Late Sodium Current Inhibition Reverses Electromechanical Dysfunction in Human Hypertrophic Cardiomyopathy

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**Background**—Hypertrophic cardiomyopathy (HCM), the most common mendelian heart disorder, remains an orphan of disease-specific pharmacological treatment because of the limited understanding of cellular mechanisms underlying arrhythmogenicity and diastolic dysfunction.

**Methods and Results**—We assessed the electromechanical profile of cardiomyocytes from 26 HCM patients undergoing myectomy compared with those from nonfailing nonhypertrophic surgical patients by performing patch-clamp and intracellular 

Conclusions—We highlighted a specific set of functional changes in human HCM myocardium that stem from a complex disease-specific pharmacological treatment. 2 HCM is the monogenic cardiac disorder, with a reported prevalence of 1 in 500 worldwide.1 Despite its epidemiological relevance, HCM is largely an orphan condition because it lacks a disease-specific pharmacological treatment.2 HCM is the most common cause of arrhythmic sudden cardiac death in young athletes.3 Enhanced ventricular arrhythmogenicity in HCM has been attributed to multiple abnormalities such as abnormal cardiomyocyte orientation and alignment (disarray), microvascular ischemia, and intramyocardial fibrosis.4 Conversely, more subtle changes occurring at the molecular and cellular levels, causing electrophysiological disturbances and likely playing a crucial role in triggering arrhythmias, have received limited attention and remain unresolved. Furthermore, by impairing intracellular calcium (Ca2+) handling, electrophysiological abnormalities are potentially implicated in diastolic dysfunction, another pathophysiologic hallmark of the disease.5 Thus, preclinical studies defining the cellular basis for the enhanced arrhythmogenesis and impaired diastolic function represent a plausible starting point in the search for disease-specific therapeutic targets in HCM.

**Clinical Perspective on p 584**

In this study, we aimed to characterize the electrophysiological profile, Ca2+ handling properties, and contractile function of isolated cardiomyocytes and trabeculae from patients

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undergoing surgical myectomy and to test the potential reversal of disease-related abnormalities by inhibition of the late Na$^+$ current (\(I_{\text{NaL}}\)). In addition, we explored the role of Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) as a potential mechanism underlying the electromechanical abnormalities in HCM. Preliminary data have been presented in abstract form.\(^6\)

**Methods**

An expanded Methods section is available in the online-only Data Supplement.

**Patients Cohorts**

The study conforms with the principles of World Medical Association’s Declaration of Helsinki for medical research involving human subjects. The experimental protocols were approved by the ethics committee of Careggi University Hospital (2006/0024713; renewed May 2009). Each patient gave written informed consent. We enrolled 26 HCM patients from the Referral Center for Cardiomyopathies in Florence, Italy, consecutively referred to surgical myectomy for relief of drug-refractory symptoms related to left ventricular outflow tract obstruction. Of the 26 patients, 20 agreed to undergo mutational screening in sarcomeric genes.

The control cohort comprised 8 patients <65 years of age who were undergoing heart surgery for mitral stenosis-insufficiency, aortic stenosis, or regurgitation. Only patients with absent left ventricular hypertrophy (septal thickness <14 mm) and normal left ventricular systolic function (ejection fraction >55%) were included. Additionally, septal specimens from 13 nontransplanted donor hearts were included as controls for Western blot and reverse transcription–polymerase chain reaction experiments.

**Tissue Processing**

Septal specimens from HCM and control patients were washed and rapidly processed. Briefly, a small portion of the tissue was frozen in liquid nitrogen and used for protein and mRNA isolation. Endocardial trabeculae suitable for mechanical measurements were dissected, and the remaining tissue was minced and subjected to enzymatic dissociation to obtain viable single myocytes.

**Protein Studies**

Immunoprecipitation and Western blot analysis were performed by a standard method\(^7\) on proteins isolated from control and HCM septal specimens using antibodies for the following native or phosphorylated proteins: CaMKII, CaV1.2, SERCA2a, PLB, NCX1, NaV1.5, KCNQ1 (KvLQT1), HERG2a, HERG2b, KCNIP2 (KChIP), KCNQ2 (Kv4.3), KCNA1.2 (CaV1.2), SCN5A (Nav1.5), NCX1, PLB, SERCA2a, RYR2, miR-1, and miR-133.

**Reverse Transcription–Polymerase Chain Reaction**

mRNA isolated from septal specimens underwent reverse transcription, and the resulting cDNA was used for quantitative real-time polymerase chain reaction using predesigned assays (Life Technologies, Carlsbad, CA) for the following genes: KCND3 (Kv4.3), KCNIP2 (KChIP), HERG2a, HERG2b, KCNQ1 (KvLQT1), CaCNA1.2 (CaV1.2), SCN5A (Nav1.5), NCX1, PLB, SERCA2a, RYR2, miR-1, and miR-133.

**Single-Cell Studies**

Perforated patch whole-cell voltage clamp and current clamp were used to measure \(I_{\text{CaL}}\) current and membrane potential, respectively. Ca$^{2+}$ variations were simultaneously monitored with the Ca$^{2+}$-sensitive fluorescent dye Fluoroforte (Enzo Life Sciences, Farmingdale, NY). Free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) was calculated from emitted fluorescence as previously described\(^8\) using 389 mmol/L as the Fluoroforte dissociation constant. Ruptured-patch voltage clamp was used to record transient outward \((I_{\text{o}})\) and inward \((I_{\text{i}})\) rectifier potassium currents and late Na$^+$ current \((I_{\text{NaL}})\) and to quantify sarcoplasmic reticulum (SR) calcium content from caffeine-activated Na$^+$/Ca$^{2+}$ exchanger (NCX) current integration. Specific protocols and solutions were used to measure each current (see the expanded Methods section in the online-only Data Supplement).

**Intact Trabeculae Studies**

Ventricular trabeculae were mounted between a force transducer and a motor for muscle length control; isometric force was recorded under various experimental conditions and stimulation protocols. In brief, inotropic responses to increased pacing frequencies, stimulation pauses, and \(\beta\)-adrenoceptor agonists were evaluated, and kinetics of isometric twitches was measured under all conditions.

**Ranolazine Studies**

For experiments on isolated cardiomyocytes and trabeculae, ranolazine was used at the concentration of 10 \(\mu\)mol/L. Test recordings in the presence of the drug were performed after >3 minutes from the beginning of drug exposure. Afterward, the drug was washed out for >5 minutes, and measurements were repeated.

**Statistics**

Data from cells and muscles are expressed as means±SEM. Statistical analysis, taking into account nongaussian distribution, inequality of variances, and within-subject correlation, was performed as detailed in the expanded Methods section in the online-only Data Supplement. For categorical data, we used the Fisher exact test. For numerical variables, \(P\) values were calculated with linear mixed models. Values of \(P<0.05\) were considered statistically significant.

**Results**

Myocardial tissue from 26 HCM patients undergoing septal myectomy was harvested and processed. Clinical and genetic features are summarized in Table I in the online-only Data Supplement. Notably, 13 patients (50%) had a history of non-sustained ventricular tachycardia on Holter monitoring, and all patients showed moderate to severe diastolic dysfunction. Clinical data for the control cohort are shown in Table II in the online-only Data Supplement.

**Action Potential Prolongation and Arrhythmogenic Mechanisms in Human HCM Cardiomyocytes**

A total of 80 cells isolated from myectomy specimens of 26 HCM patients (HCM cardiomyocytes) and 25 cells from septal specimens of 8 control patients were studied by patch-clamp technique. HCM cardiomyocytes were hypertrophic, as indicated by an increased cell volume and capacitance compared with controls (33.5±4.3 versus 17.6±3.2 pL, \(P<0.05\); and 177.8±6.2 versus 111.6±5.9 pF, \(P<0.01\)).

The action potential durations (APDs) recorded at various stimulation frequencies (0.2, 0.5, 1 Hz) were markedly prolonged in HCM compared with control cardiomyocytes (Figure 1A) and with greater APD variability among myocytes (Figure 1B). Consistent with APD prolongation, our patients often showed prolonged QTc on the ECG (Table I in the online-only Data Supplement).\(^9\) In HCM cardiomyocytes, maximum action potential upstroke velocity was slower (Figure 1I in the online-only Data Supplement).

