin inhibitors or a K^+-channel agonist, developed accentuated hypertrophy, worsened histopathology, and was at risk for early death. Despite distinct pharmacologic targets, each agent augmented diastolic Ca^{2+} concentrations in wild-type cardiac myocytes; $\alpha MHC^{403/+}$ myocytes failed to respond. Pretreatment with a Ca^{2+}-channel antagonist abrogated diastolic Ca^{2+} changes in wild-type myocytes and prevented the exaggerated hypertrophic response of treated $\alpha MHC^{403/+}$ mice. We conclude that FHC-causing sarcomere protein gene mutations cause abnormal Ca^{2+} responses that initiate a hypertrophic response. These data define an important Ca^{2+}-dependent step in the pathway by which mutant sarcomere proteins trigger myocyte growth and remodel the heart, provide definitive evidence that environment influences progression of FHC, and suggest a rational therapeutic approach to this prevalent human disease.

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Introduction

Cardiac hypertrophy, identified by increased ventricular wall thickness, is a prevalent finding that occurs in 1 of 500 healthy young individuals (1). Hypertrophy is recognized as a compensatory physiologic response to excessive hemodynamic burden (such as pressure or volume overload) or a pathologic state that compromises function and produces heart failure (reviewed in refs. 2, 3). Familial hypertrophic cardiomyopathy (FHC) is a heritable disorder of increased ventricular wall thickness that occurs in the absence of hemodynamic burden. Affected individuals can be asymptomatic or develop breathlessness, chest pain, congestive heart failure, or sudden death. Cardiac hypertrophy is the only finding of the disorder, and both the severity and anatomic distribution of this pathology varies considerably between affected individuals. Molecular genetic studies have demonstrated that dominant mutations in genes encoding β-cardiac myosin heavy chain, cardiac troponin T, myosin regulatory light chain, myosin essential light chain, cardiac actin, cardiac troponin I, α-tropomyosin, titin, or cardiac myosin-binding protein C cause hypertrophic cardiomyopathy (for a review see ref. 4). Although the genetic causes of FHC are now defined, the mechanisms by which sarcomere protein gene mutations lead to cardiac hypertrophy are not understood.

Cardiac hypertrophy can result from a variety of different causes, e.g., sarcomere protein gene mutations, pressure overload, or aberrant gene expression. To elucidate the cellular and molecular events that signal remodeling of the heart, researchers have investigated different models of cardiac hypertrophy (reviewed in ref. 5). Recent studies have exploited transgenic mice that overexpress target proteins in the heart (6, 7). Robust and unregulated transgenic expression of such unrelated proteins as α adrenergic receptors (8), calcineurin (9), and even green fluorescence protein (10) have been shown to increase myocardial mass. Significant information has also been obtained from analyses of rodent models of pressure overload produced by aortic banding. Analyses of these pressure-overload models have implicated Ca^{2+}-dependent steps in the hypertrophic process leading to this form of hypertrophy (for a review see ref. 11). Whether all of these causes of hypertrophy are signaled via the same pathways remains uncertain. Studies in murine FHC models should help address this question.

The $\alpha MHC^{403/+}$ mouse, a murine FHC model, bears a missense mutation in one allele of its endogenous

α-*cardiac myosin heavy-chain* gene (12). This defect substitutes glutamine for arginine at residue 403 and is analogous to a well-characterized human myosin mutation (13, 14). As in the human disease, cardiac pathology in $\alpha MHC^{403/+}$ mice evolves slowly; histopathology (myocyte hypertrophy and disarray) and increased ventricular wall thickness are absent in 6-week-old mice, are variably present at 15 weeks, and are established at 30 weeks. Although dissection of the hypertrophic process using pharmacologic agents has produced equivocal results in analyses of some models of hypertrophy, we hypothesized that treatment of $\alpha MHC^{403/+}$ mice, which differ from wild-type mice by only a single nucleotide, would help define mechanisms by which a sarcomere protein defect triggers cardiac hypertrophy. Therefore, we screened for pharmacologic agents that augment or inhibit the hypertrophic process in $\alpha MHC^{403/+}$ but not in wild-type mice. Three agents were identified that cause significant hypertrophy in mutant but not wild-type mice. These agents were used to unmask abnormal intracellular Ca^{2+} responses in both $\alpha MHC^{403/+}$ mice and isolated myocytes and to define a critical role for this ion in triggering the hypertrophic response due to sarcomere protein defects. The demonstration that Ca^{2+} plays a role in modulating the hypertrophic response has implications for the management and treatment of FHC patients.

Methods

Animals. Heterozygous $\alpha MHC^{403/+}$ mice bearing a missense mutation that converts codon 403 from an arginine to a glutamine has been described (12). Mouse genotypes were determined by PCR amplification and restriction enzyme digestion of genomic tail DNA from each animal. All mice were maintained according to protocols approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Drug treatment and survival. Mice were treated with cyclosporin A (CsA) (two subcutaneous injections of 15 mg/kg in PBS daily) or FK506 (two subcutaneous injections of 3 mg/kg in PBS daily) for less than 5 weeks. Minoxidil and diltiazem were added to the drinking water (200 mg/l and 450 mg/l), corresponding to 0.8 mg and 1.8 mg drug/day, respectively. Mice treated with both diltiazem and CsA or minoxidil were given diltiazem for 2 weeks before the initiation of either CsA or minoxidil treatment. L-arginine (2% wt/vol) was injected once daily.

Survival was computed by the Kaplan-Meier method (StatView software; Abacus Concepts Inc., San Francisco, California, USA).

Analyses of RNA. Northern blot analyses were performed as described previously (15) using oligonucleotide probes for α-skeletal actin (5′:TGGCTTTAATGCTTCAAGTTTTCCATTTCCTT-TCCACAGGG), BNP (5′:CAGCTTGAGATATGTGTCACCTTGGAATTTTGAGGTCTCTGC-TGGACC), ANF (AATGTGACCAAGCTGCGTGACACACCACAAGGGCTTA-GGATCTTTTGC), and GAPDH (5′GGAACATGTAGACCATGTAGTT-GAGGTCAATGAAG). Oligonucleotide probes were 5′-end labeled and hybridized to nylon membranes as described. Hybridized membranes were exposed using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, California, USA), and the hybridization signal was measured using ImageQuant software (Molecular Dynamics Inc.). Signals were normalized based on hybridization to GAPDH.

Echocardiography and blood pressure measurement. Transthoracic echocardiography was performed in adult wild-type (+/+) and $\alpha MHC^{403/+}$ mice using a 12-MHz probe and a Sonos 5500 ultrasonograph (Hewlett-Packard, Andover, Massachusetts, USA), as described (15). Mice were anesthetized with 2.5% Avertin (0.010 ml/g), warmed with a heating pad, and attached to an EKG monitor. Left ventricular (LV) parameters and heart rates were obtained from M-mode interrogation in a short-axis view. Orthogonal left atrial diameter (LAD) was obtained from two-dimensional echocardiographic images in a long-axis view. Heart rates were greater than 400 beats per minute (bpm). Echocardiographic measurements were averaged from at least three separate cardiac cycles. A single observer who was blinded to mice genotypes performed all echocardiographic measurements.

The statistical significance of differences in echocardiographic parameters between groups of wild-type (+/+) and $\alpha MHC^{403/+}$ mice were determined by unpaired Student's *t* test. Data are expressed as mean plus or minus SD. A *P* value less than 0.05 was considered significant.

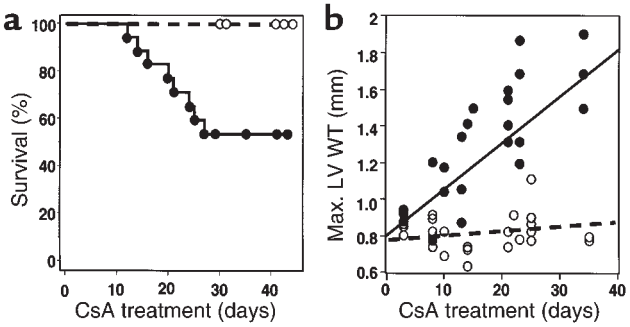
Histology and morphology. Cardiac tissue was treated for histologic and morphologic examination, as described. In brief, the heart was excised from wild-type and $\alpha MHC^{403/+}$ mice, washed in 37°C Dulbecco's PBS, and arrested in 50 mM KCl. Sections from fixed, embedded tissues were stained with either hematoxylin and eosin (H&E) or with Masson's trichrome. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assays were performed on cardiac tissue sections using a kit produced by Roche Molecular Biochemicals (Mannheim, Germany).

Ca^{2+} concentrations in cardiac myocytes. Isolated adult myocytes were prepared as described (16) and analyzed using the SoftEdge Acquisition System and IonWizard (IonOptix Corp., Milton, Massachusetts, USA). Cells were loaded with the fluorescent Ca^{2+} indicator, fura-2, and fluorescence was measured in stimulated myocytes using established procedures (16).

Results

To identify the signaling pathways for hypertrophy that are triggered by mutant sarcomere proteins, $\alpha MHC^{403/+}$ and wild-type mice (aged 6 to 12 weeks) were treated with agents implicated previously in activating or repressing myocyte growth. Pharmacologic inhibition of calcineurin activity by the immunosuppressive agent CsA has been shown previously to prevent (9, 17) and cause regression (18) of cardiac hypertrophy in

Figure 1
(**a**) Survival of 13 CsA-treated wild-type (open circles) and 18 CsA-treated $\alpha MHC^{403/+}$ (filled circles) mice. Two $\alpha MHC^{403/+}$ mice died on the same day on two occasions. Wild-type mice were sacrificed at variable times to provide control specimens for $\alpha MHC^{403/+}$ mice. (**b**) Serial assessment of LV wall thickness (measured by transthoracic echocardiography) of wild-type (open circles) and $\alpha MHC^{403/+}$ (filled circles) mice treated with CsA. The coefficient of correlation is $r = 0.83$ for LV wall thickness and duration of treatment of $\alpha MHC^{403/+}$ mice.



some rodent models. CsA was administered subcutaneously to mice at doses producing CsA serum levels of approximately 1.7 mg/ml; comparable levels are found in humans receiving immunosuppressant therapy (19). Surprisingly, approximately 40% of the $\alpha MHC^{403/+}$ mice, but no wild-type mice, died during treatment (Figure 1a). Left ventricular (LV) wall thickness was assessed in surviving mice using transthoracic echocardiography (15). Serial analyses demonstrated that LV wall thickness increased almost twofold with treatment of $\alpha MHC^{403/+}$ mice, but not wild-type mice (Figure 1b, Figure 2, Figure 3; Table 1). The extent of LV hypertrophy in $\alpha MHC^{403/+}$ mice correlated with the duration of treatment (Figure 1b; $r = 0.83$). CsA-induced LV hypertrophy was associated with significant reductions in LV chamber dimensions, increased LV fractional shortening, and left atrial dilation (Table 1).