APD prolongation leads to increased frequency of early afterdepolarizations (EADs),\(^10\) that is, spontaneous depolarizations during the plateau phase of the action potential, often associated with reopening of Na$^+$ or Ca$^{2+}$ channels.\(^11\) EADs are considered primary electrophysiological triggers for ventricular tachyarrhythmias.\(^10\) Indeed, EADs were 4 times more...
frequent in HCM cardiomyocytes than in controls (Figure 1D). Interestingly, cardiomyocytes from 13 HCM patients with a history of nonsustained ventricular tachycardia displayed increased APD and a higher occurrence of EADs compared with those from the remaining 13 patients without nonsustained ventricular tachycardia (Figure 1C). This observation highlights a possible correlation between APD prolongation and clinical history of ventricular arrhythmias in HCM patients.

Furthermore, delayed afterdepolarizations (DADs), occurring during the diastolic period and related to spontaneous Ca²⁺ release, were more frequent in HCM than in control cardiomyocytes (Figure 1E), suggesting additional arrhythmogenic mechanisms.

Interestingly, the degree of APD prolongation was similar in cardiomyocytes from HCM patients regardless of the presence or absence of sarcomeric mutations and regardless of the mutation site (Figure 1F). This observation suggests that prolongation of APD, rather than a direct consequence of the causing mutation, is the result of a complex process of hypertrophic remodeling involving multiple signaling pathways and modifying genes.

**Altered Balance Between Inward and Outward Currents Underlies APD Prolongation in HCM**

Na⁺ current was evoked by depolarizing steps to ~20 mV (from ~120-mV holding potential) and the late component (ICaL) calculated as an integral of the tetrodotoxin-sensitive inward current between 50 and 750 milliseconds from the onset of the step (Figure 2A). The density of ICaL recorded in HCM cardiomyocytes (195±35 A-ms⁻¹-F⁻¹, n=19) was larger than that recorded in control cardiomyocytes (74±27 A-ms⁻¹-F⁻¹, n=8; P<0.01) or reported for ventricular myocytes of healthy donors. In HCM versus control samples, mRNA expression and protein expression of the cardiac Na⁺ channel (NaV1.5) were unchanged (Figure 2C). As shown below, APD shortening by ranolazine, a selective blocker of ICaL, suggests that increased ICaL plays a major role in prolonging the APD of HCM cardiomyocytes.

L-type Ca²⁺ current (ICaL) density was significantly increased in HCM cardiomyocytes compared with controls at all voltages (Figure 2B). Consistently, increased expression of CaV1.2 channel, CaCNA1.2 (Figure 2F). These findings were associated with a general reduction in mRNA expression of genes coding for K⁺ channels subunits, including Ik and If subunits (Figure 2G). Selective downregulation of the K⁺ channels at the transcriptional control level was also suggested by the increased expression of their regulatory microRNA miR-151 in HCM specimens (Figure 2G).

The observed changes in Ca²⁺ late Na⁺, and K⁺ current densities were introduced into a validated mathematical model of human ventricular myocyte (see the Methods section in the online-only Data Supplement), confirming that HCM-related current changes account for APD prolongation in HCM cardiomyocytes (Figure III in the online-only Data Supplement).

**Alterations of Ca²⁺ Transients and Diastolic Ca²⁺**

The amplitude of Ca²⁺ transients evoked in current-clamp conditions was similar in HCM and control cardiomyocytes (Figure 3A and 3B). However, the kinetics of Ca²⁺ transients, as indicated by time to peak and decay time, was significantly slower in HCM compared with control cardiomyocytes.
Moreover, HCM cardiomyocytes exhibited higher intracellular diastolic Ca$^{2+}$ concentration ([Ca$^{2+}]_{i}$) at all frequencies of stimulation (Figure 3C) and a significantly greater frequency-dependent increase in diastolic [Ca$^{2+}]_{i}$ (from 0.2 to 1 Hz: 48±5 nmol/L in HCM cardiomyocytes, 10±2 nmol/L in controls; $P < 0.001$).

To exclude the contribution of prolonged APD to abnormal Ca$^{2+}$ transient kinetics, Ca$^{2+}$ transients were evoked in voltage-clamp mode by depolarizing pulses (from −80 to 0 mV) of fixed duration (200 milliseconds). Under these conditions, Ca$^{2+}$ transients still exhibited slower rise and decay and higher diastolic [Ca$^{2+}]_{i}$, in HCM compared with control cardiomyocytes (Figure 3D). This observation suggests that, besides APD prolongation, perturbations in other components of excitation-contraction coupling are responsible for the observed abnormalities of Ca$^{2+}$ handling. Factors likely involved in these perturbations were the following:

1. Loss of t tubules. In hypertrophied HCM cardiomyocytes, the ratio of capacitance to volume is reduced compared with control cardiomyocytes (5.08±0.35 versus 6.42±0.42 pF/pL; $P < 0.05$), reflecting a disproportion between surface versus volume growth. This discrepancy suggests a lower density of t tubules, in agreement with previous observations in HCM myocardium.$^{15}$ A decreased t-tubule density translates into prolongation of Ca$^{2+}$ transient rise and decay$^{16}$ owing to asynchronous Ca$^{2+}$ release from the SR.

2. SR Ca$^{2+}$ overload. SR Ca$^{2+}$ load calculated with the caffeine method (Figure 3E) was significantly increased in HCM compared with control cardiomyocytes (Figure 3F), possibly promoting diastolic Ca$^{2+}$ leakage, with potential effects on diastolic [Ca$^{2+}]_{i}$ and the rate of DADs.$^{17}$

3. Altered NCX function. In HCM myocardium, mRNA expression and protein expression of NCX are increased (Figure 3G and Figure IV in the online-only Data Supplement). In apparent contrast, caffeine-induced Ca$^{2+}$ transient decay was slower (Figure 3E and 3F), suggesting that the forward mode of the NCX (Ca$^{2+}$ extrusion) is reduced in HCM cardiomyocytes compared with controls. Changes in the dependency of NCX current ($I_{\text{NCX}}$) on [Ca$^{2+}]_{i}$, evaluated during the decay of caffeine-induced Ca$^{2+}$ transient, indicate an altered NCX electrochemical balance, reflecting increased intracellular [Na$^{+}$] ([Na$^{+}$]; Figure V in the online-only Data Supplement).
Ca²⁺ significantly affect the APD or the amplitude and kinetics of the electromechanical dysfunction of HCM cardiomyocytes.

Conversely, ranolazine markedly shortened APD in HCM cardiomyocytes (Figure 4A and 4B). Superimposed action potentials (upper traces) and corresponding Ca²⁺ transients (lower traces) from HCM cardiomyocytes showed a reduction in APD duration and a faster transient decay of Ca²⁺ transients elicited at 0.2 and 1 Hz (Figure 4C). These effects of ranolazine were more pronounced in those cells with longer APD. The relative APD shortening effect of ranolazine was linearly related to baseline APD (Figure VII in the online-only Data Supplement), with the most pronounced effects exerted on those cells with longer APD.

Inhibition of I_{NaL} Reverses Alterations of APD and Intracellular Ca²⁺ Handling

Functional analysis of HCM cardiomyocytes pointed to higher intracellular Ca²⁺ and an HCM (below) cardiomyocyte. Inhibiting INaL Reverses Alterations of APD and Intracellular Ca²⁺ Handling

As shown below, enhanced I_{NaL} leading to larger Na⁺ entry underlies the increased [Na⁺]i in HCM cardiomyocytes.

In control cardiomyocytes, ranolazine (10 µmol/L) did not affect the APD or the amplitude and kinetics of Ca²⁺_i transients (Figure 4D). In addition, ranolazine significantly reduced diastolic Ca²⁺ (Figure 4E and 4F) and attenuated its rate-dependent increase; ranolazine reduced Δ[Ca²⁺]i (0.2–1 Hz) from 48±5 to 20±3 nmol/L (P<0.001).

These effects of ranolazine were milder yet still evident in voltage-clamp mode, when Ca²⁺_i transients were elicited by 200-millisecond depolarizing steps (Figure IX in the online-only Data Supplement). This observation suggests that APD shortening largely, but not completely, accounts for the beneficial effects of I_{NaL} inhibition on Ca²⁺ handling. Indeed, ranolazine reduced...
SR Ca²⁺ load by 11±2% in HCM myocytes and significantly accelerated the decay of caffeine-induced Ca²⁺ transients, sug-

gesting an increased rate of Ca²⁺ extrusion through the NCX on exposure to the drug (Figure 4G). Accordingly, in HCM cardio-

myocytes, ranolazine caused a shift of the [Ca]²⁺ dependency of I_

NCX toward the level of control cells, suggestive of lower [Na⁺]²⁰ (Figure V in the online-only Data Supplement). Fluorescence

measurements with an Na⁺-sensitive dye confirmed that I_

NaL inhibition significantly reduced [Na⁺] in HCM cardiomyocytes at all stimulation frequencies, with a larger effect at higher rates (Figure V in the online-only Data Supplement).

In keeping with the reduction of diastolic [Ca]²⁺, the lower SR Ca²⁺ load, and the more negative diastolic potential (Figure VII in the online-only Data Supplement), ranolazine significantly reduced the occurrence of DADs in HCM cardiomyocytes (Figure VIII in the online-only Data Supplement).

All the observed effects of ranolazine were reversed on 5 minutes of washout (Figure X in the online-only Data Supplement).

**Contractile Function of HCM Trabeculae: I_

NaL Inhibition Improves Diastolic Function**

The contractile function of HCM myocardium was investigated using intact ventricular trabeculae dissected from the endocardial layer of myectomy specimens (Figure XI in the online-only Data Supplement). Measurements of active tension in these preparations consistently showed that the HCM muscle displays a positive force-frequency relationship (Figure 5A and 5B), in contrast to failing human myocardium²¹ or end-stage human HCM.²² In addition, muscle contractile reserve was preserved, as indicated by the positive inotropic response to isoproterenol. High external [Ca]²⁺, and stimula-

tion pauses (Figure 5C, 5D, and 5E), in agreement with the maintained Ca²⁺ transients and SR Ca²⁺ load in HCM cardiomyocytes. However, the previously observed kinetic abnormalities of Ca²⁺ transients and increased diastolic [Ca]²⁺, reflect into delayed and incomplete muscle relaxation. In agreement with previous findings,²³ the kinetics of isometric contractions of HCM trabeculae was slower compared with...
that reported for the myocardium of healthy donors\textsuperscript{22} and with preliminary data from control trabeculae (Figure 5B).

Ranolazine (10 µmol/L) shortened the overall twitch duration of intact HCM trabeculae, primarily by increasing the speed of force development (Figure 5F and 5G), and significantly reduced diastolic tension (Figure 5F and 5H). The faster [Ca\textsuperscript{2+}] transient kinetics and lower diastolic [Ca\textsuperscript{2+}] seen in HCM cardiomyocytes on ranolazine likely account for the shortening of twitch duration and the reduction in diastolic tension in trabeculae. Finally, ranolazine tended to reduce peak isometric (active) force and the inotropic reserve of HCM myocardium (Figure 5F and 5K).

In trabeculae from septal samples of control patients, \( I_{\text{NaL}} \) inhibition with ranolazine did not exert any significant effects on contractile parameters (Figure VI in the online-only Data Supplement), consistent with the lack of effect on Ca\textsuperscript{2+} handling observed in control cardiomyocytes.

**Mechanisms Underlying Electrophysiological and Ca\textsuperscript{2+} Handling Abnormalities in HCM: Role of Enhanced CaMKII Activity.**

CaMKII cascade plays a critical role in driving disease-related cardiomyocyte remodeling in cardiac diseases\textsuperscript{24} and, pertaining to this study, \( I_{\text{NaL}} \) gain of function.\textsuperscript{25}

We investigated the mechanistic role of CaMKII in HCM by comparing the level of CaMKII activation and phosphorylation of its downstream targets in HCM and control specimens. CaMKII autophosphorylation was increased 3.5-fold in HCM samples, indicating increased activity\textsuperscript{26} (Figure 6A). In addition, all tested targets showed increased phosphorylation at specific sites for CaMKII, including L-type Ca\textsuperscript{2+} channel, ryanodine receptor 2, and phospholamban (Figure 6A).

The 1.5-fold increase in CaMKII-dependent phosphorylation of L-type Ca\textsuperscript{2+} channel is likely responsible for the slower inactivation of \( I_{\text{CaL}} \)\textsuperscript{27} observed in HCM cardiomyocytes (Figure 2B and 2D). Similarly, 1.5-fold higher CaMKII-dependent phosphorylation of ryanodine receptor 2 may contribute to susceptibility to DADs\textsuperscript{28} (Figure 1D). The observed 3-fold-higher phosphorylation of phospholamban, by relieving SERCA inhibition, may partially counteract the effects of reduced SERCA expression and increase SR Ca\textsuperscript{2+} load in HCM cardiomyocytes\textsuperscript{29} (Figure 3G).

Furthermore, coimmunoprecipitation data suggest increased CaMKII phosphorylation of the cardiac Na\textsuperscript{+} channel Nav1.5 (Figure 6B), which is notably associated with delayed current inactivation.\textsuperscript{25} CaMKII activation may therefore be the primary determinant of \( I_{\text{NaL}} \) augmentation in HCM cardiomyocytes, albeit other mechanisms can be involved.\textsuperscript{30}

**Discussion**

In the present study, we comprehensively addressed the adaptive (and maladaptive) mechanisms occurring at the cellular
level in human HCM myocardium. We demonstrated that the functional phenotype of isolated cardiomyocytes derived from HCM patients differs significantly from that of controls. Of note, similar alterations have been described in other models of cardiac hypertrophy (although to a milder degree) but differ markedly from the global myocardial dysfunction seen in terminally failing human hearts. Interestingly, these changes appear to be significant determinants of the main pathophysiological features of HCM in patients: longer action potentials and increased incidence of EADs and DADs underlie increased arrhythmogenicity, and prolonged intracellular Ca\(^{2+}\) transients and higher diastolic [Ca\(^{2+}\)]\(_i\) may account for abnormal muscle contraction and contribute to diastolic dysfunction in patients.

Sustained activation of CaMKII-dependent signaling pathway appears to play a central role in the regulation of cardiomyocyte remodeling in HCM (Figure 7). By slowing down \(I_{Ca,L}\) inactivation and increasing \(I_{Na,L}\) amplitude, enhanced CaMKII activity contributes to APD prolongation and related arrhythmias. In addition, by altering the function of excitation-contraction coupling proteins, CaMKII might contribute to the altered Ca\(^{2+}\) transient kinetics and elevated diastolic [Ca\(^{2+}\)]\(_i\).

Sustained activation of CaMKII in diseased states is driven by increased [Ca\(^{2+}\)]\(_i\) because Ca\(^{2+}\)-bound calmodulin is the primary activator of the kinase. Of note, a sustained increase in intracellular [Ca\(^{2+}\)]\(_i\) is one of the established consequences of HCM-related sarcomeric mutations, either resulting from increased Ca\(^{2+}\) sensitivity of myofilaments or stemming from the higher energy requirements of mutant sarcomeric proteins, leading to lower ATP availability for SERCA function (Figure 7). Indeed, we found that Ca\(^{2+}\) sensitivity of force generation, measured from skinned preparations obtained from the same specimens, is increased in HCM compared with control myocardium (Figure XI in the online-only Data Supplement), possibly contributing to the slower Ca\(^{2+}\) transient decay and the increased diastolic [Ca\(^{2+}\)]\(_i\) in HCM cardiomyocytes. Additionally, increased production of reactive oxygen species, a possible consequence of myocardial energetic impairment, can contribute to CaMKII overactivation in HCM.

Figure 6. Increased Ca\(^{2+}\)/calmodulin kinase II (CaMKII) activity underlies electromechanical remodeling in hypertrophic cardiomyopathy (HCM) cardiomyocytes. A, Representative Western blots (top) for total CaMKII, phosphorylated CaMKII at threonine 287 (p-CaMKII), phosphorylated L-type Ca\(^{2+}\) channel \(\beta_2\) subunit at threonine 498 (p-LTCC\(\beta_2\)), phosphorylated phospholamban at threonine 17 (p-PLB), and phosphorylated ryanodine receptor 2 at serine 2814 (p-RyR2). Average values from septum of control (\(n=10\)) and HCM patients (\(n=10\)) are reported (bottom). B, Representative Western blots (top) and mean values (bottom) for coimmunoprecipitation of Nav1.5 with CaMKII from control (\(n=10\)) and HCM patients (\(n=10\)) probed with antibodies for Nav1.5, p-CaMKII, and total CaMKII. A and B, For each protein, 5 blots representative of the 10 are shown. Relative intensity of individual bands was quantified and normalized to GAPDH. The ratio for control was assigned a value of 1. ** \(P<0.01\).