Pathologic examination of CsA-treated $\alpha MHC^{403/+}$ hearts confirmed echocardiographic findings of markedly increased LV wall thickness and also demonstrated profound right ventricular hypertrophy (Figure 3). Reductions of ventricular cavity dimensions and massive left-atrial dilation associated with mural thrombus formation was also found in some hearts (Figure 3 and data not shown). Histologic study of myocardial sections from CsA-treated $\alpha MHC^{403/+}$ mice (Figure 4) demonstrated more extensive myocyte hypertrophy, myofibrillar disarray, and interstitial fibrosis than sections from untreated, age-matched, $\alpha MHC^{403/+}$ mice. No ultrastructural abnormalities were found in CsA-treated wild-type mice (Figure 4, a and b). Masson trichrome stain revealed increased collagen deposition and fibrosis in specimens derived from CsA-treated $\alpha MHC^{403/+}$ mice (Figure 4f) compared with hearts from untreated $\alpha MHC^{403/+}$ mice (Figure 4d); both were absent in untreated or CsA-treated wild-type mice (Figure 4a and data not shown). Tissue sections from hearts of wild-type and $\alpha MHC^{403/+}$ mice, with and without CsA treatment, were assessed using the immunohistochemical TUNEL assay. There were no significant differences in TUNEL staining between sections obtained from the four different heart samples (data not shown).

To determine whether the augmented pathology of CsA-treated $\alpha MHC^{403/+}$ mice represented an idiosyncratic response, the effects of another calcineurin inhibitor, FK506, were examined. Subcutaneous administration of FK506 to $\alpha MHC^{403/+}$ ($n = 5$) mice produced a comparable, exaggerated hypertrophic response. One $\alpha MHC^{403/+}$ mouse died suddenly 4 days after initiation of the treatment. Echocardiography of surviving mice after 1 week of treatment showed severe LV hypertrophy and histopathology similar to that observed in CsA-treated $\alpha MHC^{403/+}$ mice (Table 1). FK506 treatment of wild-type ($n = 5$) mice had no effect on LV wall thickness, cardiac hemodynamics (assessed by echocardiography; Table 1), or histopathology (data not shown).

In addition to the substantial cardiac hypertrophy found in $\alpha MHC^{403/+}$ mice treated with CsA and FK506, both the rate of development and the morphology of the drug-induced hypertrophy were unusual. Untreat-

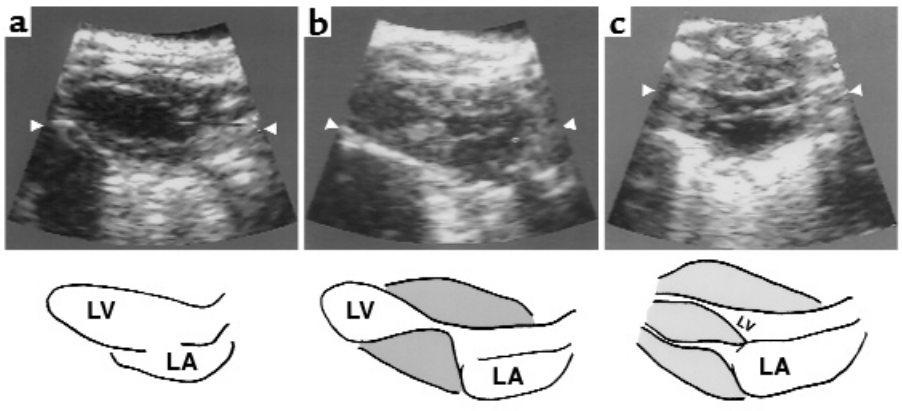


Figure 2
Echocardiographic assessment of CsA-treated and untreated $\alpha MHC^{403/+}$ hearts. Upper panel: Transthoracic echocardiograms of untreated (**a**) and CsA-treated $\alpha MHC^{403/+}$ mice (**b**, day 7; **c**, day 15). Lower panel: Cardiac morphology defined by echocardiograms of untreated (**a**) and CsA-treated $\alpha MHC^{403/+}$ mice (**b**, day 7; **c**, day 15).

Table 1
The effects of CsA, FK506, minoxidil, and diltiazem on wild-type and $\alpha MHC^{403/+}$ cardiac morphology assessed by echocardiography

	Wild-type		$\alpha MHC^{403/+}$	
Study group	Echo 1[A]	Echo 2[B]	Echo 1	Echo 2
		LVAW (mm)		
No Rx	0.88 ± 0.02	–	0.95 ± 0.02	–
CsA[B]	0.78 ± 0.09	0.85 ± 0.11	1.03 ± 0.23[C]	1.54 ± 0.23[D]
FK506	0.81 ± 0.01	–	1.43 ± 0.13[C,E]	–
Minox	0.81 ± 0.02	0.81 ± 0.01	1.15 ± 0.07[C]	1.35 ± 0.09[D]
CsA + dilt	0.80 ± 0.02	0.81 ± 0.01	0.84 ± 0.03	0.83 ± 0.01
FK506 + dilt	0.80 ± 0.01	0.81	0.87 ± 0.05	0.94 ± 0.01
Minox + dilt	0.87 ± 0.03	0.86 ± 0.01	0.95 ± 0.06	0.99 ± 0.06
		LVDD (mm)		
No Rx	3.02 ± 0.42	–	2.67 ± 0.17	–
CsA	2.92 ± 0.14	2.96 ± 0.23	2.72 ± 0.36	1.73 ± 0.53[D]
FK506	2.99 ± 0.09		1.62 ± 0.28[F]	
Minox	3.01 ± 0.05	2.98 ± 0.06	2.63 ± 0.22[C]	2.61 ± 0.18[C]
CsA + dilt	2.73 ± 0.04	2.90 ± 0.02	2.78 ± 0.06	2.89 ± 0.12
FK506 + dilt	2.86 ± 0.11	2.80	2.82 ± 0.06	2.64 ± 0.11
Minox + dilt	2.90 ± 0.01	2.92 ± 0.05	2.95 ± 0.06	2.94 ± 0.09
		LVFS (%)		
No Rx	38 ± 6	–	48 ± 6	–
CsA	45 ± 9	47 ± 6	55 ± 10	71 ± 12[D]
FK506	45 ± 8	–	70 ± 11[C,E]	
Minox	42 ± 6	41 ± 6	55 ± 5[C]	56 ± 6[C]
CsA + dilt	48 ± 1	50 ± 3	55 ± 4	55 ± 7
FK506 + dilt	51 ± 1	50	51 ± 2	54 ± 2
Minox + dilt	50 ± 1	51 ± 1	58 ± 5	56 ± 5
		LA (mm)		
No Rx	1.54 ± 0.09	–	1.43 ± 0.07	–
CsA	1.51 ± 0.07	1.52 ± 0.06	1.69 ± 0.24[C]	2.13 ± 0.33[D]
FK506	1.51 ± 0.04	–	1.99 ± 0.12[C,E]	
Minox	1.51 ± 0.08	1.52 ± 0.02	1.56 ± 0.03	1.58 ± 0.08
CsA + dilt	1.50 ± 0.01	1.52 ± 0.01	1.54 ± 0.03	1.55 ± 0.01
FK506 + dilt	1.51 ± 0.01	1.50 ± 0.00	1.54 ± 0.01	1.54 ± 0.02
Minox + dilt	1.51 ± 0.00	1.53 ± 0.03	1.57 ± 0.02	1.61 ± 0.02