Figure 7. Electromechanical remodeling in hypertrophic cardiomyopathy (HCM) cardiomyocytes. Sarcomeric mutations may cause a primary sustained increase in intracellular Ca\(^{2+}\) with multiple mechanisms. Intracellular Ca\(^{2+}\) overload (combined with increased production of reactive oxygen species) leads to sustained activation of Ca\(^{2+}\)/calmodulin kinase II (CaMKII); increased phosphorylation of its downstream targets (Ca\(^{2+}\) channel, ryanodine receptor, phospholamban, Na\(^+\) channel) is responsible for the abnormalities observed in HCM cardiomyocytes, including increased \(I_{Na,L}\). Overall, these changes aggravate intracellular Ca\(^{2+}\) overload. The enhanced \(I_{Na,L}\) is responsible for intracellular Na\(^+\) overload, which favors reverse over forward Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) mode. The latter contributes to cytosolic Ca\(^{2+}\) overload, further promoting CaMKII activation, thus setting up a vicious circle.
function, increased CaMKII activity may be responsible for activation of the hypertrophic gene expression program and therefore sustain the structural changes occurring in HCM hearts, including cell hypertrophy and intramyocardial fibrosis. Taken together, these observations suggest that altered CaMKII activity may link the direct effects of causal mutations with the final HCM phenotype (Figure 7).

The striking effects of ranolazine in HCM cardiomyocyte compared with the relative lack of effects in control cardiomyocytes highlight the central role of CaMKII, in particular compared with the relative lack of effects in control patients, and Annibale Biggeri for the statistical analysis. We thank Alessandra Rossi, Pierluigi Stefano, Georges Popoff, and the personnel of the cardiac surgery units of Careggi University-Hospital and Villa Maria-Beatrice Hospital for the surgical work and their assistance with specimen collection.

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Hypertrophic cardiomyopathy (HCM), despite being the most prevalent monogenic cardiac disorder, remains an orphan of fundamental pathophysiological feature of HCM, responsible for manifestations ranging from ventricular arrhythmogenicity to diastolic dysfunction. To date, however, the cellular basis of electromechanical dysfunction in cardiomyocytes from HCM patients and the presence of related therapeutic targets are unresolved. We compared the electromechanical profile of surgical patients without left ventricular hypertrophy. Using patch-clamp measurements and studies of intracellular calcium dynamics, and higher diastolic tension, favoring arrhythmogenesis and diastolic dysfunction. Intriguingly, all these anomalies to diastolic dysfunction. To date, however, the cellular basis of electromechanical dysfunction in cardiomyocytes from patients with terminal heart failure. Circulation. 1999;518(pt 1):371–384.


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Expanded Methods

**Tissue processing:** Septal specimens from HCM and control patients were rapidly washed in ice-cold cardioplegic solution containing (in mmol/L): KH$_2$PO$_4$ 50, MgSO$_4$ 8, HEPES 10, adenosine 5, glucose 140, mannitol 100, taurine 10 (pH 7.4 with KOH). Within 15 minutes from excision, a small portion of the tissue was frozen in liquid nitrogen and used for protein and mRNA isolation. The remaining fresh tissue is kept in ice-cold cardioplegic solution and used to isolate multicellular preparations and single cardiomyocytes. Endocardial trabeculae suitable for mechanical measurements (300-800 µm diameter) were dissected, while the remaining tissue was minced to small pieces (~1mm$^3$) and subjected to enzymatic and mechanical dissociation to obtain viable single myocytes, as described before. In brief, tissue chunks are transferred to small pieces (~1mm$^3$) in a scraping device and the bathing solution changed to Ca$^{2+}$-free dissociation buffer containing (in mM): NaCl 113, KCl 4.7, KH$_2$PO$_4$ 0.6, Na$_2$HPO$_4$ 0.6, MgSO$_4$$\cdot$7H$_2$O 1.2, NaHCO$_3$ 12, KHCO$_3$ 10, HEPES 10, taurine 20, Na pyruvate 4, glucose 10, BDM 10 (pH 7.3 with NaOH) and heated to 37 ºC. Collagenase Type V and Protease Type XXIV (Sigma) were subsequently added and tissue chunks digested for a total 2 hours’ time. During the digestion, the buffer containing dissociated myocytes was collected every 15 minutes from the scraping device and diluted with KB solution at room temperature. KB solution contained (in mM): KCl 20, KH2PO4 10, glucose 25, mannitol 5, L-glutamic acid monopotassium salt 70, β-hydroxybutyric acid 10, EGTA 10 and 2mg/mL albumin (pH 7.2 with KOH). The myocytes were left to settle and then resuspended in Ca$^{2+}$-free Tyrode solution containing (in mM): 132 NaCl, 4 KCl, 1.2 MgCl$_2$ 10 HEPES, and 11 glucose (pH 7.35 NaOH). CaCl$_2$ was added stepwise up to 0.6 mM. Cells were stored in this solution and used within 3 hours.

**Patch-clamp/intracellular Ca$^{2+}$ studies on single myocytes:** Myocytes were incubated 30’ with the Ca$^{2+}$-indicator Fluoforte (Enzo Life Sciences, Farmingdale, New York) at room temperature, washed and transferred to a temperature-controlled recording chamber (experimental temperature= 35±0.5ºC), mounted on the stage of an inverted microscope. Intracellular Ca$^{2+}$ was monitored while recording $I_{CaL}$ or membrane potential using patch-clamp. Fluoforte fluorescence was detected at 505-520nm during bright-field illumination at 492±3 nm. Absolute free intracellular Ca$^{2+}$ concentrations [Ca$^{2+}$], corresponding to Calcium dye Fluorescence values were estimated as previously done by Trafford et al. on ferret myocytes and by Voigt et al. in human atrial cardiomyocytes.

Absolute free Ca$^{2+}$ concentration ([Ca$^{2+}$]) corresponding to Fluoforte fluorescence (F) was calculated as follows:

$$[Ca^{2+}]_i = \frac{K_d \cdot F}{F_{max} - F}$$

$K_d$ is the dissociation constant of Fluoforte (389 nmol/L), F is Fluoforte fluorescence, and $F_{max}$ is Ca$^{2+}$ saturated fluorescence obtained at the end of each experiment by damaging the cell with
the patch pipette. The $K_d$ we used for Fluoforte (389 nmol/L) is the value published on the company’s datasheet (www.enzolifesciences.com).

Action potentials (APs) and $I_{\text{CaL}}$ were measured using the perforated-patch configuration (amphotericin-B method), while ruptured-patch was used to measure $I_{\text{NaL}}$, K⁺ currents and to quantify SR Ca²⁺ load. Experimental temperature was 35±5 °C for all protocols except $I_{\text{CaL}}$ recordings (see below). Specifically, for AP recordings, the pipette solution contained (in mM) 115 K⁺ methanesulfonate, 25 KCl, 10 HEPES, 3MgCl₂ and cells were superfused with Tyrode buffer (see above) containing 1.8mM CaCl₂. APs were elicited with short depolarizing stimuli (<3ms) at different frequency of stimulation (0.2Hz, 0.5Hz and 1Hz, 1 minute at each frequency). For $I_{\text{CaL}}$ recordings, the pipette solution contained (in mM) 80 CsMES (Cesium methanesulfonate), 40 CsCl, 10 HEPES, 1 KCl, 1 CaCl₂, pH 7.4 (CsOH) and the external solution contained (in mM) 140 NaCl, 6 CsCl, 10 glucose, 10 HEPES, 1 MgCl₂, 2 CaCl₂, pH 7.35 (with CsOH). $I_{\text{CaL}}$-voltage (I-V) relationships were measured as described before. $I_{\text{CaL}}$ deactivation kinetics was assessed by bi-exponential fitting, calculating fast ($\tau_f$) and slow ($\tau_s$) time constants.