There were significant differences between CsA-treated and FK506-treated $\alpha MHC^{403/+}$ mice at Echo 1 (see [E] and [F]). [A]Echo 1 was performed after 7–10 days of treatment. [B]Echo 2 was performed after 18–20 days of treatment. [C]$P < 0.05$. [D]$P = 0.0001$. [E]$P < 0.05$. [F]$P = 0.0001$. AW, anterior wall; dilt, diltiazem; DD, end-diastolic diameter; FS, fractional shortening; LA, left atrium; LV, left ventricle; Rx, treatment.

ed 20-week-old $\alpha MHC^{403/+}$ mice exhibit only modest LV hypertrophy (<10% increase in maximum LV wall thickness; see ref. 12 and our unpublished data). In contrast, CsA or FK506 treatment of 8- to 10-week old mutant mice produced rapidly progressive severe LV hypertrophy (>50% increase in maximum LV wall thickness) within 16 days of treatment (Figures 1b, Figure 2, and Figure 3). The morphologic pattern of hypertrophy in untreated $\alpha MHC^{403/+}$ mice is uniformly concentric (data not shown), whereas CsA- or FK506-treated $\alpha MHC^{403/+}$ mice developed proximal thickening of anterior and posterior LV walls that caused dynamic midcavity obliteration and outflow tract obstruction (Figure 2). Such features are highly reminiscent of the pathology seen in humans with severe obstructive hypertrophic cardiomyopathy (4).

Long-term treatment with minoxidil, a K^+-channel agonist, can cause midcavity hypertrophy in humans and rodents (20–22). To determine whether a molecule with distinct pharmacologic actions could also augment the hypertrophic response of $\alpha MHC^{403/+}$ mice, minoxidil was orally administered through drinking water to wild-type ($n = 3$) and mutant mice ($n = 7$). Echocardiographic studies at day 7 demonstrated significant LV hypertrophy only in $\alpha MHC^{403/+}$ mice that progressively became more severe through treatment day 14 (Table 1). The morphologic pattern of hypertrophy of minoxidil-treated $\alpha MHC^{403/+}$ mice mimicked that produced by CsA and FK506.

Molecular markers of cardiac hypertrophy were measured in the CsA-, FK506-, and minoxidil-treated $\alpha MHC^{403/+}$ mouse hearts. Northern blot analyses (15) demonstrated 14.4-, 2.57-, and 3.4-fold increases in atrial natriuretic factor, brain natriuretic factor, and skeletal α actin RNAs, respectively, in CsA-treated $\alpha MHC^{403/+}$ hearts compared with CsA-treated wild-type hearts. Both FK506 and minoxidil also elevated these RNA markers of cardiac hypertrophy at least twofold in hearts from $\alpha MHC^{403/+}$ compared with treated wild-type mice. Levels of these transcripts were the same in ventricles of untreated mutant mice (age less than 12 weeks) and wild-type mice receiving CsA or minoxidil (data not shown).

To determine whether increased hemodynamic load (due to systemic vasoconstriction) accounted for the accelerated hypertrophic response of $\alpha MHC^{403/+}$ mice, blood pressures were measured (23). Mean arterial blood pressures of untreated wild-type and $\alpha MHC^{403/+}$ mice were similar (117 ± 15 versus 112 ± 16 mmHg, respectively; P = NS). CsA is known to increase blood pressure in rats (24) and humans (19), an effect abrogated by coadministration of the nitric oxide donor L-arginine (24). CsA increased blood pressure in wild-type mice (days 1–10: 124 ± 15 mmHg, $P < 0.05$; days 11–30: 129 ± 15 mmHg, $P < 0.05$), but coadministration of L-arginine with CsA normalized arterial pressures (days 11–30: 119 ± 13 mmHg; P = NS, compared with untreated wild-type mice). Mean arterial pressures in $\alpha MHC^{403/+}$ mice did not increase during 1–10 days of CsA treatment (115 ± 12 mmHg; P = NS) and subsequently pressures significantly declined (days 11–30: 107 ± 13 mmHg, treated vs. untreated $\alpha MHC^{403/+}$ mice; $P < 0.05$), presumably because cardiac output was reduced by development of marked ventricular hypertrophy. Coadministration of CsA plus L-arginine had no effect on the augmented hypertrophic response of mutant mice. Minoxidil, an agent with antihypertensive properties, lowered the mean arterial pressures in all groups of mice (97 ± 3.8 mmHg, treated, vs. 117 ± 15 mmHg, untreated; $P < 0.02$).

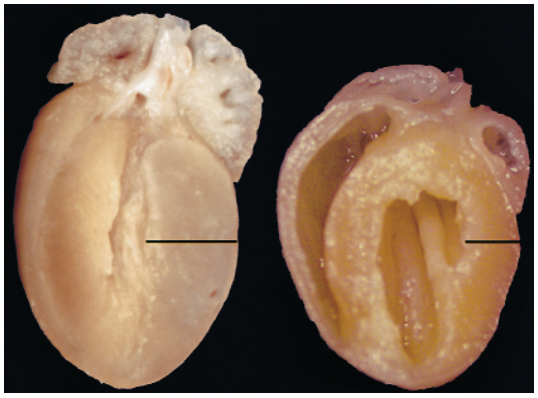


Figure 3
Gross morphology of hearts from CsA-treated $\alpha MHC^{403/+}$ (left) and wild-type (right) mice. The $\alpha MHC^{403/+}$ specimen exhibits marked left- and right-ventricular hypertrophy with cavity obliteration after 30 days of CsA treatment.

CsA has been shown to alter Ca^{2+} concentration in multiple cell types (25, 26), although this effect has not been examined in cardiac myocytes. Since changes in the cyclical variation of Ca^{2+} concentrations that normally occur throughout the cardiac cycle have been proposed as one signal for LV hypertrophy (27), we examined whether diastolic or systolic Ca^{2+} levels were altered in wild-type and $\alpha MHC^{403/+}$ mice. No significant differences in diastolic or systolic Ca^{2+} concentration were found between cardiac myocytes isolated from untreated wild-type and $\alpha MHC^{403/+}$ adult mice (data not shown). However myocytes isolated from CsA-treated wild-type adult mice had elevated diastolic Ca^{2+} concentration (untreated, n = 16, 113 ± 8 nM vs. treated, n = 30, 78 ± 8 nM; $P < 0.001$). In contrast, diastolic Ca^{2+} concentrations were not increased in myocytes from CsA-treated $\alpha MHC^{403/+}$ mice (untreated, n = 11, 124 ± 11 nM vs. treated, n = 21, 114 ± 7 nM; P = NS).

To determine whether the altered Ca^{2+} levels in myocytes reflected a direct effect of CsA on myocyte physiology versus indirect, systemic effects of this agent, myocytes were isolated, treated ex vivo with CsA, and Ca^{2+} transients were measured (Figure 5). Within 3 minutes of exposure to CsA, wild-type myocytes exhibited a 30% increase in diastolic Ca^{2+} concentrations (Figure 6), maximum systolic Ca^{2+} concentrations remained unchanged (before CsA, 222.9 ± 36 nM; after CsA, 233.3 ± 50 nM; n = 10, P = NS). The Ca^{2+} concentration differential between diastole and systole was therefore diminished by 23%. In contrast, $\alpha MHC^{403/+}$ myocytes exhibited little ex vivo response to CsA; diastolic Ca^{2+} concentration increased less than 8% and neither systolic Ca^{2+} concentrations nor the systolic/diastolic differential changed (Figures 5, 6).

The effects of ex vivo minoxidil on myocyte Ca^{2+} transients were also examined. Surprisingly, the responses of wild-type and $\alpha MHC^{403/+}$ myocytes to minoxidil mirrored responses to CsA. That is, wild-type myocytes increased diastolic Ca^{2+} concentrations in response to minoxidil, whereas diastolic Ca^{2+} concentrations remained unchanged in mutant myocytes (Figure 5 and Figure 6).

To determine if aberrant Ca^{2+} responses accounted for the dramatic hypertrophic response of $\alpha MHC^{403/+}$ mice to CsA, FK506, or minoxidil, animals were pretreated with the L-type Ca^{2+}-channel blocker, diltiazem (28–30). After 2 weeks of diltiazem pretreatment, coadministration of CsA, FK506, or minoxidil for 3 weeks had no effect on $\alpha MHC^{403/+}$ mouse hearts. Hearts from $\alpha MHC^{403/+}$ mice treated with diltiazem and CsA, FK506, or minoxidil had LV wall thickness, myocyte hypertrophy, and fibrosis comparable to that found in untreated mutant mouse hearts (Table 1, Figure 1b, and Figure 4e). No deaths occurred in $\alpha MHC^{403/+}$ mice pretreated with diltiazem (data not shown). Ex vivo coadministration of diltiazem and CsA abrogated the diastolic Ca^{2+} increases in isolated wild-type myocytes (Figure 6).

Discussion

Human genetic studies defined the molecular cause of hypertrophic cardiomyopathy as heterozygous