For SR Ca²⁺-load quantification, the pipette solution contained (in mM) 110 K⁺-aspartate, 23 KCl, 0.4 CaCl₂ (free-Ca²⁺=10⁻⁷M), 3 MgCl₂, 5 HEPES-KOH, 1 EGTA-KOH, 0.4 GTP-Na⁺ salt, 5 ATP-Na⁺ salt, 5 creatine-phosphate, pH 7.3 with KOH. The SR Ca²⁺-load was quantified by releasing all of the SR Ca²⁺ with rapid exposure to 20mM caffeine after 10 conditioning 150ms depolarizing steps to 0mV (-80mV holding potential) at 1Hz. Integral of the caffeine-induced NCX-mediated current was used to calculate the amount of extruded Ca²⁺, matching total SR Ca²⁺-load after correction for non-electrogenic Ca²⁺ removal. SR Ca²⁺-load was normalized for cell volume.

Ruptured-patch voltage-clamp was employed for $I_{\text{NaL}}$ and $I_{\text{k1}}$ measurements: pipette solution contained (mM): K-L-Aspartic acid 130, HEPES 10, Na₂-ATP 5, Na₂-GTP 0.1, EGTA 11, MgCl₂ 2.0, CaCl₂ 5.0, pH adjusted to 7.2 with KOH. For $I_{\text{NaL}}$, 0.3mM CdCl₂ and 30µM TTX were added to bathing Tyrode solution. For $I_{\text{k1}}$ recordings, only 0.3mM CdCl₂ was added. $I_{\text{NaL}}$ recordings were carried out 21-23°C using depolarizations steps as described before. To measure $I_{\text{k1}}$, voltage ramps from -120mV to +40mV were applied at 35°C. $I_{\text{k1}}$ I-V relationships were estimated from the difference between total current in the absence and presence of 2mM BaCl₂.

Late sodium current ($I_{\text{NaL}}$) was measured as described before. In brief, $I_{\text{NaL}}$ was elicited using a 0.25Hz train of pulses to -20mV from -120mV holding potential: 10 subsequent episodes were averaged. The current was then elicited 10 times in the presence of 10µM ranolazine. Afterwards, tetrodotoxin (TTX, 30µM) was added and the protocol repeated. To block the cardiac Na⁺ channels completely, we employed a (over)maximal TTX concentration (30 µM), which allowed us to obtain a “zero Na⁺ current level” that is used as reference. $I_{\text{NaL}}$ was then estimated as the difference between the traces recorded in the absence and in the presence of TTX (TTX-corrected traces). The integral of TTX-corrected $I_{\text{NaL}}$ between 50 and 750ms after the onset of depolarization was finally calculated for each myocyte in each condition (e.g. presence or absence of ranolazine).
Potential and current signals were measured with a Multiclamp 700B amplifier. Patch-clamp and fluorescence signals were simultaneously digitized through using Digidata 1440A. Acquisition and analysis was controlled by dedicated software (pClamp10.0). All products from Molecular Devices, Sunnyvale, California.

EADs and DADs: EAD and DAD events were considered when a spontaneous depolarization larger than 20mV was detected during the plateau of an AP or during the diastolic period, respectively. A cardiomyocyte was scored positive for EADs or DADs if it displayed > 2 events during 3’ of stimulated activity.

Ranolazine cell studies: test recordings in presence of 10μM ranolazine were performed after >3 minutes from the beginning of drug exposure and repeated after >5 minutes of washout.

[Ca$^{2+}$]/I$_{NCX}$ relationship: ruptured patch configuration was used to assess the [Ca$^{2+}$]/I$_{NCX}$ relationship through simultaneous recording of Ca$^{2+}$ fluorescence signals (using Fluoforte dye) and transmembrane current. All of the SR Ca$^{2+}$ was released with a rapid exposure to 20mM caffeine at -80mV: of note, the resulting inward current is only due to electrogenic Ca$^{2+}$ removal through the NCX$^6$. To assess the relationship between [Ca$^{2+}$]$_i$ and NCX current during caffeine exposure, [Ca$^{2+}$]$_i$ was calculated from Fluoforte fluorescence as previously described. Calculated [Ca$^{2+}$]$_i$ is plotted against I$_{NCX}$ recorded simultaneously during the decay of caffeine-induced transients. Each curve is linearly fitted to calculate the intercept, that is the [Ca$^{2+}$]$_i$ value when I$_{NCX}$ is 0.

Intracellular Na$^+$ studies on single myocytes: For intracellular Na$^+$ measurements, cardiomyocytes were incubated with fluorescent dye Asante NaTRIUM Green-2 AM (Teflabs, Austin, Texas) for 45 min at room temperature and left settling for another 30 min after dye withdrawal to allow complete dye de-esterification$^{10}$. Excitation light was set at 514 nm, and fluorescence was acquired at wavelengths >527 nm. Measurements were performed at 35 ±0.5 °C. All fluorescence measurements were normalized by the maximal fluorescence value in each cell, obtained by mechanically permeabilizing the cell at the end of the experiment. Intracellular Na$^+$ was monitored during stimulation at 3 different pacing rates (0.1, 0.5 and 1Hz) in current-clamp mode while recording membrane action potential. Of note, the relatively large dynamic interval of this novel dye combined with the high response speed (when compared to older dyes such as SBFI) allowed recording of intracellular sodium movements during the course of a single activation cycle, as previously observed$^{11}$.

Intact trabeculae studies: Intact ventricular trabeculae were mounted between a basket-shaped end of a force transducer (KG7A, Scientific Instruments Heidelberg, Germany) and a motor (Aurora Scientific Inc., Aurora, Canada), controlled by a custom Labview (National Instruments, Austin,Texas) program. Muscles were mounted in cold cardioplegic solution and then perfused with Krebs/Henseleit buffer, containing (in mM) 119 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25 NaHCO$_3$; pH 7.4 with 95%O$_2$:5%CO$_2$. Muscles were allowed to stabilize for at least 30 min before recordings. Diastolic sarcomere
length was assessed by calculating the average distance of striations and set at 2.10-2.20 µm. Isometric force
was recorded at 35±2°C under various conditions. In brief, inotropic responses to increased pacing
frequencies, stimulation pauses and beta-adrenoceptor agonist isoproterenol (10⁻⁷M) were evaluated and
kinetics of isometric twitches was assessed under all conditions. The trabecula was stimulated at increasing
pacing rates (0.1-2.5 Hz): at each frequency, force was allowed to reach steady-state before recordings.
Stimulation pauses (30s) were inserted after the last contraction of a steady series (at 0.5 Hz) and post-rest
potentiation was evaluated at the first stimulated beat after the pause. Large (>10% of muscle length) motor-
induced shortening steps allowed assessment of diastolic tension. The effects of drugs (Ranolazine 10µM,
isoproterenol 0.1 µM) were evaluated >20 minutes after adding them to the recirculating buffer and
measurements repeated after >15 minutes of washout. Finally, muscle section was measured for force
normalization.

Mechanical studies on skinned trabeculae: In order to assess myofilament function, muscle strips
and trabeculae were skinned by overnight incubation in relaxing solution added with 0.5% Triton X100.
Triton was then removed and the skinned preparations were mounted horizontally between a force transducer
and a motor by means of T-clips. Muscles were activated by transferring them manually between baths
containing different pCa solutions and the pCa-force relationship was determined. Three types of solutions
were employed: relaxing solution (pCa 9) with 5 mM EGTA, pre-activating solution with 0.5 mM EGTA
and 4.5 mM 1,6-diamino hexane-N,N,N',N'-tetraacetic acid (HDTA), and a maximal activating (pCa 4.5)
solution with 5 mM CaEGTA. Relaxing and maximal activating solutions were mixed in different
proportions to obtain activating solutions with various pCa’s. Solutions were applied in the sequence:
relaxing, pre-activating, activating, relaxing. All solutions contained: 60 mM BES (N,N-bis[2-hydroxyethyl]-
2-aminoethane sulphonic acid); 5.83 mM Na₂ATP₂, 7.4 mM MgCl. Potassium propionate was added to
adjust the final ionic strength to 0.20 M. pH was adjust to 7.1 with KOH at 20°C.