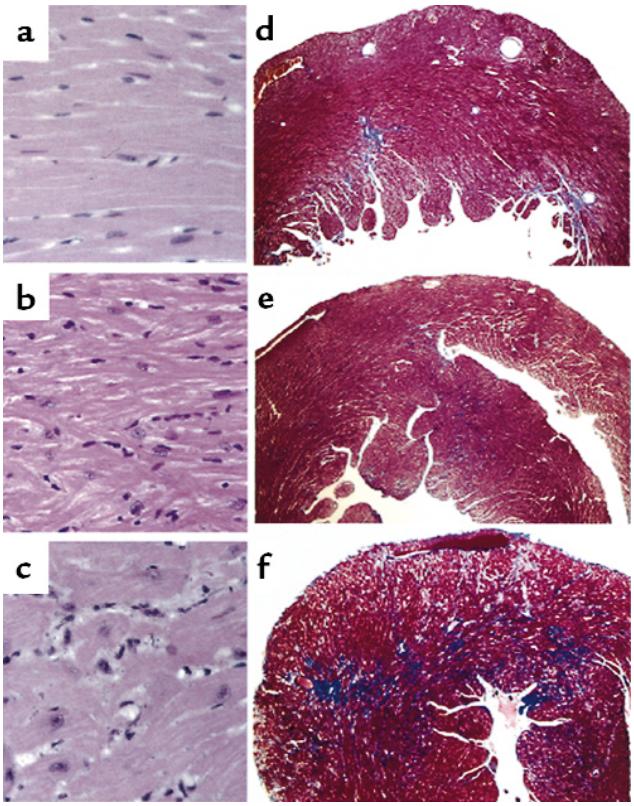


Figure 4
Cardiac histopathology in hypertrophic and normal mice. H&E-stained sections from CsA-treated wild-type mice (**a**) appear normal. Myocyte hypertrophy and disarray is mild in sections (×40 objective lens) from untreated $\alpha MHC^{403/+}$ mice (**b**), but marked in sections from CsA-treated $\alpha MHC^{403/+}$ mice (**c**). Masson trichrome was used to stain fibrosis (blue) in cardiac sections (×5 objective lens) from $\alpha MHC^{403/+}$ mice receiving no treatment (**d**), CsA plus diltiazem (**e**), or CsA alone (**f**).

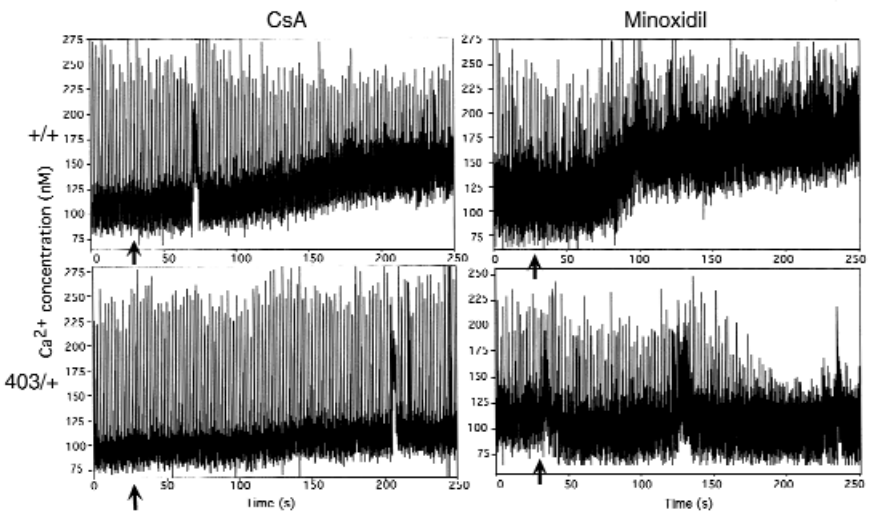


Figure 5
Ca^{2+} concentrations, assessed in fura-2–loaded $\alpha MHC^{403/+}$ and wild-type myocytes, before and after CsA or minoxidil treatment. Isolated myocytes from wild-type (+/+) and $\alpha MHC^{403/+}$ (403/+) mice have comparable calcium concentrations at base line. Addition of CsA (15 μg/ml) or minoxidil (200 μg/ml) (vertical arrows) increases diastolic Ca^{2+} concentrations (vertical bars) in wild-type, but not mutant, myocytes.

mutations in genes encoding sarcomere proteins. The development of cell and animal model systems to study the cellular consequences of these gene defects have shown incorporation of mutant peptides into the contractile apparatus with dominant-negative effects (12, 31–33). However, the mechanism by which sarcomere mutations trigger hypertrophic growth of the myocardium remains unknown.

We demonstrate here that minoxidil, FK506, and CsA dramatically increase the hypertrophic response of $\alpha MHC^{403/+}$ mice (Figures 1–3). We have compared the mutant and wild-type myocyte response to these agents as a first step toward defining the mechanism by which the hypertrophic response is signaled in these cells. Myocytes from wild-type mice increase their diastolic Ca^{2+} concentration in response to either minoxidil or CsA (Figure 5 and 6) by approximately 30%. However, $\alpha MHC^{403/+}$-derived myocytes increased their diastolic Ca^{2+} concentration in response to these drugs by less than 10%. We conclude that mutant myocytes do not regulate their Ca^{2+} concentration appropriately.

The conclusion that inappropriate myocyte regulation of Ca^{2+} concentration plays a role in the hypertrophic response of $\alpha MHC^{403/+}$ mice is confirmed by the observation that diltiazem, an L-type Ca^{2+}-channel inhibitor blocks this response (Figures 5 and 6). Further, diltiazem blocks the wild-type myocytes' increase in diastolic Ca^{2+} concentration due to CsA and minoxidil (Figure 6). We conclude that sarcomere protein defects trigger hypertrophic remodeling of the heart through a novel Ca^{2+}-dependent event.

Previous studies have suggested a role for calcineurin-dependent dephosphorylation of the transcription factor NFAT in hypertrophic growth of the heart (9, 17). Our data indicate this inhibition of calcineurin does not prevent, and indeed severely enhances, hypertrophy caused by the cardiac myosin heavy-chain Arg403Gln mutation. CsA has been reported to provide variable degrees of benefit in transgenic models of heart disease (for a review see ref. 34). However, we demonstrate here that CsA treatment of mice with sarcomere defects similar to those found in the human disease showed definitive adverse effects, including dramatic worsening of histopathology and sudden death. The histopathologic changes are not due to CsA-induced apoptosis (see Results). Several findings indicate this to be a direct effect on myocyte physiology. First, significant increases in arterial blood pressure were not observed in CsA-treated $\alpha MHC^{403/+}$ mice, and coadministration of L-arginine, an agent that inhibits multiple systemic effects of CsA, failed to block the enhanced hypertrophic effect. Second, minoxidil, an antihypertensive agent that reduced blood pressure in wild-type mice, also exacerbated the pathology of $\alpha MHC^{403/+}$ mice. Third, ex vivo CsA or

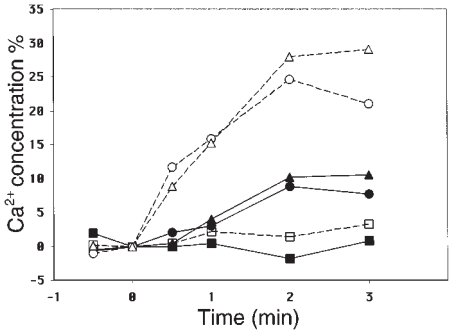


Figure 6
The change (%) in diastolic Ca^{2+} concentrations in myocytes derived from wild-type (open symbols, dashed lines) or $\alpha MHC^{403/+}$ (closed symbols, solid lines) mice treated with minoxidil (triangles), CsA (circles), or CsA plus diltiazem (squares). Diltiazem (28 μg/ml) administration began 20 seconds before addition of CsA. Each data point represents the average Ca^{2+} concentration from ten myocytes. After 2 and 3 minutes of treatment with either CsA or minoxidil, the Ca^{2+} concentration in wild-type myocytes was significantly different from the Ca^{2+} concentration in mutant myocytes treated with the same drug ($P < 0.02$) and was significantly different from the Ca^{2+} concentration in wild-type myocytes treated with diltiazem and CsA or minoxidil ($P < 0.01$).

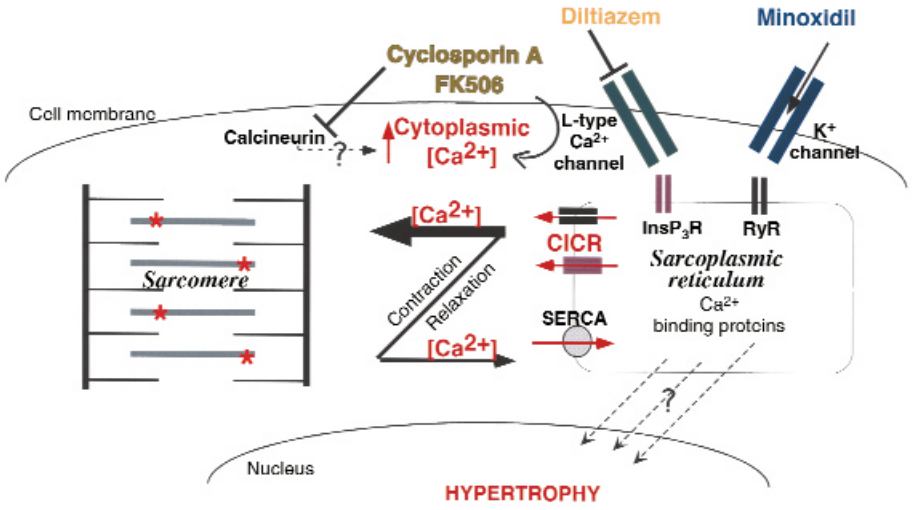


Figure 7
The pathway leading from sarcomere protein gene mutation to hypertrophic cardiomyopathy and the role of an abnormal Ca^{2+} response. CsA and FK506 may increase cytoplasmic Ca^{2+} through interaction with calcineurin or by activation of the L-type Ca^{2+} channel. Cytoplasmic Ca^{2+} enters the sarcoplasmic reticulum by way of an ATPase-dependent calcium pump (SERCA) and exits by way of the inositol triphosphate receptor ($InsP_3R$) and the ryanodine receptor (RyR). Small increases in Ca^{2+} trigger Ca^{2+}-induced Ca^{2+} release (CICR) primarily through the RyR. Considerable Ca^{2+} is stored in the sarcomere. We hypothesize that sarcomeres containing mutant myosins (denoted by asterisks) store more Ca^{2+} than normal sarcomeres, causing a reduction in sarcoplasmic reticulum Ca^{2+} that signals a hypertrophic response. Most Ca^{2+} in the sarcomere and sarcoplasmic reticulum is bound to carrier proteins, whereas most Ca^{2+} in the cytoplasm is free. Diltiazem is an inhibitor of the L-type Ca^{2+} channel, whereas minoxidil is an activator of the K^+ channel.

minoxidil treatment of wild-type, but not $\alpha MHC^{403/+}$, myocytes produced the same intracellular response. We also hypothesize, because both minoxidil and CsA appear to alter the hypertrophic response through a Ca^{2+}-mediated response and because myocyte alterations in Ca^{2+} concentration occur within a few minutes after administration of these agents, that these agents are not acting through the dephosphorylation of the transcription factor NFAT.

Studies of CsA effects on other cells types (25–27) demonstrated that an early event was increased cytosolic Ca^{2+} concentrations. Our data show that myocytes respond similarly to this agent by augmenting cytosolic Ca^{2+}. In the myocyte, as in other contractile cells, this should prompt sarcoplasmic reticulum release of Ca^{2+} (termed calcium-induced calcium release) predominantly through ryanodine receptors, perhaps with some contribution from inositol triphosphate receptors (Figure 7; see refs. 35, 36). Like CsA, the K^+-channel agonist minoxidil also elevated myocyte Ca^{2+} concentrations. Activation of the ryanodine receptor by K^+ influx is one mechanism by which this might occur (Figure 7; ref. 37).