RT-PCR: mRNA isolated from septal specimens underwent reverse transcription and the resulting
cDNA was employed for quantitative real time PCR using predesigned assays for the following genes:
Kv4.3, KChIP, HERG2b, KCNQ1, CaCNA1.2, NaV1.5, NCX1, PLB, SERCA2a and RYR2.Total RNA
from each frozen cardiac sample was isolated and DNase-treated with the RNeasy Fibrous Tissue Mini Kit
(Qiagen) following manufacturer's instructions. Single-stranded cDNA was synthesized from 2 µg total RNA
using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described before. The
genes selected for quantification were investigated using predesigned TaqMan® Gene Expression assays
(Applied BioSystems, USA). All reactions were performed in triplicate and included a negative control.
Relative quantification of the mRNA level for the different genes was determined by the 7500 system
software (Applied BioSystems, USA), using the comparative method (ΔΔCt). In brief, the threshold cycle
(Ct) difference of the index gene and the reference gene, calculated from each specimen, is subtracted from
the average Ct of the control group; this value is used as the exponent of 2 to calculate ∆∆Ct for each specimen. For all mRNA quantification assay, GAPDH was used as reference gene. In order to validate GAPDH as a reference gene, GAPDH mRNA was compared with ribosomal RNA 18S and expression level calculated as ∆∆Ct. No differences were noted among the control and HCM groups (0.99±0.06 vs 1.01±0.06). Quantitative expression of micro RNAs miR-1 and miR-133 in control and HCM samples was evaluated as relative to RNU48 (small nuclear RNA 48).

**Protein studies:** Immunoprecipitation and Western blot analysis were performed by a standard method on proteins isolated from control and HCM septal specimens. For immunoprecipitation studies, 5µg of CaMKIIδ antibody was incubated with 50 μl of protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Antibody-bound beads were then washed with PBS and blocked with 3% BSA for 2h at 4°C. The cytosolic fraction was incubated with the antibody-bound beads overnight at 4°C and thoroughly washed with PBS. Bound material was eluted with SDS sample buffer, run on a 10% SDS/PAGE, transferred and probed for Na,1.5, phospho-CaMKII, and CaMKIIδ. Secondary antibody was horseradish peroxidise (HRP)-linked goat anti-rabbit (1:1000) (PerkinElmer Life and Analytical Sciences) or Protein A/G-HRP (Thermo Fisher Scientific, Waltham, MA). The following antibodies were used: phospho-CaMKII at threonine 287 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); CaMKIIδ (Abcam,Cambridge, MA); RyR2, CaV1.2, NCX1 (Millipore,Billerica, MA); phospho-PLB at threonine 17, PLB, SERCA2a, phospho-RyR2 at serine 2814 (Badrilla,Leeds, UK); NaV1.5 (Alomone,Jerusalem, Israel). Relative intensity of individual bands from Western blots was quantitated using ImageJ software and normalized to GAPDH. The ratio for control was assigned a value of 1.

**Chemicals:** unless otherwise specified, all chemicals were purchased from Sigma-Aldrich,St. Louis, MO.

**Mathematical model:** To obtain a control human cardiomyocyte model, we used a validated model of human ventricular cardiomyocyte, recently developed by Grandi and Bers. Simulation of INaL was added to the original model (as done by Wu et al.) using a Hodgkin-Huxley formalism, similar to that used by Hund et al.:

\[
I_{NaL} = g_{NaL} \cdot m^3 \cdot h_L \cdot (V - E_{Na})
\]

INaL activation (gating variable, m) is similar the activation of the fast INa, whereas inactivation of INaL (gating variable, h) was formulated as shown below:

\[
h_L = \frac{1}{1 + e^{\frac{V_{th}+91}{51}}}
\]

INaL maximal conductance (GNaL) was set to 0.085 mS/µF to simulate a larger endogenous INaL in human ventricular myocytes compared to dog myocytes.
To simulate HCM cardiomyocytes in agreement with our results, we introduced the following changes to the control cell model:

1. 107% increase of $I_{\text{NaL}}$ maximal conductance;
2. 34% decrease of $I_{kr}$ maximal conductance;
3. 27% decrease of $I_{ks}$ maximal conductance;
4. 85% decrease of $I_{to}$ maximal conductance;
5. 15% decrease of $I_{k1}$ conductance;
6. 19% increase of $I_{CaL}$ maximal conductance;
7. 34% increase of NCX activity;
8. 43% reduction of SERCA activity.

Model differential equations were implemented in COR$^{16}$ and solved numerically with the 4th order Runge-Kutta method. The digital cardiomyocyte was stimulated with a current pulse (10 A/F; 4 milliseconds) at the cycle length of 1s, and APD was measured as the time between AP onset and 90% repolarization level (APD90%).

**Statistical analysis:** Clinical data are expressed as means±SD. Data from studies on isolated myocytes, trabeculae and proteins are expressed and plotted as means±SEM (Standard Error of Mean) values obtained from a number of independent determinations on different myocytes or muscles: number of cells/trabeculae (n) and number of patients (N) are indicated in the figure legends for each set of measurements.

To faithfully compare different sets of measurements, sensitivity analysis was performed for each statistical comparison, in order to account for:

1. non-Gaussian distribution
   The data were tested for normality using the Skewness/Kurtosis test$^{17}$.
2. heteroscedasticity (inequality of variances)
   We used the F test for equality of variances in two-group comparison studies and the Bartlett’s test for variance homogeneity in the multiple comparison design.
   Non-parametric test based on rank transformation (Wilcoxon’s sum of rank) was used to check robustness of results under violation of condition 1- or 2-.
3. within-subject correlation
   Most of the average data derives from multiple myocytes or trabeculae from different patients. We estimated within-subject correlation for each variable with One-way ANOVA. In order to account for the correlation among different cells/muscles from the same patient, we used linear mixed models$^{18}$ to compare couples of data groups, both paired and unpaired. Correction for heteroscedasticity was applied to linear mixed models in unpaired comparisons whenever the variances of the two groups were unequal (as calculated by F-test).
   All the results of the new statistical analysis are available on request. The Probability ($P$) values that are shown in the manuscript and in the online supplement were calculated with linear mixed models according to the aforementioned procedure, both when comparing repeated measurements on the same samples (e.g. effect of ranolazine) and when comparing unpaired datasets. $P$-values <0.05 were considered statistically significant.
To quantify EADs and DADs occurrence and its confidence intervals, the binomial proportion confidence interval was calculated as approximating the binomial distribution with a normal distribution (central limit theorem). The confidence interval was calculated as:

\[ \hat{p} \pm \sqrt{\frac{\hat{p}(1 - \hat{p})}{N}} \]

where \( \hat{p} \) is the fraction of successes in a Bernoulli trial process estimated from the statistical sample and \( N \) is the sample size. In our case \( \hat{p} \) is the fraction of myocytes scored positive for EAD or DAD events as described above.

The central limit theorem was not applied to a binomial distribution where the fraction \( \hat{p} \approx 0 \). In these cases the Wilson score interval was applied:

\[ \left( \frac{\hat{p} + \frac{1}{2N}}{1 + \frac{1}{N}} \right) \pm \frac{\hat{p}(1 - \hat{p}) + \frac{1}{4N^2}}{1 + \frac{1}{N}} \]

The statistical significance of differences in DAD or EAD occurrence was assessed using the Fisher exact test. Statistical analysis was performed using Stata 12 software (StataCorp LP, College Station, Texas, USA).
Supplementary Tables and Figures
### Clinical / Demographic Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Female 11/26 (42%)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>38 ± 10 yrs</td>
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<tr>
<td>Age at surgery</td>
<td>47 ± 14 yrs</td>
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<tr>
<td>Fam. history of HCM</td>
<td>7/26 (27%)</td>
</tr>
<tr>
<td>Fam. history of SCD</td>
<td>13/26 (50%)</td>
</tr>
<tr>
<td>NYHA Class II</td>
<td>13/26 (50%)</td>
</tr>
<tr>
<td>NYHA Class III</td>
<td>13/26 (50%)</td>
</tr>
<tr>
<td>Angina pectoris</td>
<td>7/26 (27%)</td>
</tr>
<tr>
<td>Syncope</td>
<td>14/26 (23%)</td>
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<tr>
<td>History of Atrial Fibrillation</td>
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</tr>
<tr>
<td>NSVT</td>
<td>13/26 (50%)</td>
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<tr>
<td>ICD implanted</td>
<td>8/26 (31%)</td>
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<tr>
<td>Mutational screening</td>
<td>20/26</td>
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<tr>
<td>Mutations</td>
<td>6/20 Negative for sarcomeric mut. 7/20 MyBPC 4/20 MHC 3/20 Multiple Genes</td>
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### Medical Treatment