Ca^{2+} flux is essential for excitation-contraction coupling in the heart and skeletal muscle (reviewed in 38). Depolarization triggers entry of small amounts of Ca^{2+} through L-type Ca^{2+} channels located on the cell membrane. Calcium-induced calcium release from the sarcoplasmic reticulum then rapidly raises cytosolic Ca^{2+}, which fosters Ca^{2+} -troponin-C interactions and triggers sarcomere contraction. Ca^{2+} recycling into the sarcoplasmic reticulum then occurs by an ATPase-dependent calcium pump (SERCA), resulting in sarcomere relaxation.

Wild-type myocytes treated with CsA or minoxidil promptly responded with an elevation of diastolic Ca^{2+}. Because this response caused no demonstrable effect on either cardiac hemodynamics or cardiac morphology in wild-type mice, we suspect that Ca^{2+} reservoirs in healthy myocytes adequately compensate to accommodate this demand. In contrast, $\alpha MHC^{403/+}$ myocytes failed to raise diastolic Ca^{2+} concentration in response to these agents. We interpret this result to indicate a relative depletion of intracellular Ca^{2+} reserves. The finding that wild-type myocytes respond to CsA or minoxidil in the same fashion, even when extracellular Ca^{2+} is significantly reduced, supports this interpretation (B. McConnell and J.G. Seidman, unpublished data). In addition to the cytoplasm, myocyte Ca^{2+} is enriched in the sarcoplasmic reticulum and the sarcomere. Since $\alpha MHC^{403/+}$ myocytes differ from wild-type only by expression of a single amino acid difference in the myosin heavy-chain polypeptide, the mutant sarcomere is likely to account for the observed Ca^{2+} deficit. We suggest that the contractile apparatus in $\alpha MHC^{403/+}$ myocytes functions like an unregulated ion trap (Figure 7). Two previous observations of $\alpha MHC^{403/+}$ muscle physiology support this model: (a) mutant fibers show greater than normal isometric tension development at submaximal Ca^{2+} levels (39), and (b) mutant hearts exhibit decreased rates of relaxation (40). Chronic elevation in sarcomere Ca^{2+} levels could account for both findings. The beneficial response of L-type channel inhibition by diltiazem is

also explained by this model; chronic reduction of calcium-induced calcium release might limit Ca^{2+} sequestration by the mutant sarcomere.

A corollary to this hypothesis is that altered Ca^{2+} is the inciting trigger for the hypertrophic response. We presume this effect requires interaction with a protein that functions like a Ca^{2+} thermostat. Of the many known Ca^{2+}-binding proteins within myocytes (reviewed in ref. 38), those located in the sarcoplasmic reticulum are particularly well positioned for this function. The unaltered levels of cytoplasmic Ca^{2+} levels in $\alpha MHC^{403/+}$ myocytes (Figure 7) after either calcineurin inhibition or K^+-channel agonist treatment, despite the induction of dramatic hypertrophy, suggests a compartmentalized location for this putative Ca^{2+} sensor. Furthermore, model systems that alter expression of sarcoplasmic reticulum proteins such as calsequestrin have been shown to cause marked cardiac hypertrophy (41).

The conclusion that FHC is mediated through a Ca^{2+}-dependent event has implications for the management and treatment of patients with this condition. First, these studies explain, at least in part, the variable severity of disease, long recognized in patients with the same mutation (4, 42, 43). The finding that agents that modify myocyte intracellular Ca^{2+} concentration can dramatically augment the hypertrophic response in FHC provides the first demonstration that an environmental factor can alter the disease severity. Given that agents as diverse as calcineurin inhibitors and a K^+-channel agonist affect this ion, environmental factors such as lifestyle, diet, exercise, and pharmacologic agents are likely to substantially influence responses to a sarcomere protein gene mutation. Second, a rational therapeutic approach to FHC can now be considered. Patients with hypertrophic cardiomyopathy are routinely treated with Ca^{2+}-channel blockers and/or beta-adrenergic receptor blockers because these drugs alter heart rate and/or cardiac contractility. The results presented here suggest the mechanism by which Ca^{2+}-channel blockers may alter symptoms in FHC patients. Third, these studies may suggest a therapy for a recently identified class of patients—individuals bearing sarcomere protein gene mutations who do not yet have clinical signs of FHC, but who are genetically programmed to develop hypertrophic cardiomyopathy. Perhaps treatment of "presymptomatic" FHC patients (i.e., individuals bearing sarcomere protein gene mutations but who do not yet display signs of their disease) with diltiazem should be considered.

These studies have significant implications for understanding muscle biology and the pathology of hypertrophic cardiomyopathy. A central role for the sarcomere is defined in which the contractile apparatus regulates cell growth through Ca^{2+}. In the healthy myocyte, Ca^{2+} equilibrium between the sarcomere, sarcoplasmic reticulum, and cytoplasm is critically maintained. In genetic and possibly acquired forms of sarcomere dysfunction, Ca^{2+} balance shifts (Figure 7), triggering events that remodel the myocyte and ultimately perturb cardiac structure. For patients with hypertrophic cardiomyopathy the immediate ramifications of these data are that treatment with CsA, as has been proposed elsewhere (refs. 17, 18; see also www.clinicaltrials.gov), should be reconsidered. Further, environmental factors that alter Ca^{2+} homeostasis should be considered modifiers of disease expression. Finally, Ca^{2+} channel-blocking agents may have beneficial effects, particularly as a "preventative" treatment, in contrast to the common use of such agents in the management of symptoms. Identification of the effectors through which Ca^{2+} modulates the hypertrophic response should provide further insights into the mechanism by which sarcomere gene defects produce the striking pathophysiology of hypertrophic cardiomyopathy.

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