<table>
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<tr>
<th>Treatment</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta blockers</td>
<td>26/26 (100%), mostly nadolol</td>
</tr>
<tr>
<td>Disopyramide</td>
<td>12/26 (42%)</td>
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<tr>
<td>Amiodarone</td>
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<td>Diuretics/ACE-Inhibitors</td>
<td>17/26 (65%)</td>
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### ECG features

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<th>Parameter</th>
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<tr>
<td>Sinus Rhythm</td>
<td>26/26 (100%)</td>
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<tr>
<td>PQ (ms)</td>
<td>168 ± 21</td>
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<tr>
<td>QRS (ms)</td>
<td>104 ± 11</td>
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<tr>
<td>QTc</td>
<td>473 ± 27 ms</td>
</tr>
<tr>
<td>QTc &gt; 480ms</td>
<td>12/26 (40%)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>64 ± 8</td>
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### Echo features

<table>
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<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Maximal thickness site</td>
<td>26/26 (100%) upper septum</td>
</tr>
<tr>
<td>Maximal septal thickness</td>
<td>26 ± 5 mm</td>
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<tr>
<td>LA end-systolic diameter</td>
<td>46 ± 7 mm</td>
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<tr>
<td>LA end-systolic volume</td>
<td>105 ± 41 mL</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>67 ± 9 %</td>
</tr>
<tr>
<td>LVOT gradient &gt;30mmHg</td>
<td>26/26 (100%)</td>
</tr>
<tr>
<td>LVOT gradient at rest</td>
<td>73 ± 30 mmHg</td>
</tr>
<tr>
<td>Severe mitral regurgitation</td>
<td>10/26 (38%)</td>
</tr>
<tr>
<td>Septal E</td>
<td>4.3 ± 1.3 cm/s</td>
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<tr>
<td>Lateral E’</td>
<td>7 ± 2.3 cm/s</td>
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<tr>
<td>Diastolic pattern</td>
<td>0/26</td>
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<tr>
<td>Normal</td>
<td>12/26 (46%)</td>
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<tr>
<td>Delayed relaxation</td>
<td>13/26 (50%)</td>
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<tr>
<td>Pseudonormalized</td>
<td>1/26 (4%)</td>
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### Cardiac Magnetic Resonance

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<th>Parameter</th>
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</thead>
<tbody>
<tr>
<td>Body surface area</td>
<td>1.9 ± 0.2 m²</td>
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<tr>
<td>LV end-diastolic volume</td>
<td>139 ± 41 ml</td>
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<td>LV end-diastolic volume index</td>
<td>75 ± 19 ml/m²</td>
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<tr>
<td>LV end-systolic volume</td>
<td>42 ± 23 ml</td>
</tr>
<tr>
<td>LV end-systolic volume index</td>
<td>22 ± 12 ml/m²</td>
</tr>
<tr>
<td>LV mass</td>
<td>237 ± 85 g</td>
</tr>
<tr>
<td>LV mass index</td>
<td>121 ± 42 g/m²</td>
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<tr>
<td>LV mass/volume ratio</td>
<td>1.76 ± 0.7 g/ml</td>
</tr>
<tr>
<td>LV ejection fraction</td>
<td>74 ± 8 %</td>
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**Supplementary Table 1. Clinical features of HCM patients.**

The table includes pre-operative data from the 26 patients from whom myocardial tissue specimens were obtained and studied. All values and test results were obtained within the 3 months prior to the elective surgery. Data are indicated as Mean±SD.

SCD = sudden cardiac death  
NSVT = hystory of non-sustained ventricular tachycardia documented at Holter ECG  
ICD = implantable cardiac defibrillator  
MyBPC = Myosin-binding protein C  
MHC = Myosin heavy chain  
QTc = QT corrected  
LA = left atrium  
LVOT = left ventricular outflow trait  
Septal/lateral E’ = Septal/Lateral mitral annulus early diastolic velocity
Supplementary Table 2. Clinical features of control patients

The table includes pre-operative data from the 8 patients from whom myocardial tissue specimens were obtained and studied. Data are indicated as Mean ± SD.

LV = left ventricle
LA = left atrium
LVOT = left ventricular outflow trait

<table>
<thead>
<tr>
<th>Clinical / Demographic Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
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<tr>
<td>Female 3/8 (38%)</td>
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<tr>
<td>Age at surgery</td>
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<tr>
<td>58 ± 4 yrs</td>
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<tr>
<td>Presence of known cardiomyopathies</td>
</tr>
<tr>
<td>0/8 (0%)</td>
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<tr>
<td>NYHA Class I</td>
</tr>
<tr>
<td>6/8 (75%)</td>
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<tr>
<td>NYHA Class II</td>
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<td>2/8 (25%)</td>
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<tr>
<td>Atrial Fibrillation</td>
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<td>0/8 (0%)</td>
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<tr>
<th>Medical Treatment</th>
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<tbody>
<tr>
<td>Beta blockers</td>
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<tr>
<td>5/8 (75%)</td>
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<tr>
<td>Diuretics/ACE-Inhibitors</td>
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<td>3/8 (25%)</td>
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<table>
<thead>
<tr>
<th>Echo features</th>
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<tbody>
<tr>
<td>Maximal LV wall thickness</td>
</tr>
<tr>
<td>12 ± 1 mm</td>
</tr>
<tr>
<td>LA end-systolic volume</td>
</tr>
<tr>
<td>81 ± 15 mL</td>
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<tr>
<td>1/8 (12%)</td>
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<td>insufficiency</td>
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<tr>
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Supplementary Figure 1: Additional action potential characteristics in HCM and control cardiomyocytes.

(a) Superimposed representative action potentials elicited at 0.2Hz, 0.5Hz and 1Hz in a control cardiomyocyte and an HCM cardiomyocyte. (b) Mean Diastolic Potential (MDP), Action Potential Amplitude (APA), Action Potential Duration at 20% and 50% of repolarization (APD20% and APD50%) and maximum upstroke speed of action potential phase 0 in HCM and control cardiomyocytes. Means ±SD from 70 HCM and 22 control myocytes. **= P <0.01, * = P <0.05.
Supplementary Figure 2. Properties of L-Type Ca\textsuperscript{2+} current (I\textsubscript{CaL})

(a) Ca\textsuperscript{2+} dependent facilitation of I\textsubscript{CaL} channels in control and HCM cardiomyocytes was evaluated by repeatedly imposing 200 ms depolarization steps at 1Hz rate after 10s of stimulation pause. Calcium current at steady state 1Hz stimulation (step 10, dashed traces) was compared with the first I\textsubscript{CaL} current after a 10s pause (step 1, dotted traces).

(b) Top: the ratio between amplitude at step 10 and at step 1. Bottom: the ratio between inactivation time constant $\tau_{\text{slow}}$ at step 10 and at step 1. While control septal myocytes displayed slower late decay kinetics and a slightly bigger amplitude at steady state, HCM myocytes maintained the same time constants of inactivation with smaller peak, consistent with lack of Ca\textsuperscript{2+} dependent facilitation.

(c) Voltage dependency of I\textsubscript{CaL} inactivation. From -80 mV resting voltage, after a 250ms prepulse to voltages ranging from -40mV to +70mV, a 200 ms test pulse at 0mV at is imposed and remaining inward current is measured and normalized for its maximum. Representative trace from a HCM myocyte is shown.

(d) Voltage dependency of I\textsubscript{CaL} activation and inactivation. No difference is noted between control and HCM myocytes. Means ±SE from 12 HCM and 6 control myocytes. *= P<0.05.
Supplementary Figure 3. Mathematical model of human HCM ventricular cardiomyocyte and simulated effect of ranolazine on the Action Potential.

(a) Left: Superimposed traces from the control and the HCM cell models showing different transmembrane currents during a regular action potential: Ca\(^2+\) current, Na\(^+\) current, I\(_{to}\) and I\(_{k1}\) potassium currents. The model of HCM cardiomyocyte was generated from the control cell model by changing transmembrane current densities in agreement with our experimental results (see supplemental methods). Right: Superimposed traces from the control and the HCM models showing action potentials. APD\(_{90\%}\) = 381 ms control, 689 ms HCM.

(b) Superimposed traces from the control cell model showing action potentials elicited at 1Hz at baseline and during simulated ranolazine exposure. APD\(_{90\%}\) = 381 ms in the absence, 369 ms in the presence of ranolazine.

(c) Superimposed traces from the HCM cell model showing action potentials elicited at 1Hz at baseline and with ranolazine. APD\(_{90\%}\) = 689 ms in the absence, 496 ms in the presence of ranolazine.
Supplementary Figure 4: Protein and mRNA levels of EC-coupling molecular components

(a) Representative Western blots for protein expression of LTCC α subunit 1.2 (Cav1.2), Na⁺-Ca²⁺ exchanger 1 (NCX1), phospholamban (PLB), sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a) and ryanodine receptor (RyR2) from septum of control and HCM patients. Relative intensity of individual bands from Western blots was quantitated using ImageJ software and normalized to GAPDH. The ratio for control was assigned a value of 1. Mean values are in Fig. 3e of the main text.

(b) mRNA expression of Na⁺/Ca²⁺ exchanger (NCX), sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2a) and phospholamban (PLB) genes in HCM (n=14) and control (n=14) samples. *=P<0.05 unpaired t-test; **=P<0.01 unpaired t-test.
Supplementary Figure 5. Changes of NCX function as a consequence of [Na⁺]i variations.

(a) Representative traces showing simultaneous [Ca²⁺]i and I_{NCX} current during exposure to caffeine 10mM at -80mV.

(b) Current density is plotted against [Ca²⁺]i during the decay phase of caffeine-induced transient. Each curve is linearly fitted to calculate the [Ca²⁺]i level at which NCX current is 0. The representative superimposed traces are derived from the examples in a. In this example, NCX equilibrium is reached when [Ca²⁺]i is 141 nM in the control cardiomyocyte and 231 nM in the HCM cardiomyocyte. The bar graph represents average [Ca²⁺]i at NCX equilibrium (current=0 at -80mV) calculated from the fitted curves (as in b) from 16 HCM and 10 control cardiomyocytes.

(c) Average [Ca²⁺]i at NCX equilibrium (current=0 at -80mV) calculated from the fitted curves (as in b) from 11 HCM cardiomyocytes in the absence and presence of ranolazine.

(d-e) Effects of ranolazine on intracellular Na⁺. Representative traces of ANG fluorescence during diastole, recorded at steady state stimulation of 0.2 Hz, 0.5 Hz and 1 Hz in the absence (black) and presence (red) of 10µM Ran. (e) means±SE of diastolic Na⁺ (estimated from normalized diastolic fluorescence levels) in 8 myocytes from 3 HCM patients. Diastolic Na⁺ levels recorded from a single control cardiomyocyte (grey) are also shown for comparison. *=P<0.05; **=P<0.01.
Supplementary Figure 6. Effects of 10 µM ranolazine on control cardiomyocytes and trabeculae.

(a) Superimposed representative action potentials at baseline (Basal) and during ranolazine exposure (Ran) from a control (CTR) cardiomyocyte paced at 0.2Hz, 0.5Hz and 1Hz. (b) APD90% in control cardiomyocytes at 3 pacing rates at baseline and with ranolazine. (c) Superimposed Ca$^{2+}$ transients from a control myocyte at baseline and with ranolazine. (d) Time to peak (TP), time from peak to 50% decay (T50%) and time from peak to 90% decay (T90%) of Ca$^{2+}$ transients at 0.2Hz in the absence and presence of Ran. (e) Ca$^{2+}$ transient amplitude at 0.2Hz in the absence and presence of Ran (means±SE from 9 control cardiomyocytes). (f) Representative superimposed force twitches during excitation at 0.5Hz from a control trabecula in the absence of ranolazine (Grey) and in presence of ranolazine (Blue). (g) Average time from peak to 50% relaxation in the absence of ranolazine and in presence of ranolazine in control trabeculae. (h) Example data from a control trabecula showing diastolic tension at different frequencies in the absence of ranolazine and in presence of ranolazine. All differences are non-significant.
Supplementary Figure 7. Effects of ranolazine on action potentials in HCM cardiomyocytes.

(a) Superimposed representative action potentials at baseline (black traces) and during ranolazine exposure (red traces) from a HCM cardiomyocyte paced at 0.2Hz (top), 0.5Hz (centre) and 1Hz (bottom). (b) Average action potential duration at 50% repolarization (APD50%) in HCM cardiomyocytes at the 3 frequencies tested at baseline (black) and with ranolazine (red). (c) Mean AP upstroke speed at baseline and with Ran. Means±SE of 29 myocytes from 10 HCM patients. * = P<0.05 at paired t-test; ** = P<0.01 at paired t-test. APD- shortening effect of ranolazine is larger in cardiomyocytes with longer APDs. (c-d) APD- shortening effect of ranolazine is larger in cardiomyocytes with longer APDs. (c) Representative superimposed action potentials excited at 0.2Hz from a HCM myocyte with APD> 1000ms at baseline (black) and with ranolazine (red). (d) Correlation between the reduction of APD90% by ranolazine (% change from baseline) and the baseline APD90% in 28 HCM cardiomyocytes; of note, the APD-shortening effect of ranolazine is linearly correlated with the degree of APD prolongation. * = P<0.05; ** = P<0.01.
Supplementary Figure 8. Effect of Ranolazine on cellular arrhythmias.

(a) Top: representative trace from a HCM cardiomyocyte in the absence of ranolazine showing multiple early after depolarizations (EADs). Bottom: trace from the same cardiomyocyte in the presence of ranolazine: the drug abolishes EADs. Arrowheads mark stimuli. (b) % of HCM cardiomyocytes at baseline (black) and with ranolazine (red) showing EADs. (c) Representative superimposed traces from an HCM cardiomyocyte in the absence and in the presence of ranolazine. Of note, DADs disappear upon drug exposure. (d) % of HCM cardiomyocytes showing DADs in the absence and in presence of ranolazine. EAD and DAD occurrence was assessed in 22 HCM cardiomyocytes. **=P<0.01.
Supplementary Figure 9. Effects of ranolazine on Ca\textsuperscript{2+}\textsubscript{i} transients evoked by short depolarizing pulses.

(a) Representative superimposed current traces (above) and Ca\textsuperscript{2+}\textsubscript{i} fluorescence in a voltage clamped HCM cardiomyocyte subject to 100ms depolarization steps, in the absence (black) and in presence of ranolazine 10\(\mu\)M (red).

(b) Above: average time to peak (TP), time from peak to 50% decay (T50%) and time from peak to 90% decay (T90%) of depolarization-evoked Ca\textsuperscript{2+}\textsubscript{i} transients from HCM myocytes in the absence (black columns) and in presence of Ran (red columns). Below: amplitude of Ca\textsuperscript{2+}\textsubscript{i} transients evoked by square depolarization steps in the absence and in presence of Ran. *=P<0.05
Supplementary Figure 10. Effects of ranolazine on AP duration and Ca^{2+} revert after 5 minutes of wash-out.

(a) Representative superimposed action potentials excited at 0.5Hz from a HCM myocyte in the absence of ranolazine (Basal, black), in presence of ranolazine (Ran, red) and after 5 minutes of wash-out (Wash, violet). (b) Correspondent superimposed Ca^{2+} transients from the same HCM myocyte in the absence of ranolazine, in presence of ranolazine and upon washout.
Supplementary Figure 11. Additional information on the mechanics of HCM myocardium

(a) representative photomicrographs showing the endocardial side of a septal myectomy sample with free running trabeculae (left) and two examples of trabeculae dissected from the same sample (right). Calibrations bars = 1 mm.

(b) Average pCa–force relationship of skinned HCM and control preparations. HCM strips and trabeculae were obtained from 5 patients of those included in the cellular and intact muscle studies; preparations from 4 non-failing non-hypertrophic cardiac patients were used for comparison. Force values are normalized to those measured at pCa 4.5. Data points are means±SE from all preparations of each group. The lines are drawn according to the parameters estimated by fitting the data to the Hill equation: \( \frac{F}{F_{\text{max}}} = \frac{1}{1 + 10^{-n_H(p\text{Ca}_{50} - p\text{Ca})}} \); pCa\(_{50}\) was 5.93±0.07 and 5.61±0.02 in HCM and control preparations respectively (p<0.05); n\(_H\) was 1.99±0.41 and 2.98±0.25 in HCM and control preparations respectively (p=0.08). The left-shifted pCa–force relationship in HCM patients indicates that changes in myofilament function (e.g. increased Ca\(^{2+}\)-sensitivity) may also contribute to altered intracellular Ca\(^{2+}\) levels and E-C coupling abnormalities in HCM myocardium.
Supplementary references:


