ORIGINAL RESEARCH ARTICLE

Genotype-Dependent and -Independent Calcium Signaling Dysregulation in Human Hypertrophic Cardiomyopathy

BACKGROUND: Aberrant calcium signaling may contribute to arrhythmias and adverse remodeling in hypertrophic cardiomyopathy (HCM). Mutations in sarcomere genes may distinctly alter calcium handling pathways.

METHODS: We analyzed gene expression, protein levels, and functional assays for calcium regulatory pathways in human HCM surgical samples with (n=25) and without (n=10) sarcomere mutations compared with control hearts (n=8).

RESULTS: Gene expression and protein levels for calsequestrin, L-type calcium channel, sodium-calcium exchanger, phospholamban, calcineurin, and calcium/calmodulin-dependent protein kinase type II (CaMKII) were similar in HCM samples compared with controls. CaMKII protein abundance was increased only in sarcomere-mutation HCM (P<0.001). The CaMKII target pT17-phospholamban was 5.5-fold increased only in sarcomere-mutation HCM (P=0.01), as was autophosphorylated CaMKII (P<0.01), suggestive of constitutive activation. Calcineurin (PPP3CB) mRNA was not increased, nor was RCAN1 mRNA level, indicating a lack of calcineurin activation. Furthermore, myocyte enhancer factor 2 and nuclear factor of activated T cell transcription factor activity was not increased in HCM, suggesting that calcineurin pathway activation is not an upstream cause of increased CAMKII protein abundance or activation. SERCA2A mRNA transcript levels were reduced in HCM regardless of genotype, as was sarcoplasmic endoplasmic reticular calcium ATPase 2/phospholamban protein ratio (45% reduced; P=0.03). 45Ca sarcoplasmic endoplasmic reticular calcium ATPase uptake assay showed reduced uptake velocity in HCM regardless of genotype (P=0.01). The cardiac ryanodine receptor was not altered in transcript, protein, or phosphorylated (pS2808, pS2814) protein abundance, and [3H]ryanodine binding was not different in HCM, consistent with no major modification of the ryanode receptor.

CONCLUSIONS: Human HCM demonstrates calcium mishandling through both genotype-specific and common pathways. Posttranslational activation of the CaMKII pathway is specific to sarcomere mutation–positive HCM, whereas sarcoplasmic endoplasmic reticular calcium ATPase 2 abundance and sarcoplasmic reticulum Ca uptake are depressed in both sarcomere mutation–positive and –negative HCM.
intracellular calcium mishandling has been implicated in hypertrophic cardiomyopathy (HCM), but limited data are available in humans, and it is unclear whether calcium mishandling is specific to sarcomere mutation carriers. In surgical myectomy samples from patients with HCM, a marked reduction in sarcoplasmic endoplasmic reticular calcium ATPase 2 (SERCA2) abundance correlated with reduced SERCA2 function in HCM compared with control hearts regardless of the underlying genetic pathogenesis. Calcium/calcmodulin-dependent protein kinase type II (CaMKII), a calcium-sensing kinase, was activated in HCM, but only in sarcomere gene mutation carriers. Activation of CaMKII was associated with an increase in phospholamban phosphorylation at threonine-17 in HCM, but myocyte enhancer factor 2 activity was not increased.

What Are the Clinical Implications?
- Calcium mishandling may be a potential link between the primary genetic causes of HCM and the downstream signaling cascade that leads to hypertrophy and arrhythmias.
- This study showed that 2 major calcium modulators, SERCA2 and CaMKII, are dysregulated in human HCM.
- SERCA2 function is also reduced in dilated cardiomyopathy, and gene therapy to increase SERCA2 activity is currently being investigated in clinical trials.
- CaMKII pathway inhibition may improve aberrant calcium cycling through modulation of multiple CaMKII phosphorylation targets.
- Because we found no evidence for CaMKII activation of transcriptional targets, prohypertrophic signaling may not be affected by CaMKII inhibition.

Medical Perspective

What is New?
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- In surgical myectomy samples from patients with HCM, a marked reduction in sarcoplasmic endoplasmic reticular calcium ATPase 2 (SERCA2) abundance correlated with reduced SERCA2 function in HCM compared with control hearts regardless of the underlying genetic pathogenesis.
- Calcium/calcmodulin-dependent protein kinase type II (CaMKII), a calcium-sensing kinase, was activated in HCM, but only in sarcomere gene mutation carriers.
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Ventricular arrhythmias can be a fatal consequence of hypertrophic cardiomyopathy (HCM), but whether this risk is the result of structural remodeling, dysregulated calcium handling, or other electrophysiological consequences is not clear. Extensive data from animal, in vitro, and human tissue studies have shown that mutations causative of HCM in both the thick and thin sarcomere filaments result in abnormal calcium handling, possibly contributing to the arrhythmia risk in HCM. In patients with HCM, arrhythmia risk has been linked to hypertrophy burden and scar, but the predictive value of these variables is low, raising the possibility that macro-reentry from remodeling does not fully explain clinical arrhythmias.6,7 Furthermore, resting ECG findings such as prolonged QT interval have not been demonstrated to be independent predictors of sudden death but do not necessarily reflect abnormal calcium handling. In human surgical samples, calcium dysregulation has been observed independently of genotype, and altered Ca2+/calmodulin-dependent protein kinase II (CaMKII) activity was proposed as a possible link between HCM-causal mutations and abnormal calcium signaling, implicating a potential single pathway for the induction of both hypertrophy and arrhythmias. The implication of a single node, CaMKII, modulating diverse disease effects in HCM has important relevance for therapeutic development. Here, we find that sarcomere-mutation HCM is strongly associated with constitutive activation of CaMKIIδ protein in the lack of transcriptional dysregulation of CAMK2D or the calcineurin pathway. We do not find direct evidence of activation of myocyte enhancer factor 2 (MEF2) as a link between CaMKII-induced calcium mishandling and hypertrophic/profibrotic signaling. In contrast, we find genotype-independent downregulation of SERCA2A gene expression and activity in HCM. Altered sarcoplasmic endoplasmic reticular calcium ATPase (SERCA) function has been demonstrated in mouse models of HCM but has not previously been explored in human HCM.2

METHODS

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

Human Heart Tissue Procurement

Myocardial tissue from the interventricular septum was obtained from unrelated subjects with HCM at the time of surgical myectomy for symptomatic left ventricular outflow tract obstruction (n=33) or at time of heart transplantation for severe refractory heart failure (n=2). HCM was diagnosed on the basis of standard criteria and in the absence of any cause for secondary hypertrophy. Summary subject clinical data are shown in the Table, and detailed per-subject clinical information and genetic testing results are shown in Table I in the online-only Data Supplement. Genetic testing was performed on a clinical basis by Clinical Laboratory Improvement Amendments–certified laboratories using standard HCM gene panels (including at least all standard sarcomere genes and infiltrative genetic causes). Because of limited tissue amount depending on the extent of surgical myectomy, not all assays were performed in all patients. When tissue was limited, the number assayed for each group is listed in the text or figure legend. Patient demographic data were recorded at the time of tissue collection, including genotype status. Nomenclature for sequence variants conforms to the recommendations of the Human Genome Variation Society. Tissue was snap-frozen in liquid N2 immediately on excision. Ventricular septal tissue was obtained from control donor hearts at time of explantation (n=8). Donor hearts were perfused with cardioplegia solution before removal, and tissue was snap-frozen in liquid N2 immediately on dissection. This study had the approval of the University of Michigan Institutional Review Board, and subjects gave informed consent.
Table. Summary of Subject Clinical Data

| Control subjects | 8 | 56±6 | 13 | 11.6±0.8 | 64±2 | — |
| Patients with hypertrophic cardiomyopathy | | | | | |
| No sarcomere mutation | 10 | 49±3 | 80 | 20.4±0.9* | 67±2 | 73±18 |
| MYH7 mutations | 8 | 44±6 | 50 | 18.3±0.8 | 73±4* | 68±8 |
| MYBPC3 mutations | 17 | 39±3* | 41 | 28.0±3.3* | 75±2† | 77±11 |
| Overall P value | | | | | | 0.038 | <0.001 | 0.006 | 0.9 |

*P<0.05 vs control subjects.
†P<0.05 vs patients with hypertrophic cardiomyopathy with no sarcomere mutation.

Transcript and Protein Analysis

Reverse transcription–polymerase chain reaction, cDNA sequencing, and quantitative reverse transcription–polymerase chain reaction were performed by standard techniques (Table II in the online-only Data Supplement). Immunoblotting of protein isolated from human surgical samples was performed by established methods (online-only Data Supplement).

SERCA Uptake Assay

SERCA Ca²⁺ uptake was determined as previously described with 50 mg homogenized frozen human heart tissue per sample. Both SERCA Ca²⁺ uptake velocity and total uptake capacity at 1 hour were determined for a range of pCa values from 8 to 5. Each measurement was performed in triplicate. Measurements were repeated for a subset of samples after pretreatment with lambda phosphatase inhibitor.

[³H]Ryanodine Binding

[³H]ryanodine binding was performed as previously described. To maximize signal from limited quantity of surgical myectomy tissue, total binding was determined at an optimal calcium concentration (pCa 5) to reflect maximal [³H]ryanodine binding activity. Bindings were also performed in the presence of spermine NONOate and H₂O₂ to determine posttranslational modification by nitrosylation or oxidation, respectively. Each measurement was repeated in triplicate.

Transcription Factor Studies

Activation of nuclear factor of activated T cell 1 (NFATc1) and MEF2 was assessed by an ELISA-based method per the manufacturer’s recommended conditions (Active Motif, Carlsbad, CA). Nuclear extracts from frozen surgical samples were prepared, and 10 µg of nuclear extracts were applied to wells coated with oligonucleotides containing transcription factor consensus binding sites. After incubation with the primary antibody and a horseradish peroxidase–conjugated secondary antibody, quantification was performed using a microplate reader at 450 nm with a reference wavelength of 655 nm. C2C12 nuclear extract was used as a positive control for MEF2, and nuclear extract from Jurkat T cells was a positive control for NFATc1.

Statistical Analysis

Values are expressed as mean±SEM unless otherwise indicated. Normality was determined by the Shapiro-Wilk test. Normally distributed data were analyzed by 1-way ANOVA, and the Tukey post hoc test was used for multiple comparisons between HCM subgroups and controls if the overall result was statistically significant. Western blot, quantitative reverse transcription–polymerase chain reaction, and ryanodine binding data were not normally distributed and are presented as box plots (boxes denoting first, second, and third quartiles; fences denoting the minimum and maximum data within 1.5 times the interquartile range of the first and third quartiles, respectively) and were analyzed with the Kruskal-Wallis test for overall statistical significance. Pairwise significance was tested if the overall value was P<0.05. When noted, distributions between groups with small sample sizes were compared with the Mann-Whitney test with exact P value calculations. Statistical analysis was performed with IBM SPSS Statistics. Statistical significance for all tests was defined by a 2-sided value of P<0.05. MYBPC3 and MYH7 mutation carriers were combined as a single sarcomere-mutation HCM group to increase statistical power compared with patients with sarcomere mutation–negative HCM and control subjects unless otherwise stated.

RESULTS

Clinical Characteristics

Summary clinical characteristics of subjects are shown in the Table, and detailed clinical information is given in Table I in the online-only Data Supplement. MYBPC3 mutation carriers were younger than control subjects and had higher ejection fractions than patients with HCM without sarcomere mutations. The ejection fraction was higher in both MYH7 and MYBPC3 mutation carriers compared with control subjects and was higher in MYBPC3 carriers compared with sarcomere-negative patients. The magnitude of left ventricular outflow tract obstruction was similar among sarcomere-negative and sarcomere-positive patients. Calcium channel blocker and β-blocker prescriptions were also similar among groups (Table I in the online-only Data Supplement).
Shared and Divergent Calcium Handling Gene Expression and Protein Levels in HCM

Analysis of most mRNA transcript levels of calcium handling and CaMKII pathway genes showed no statistically significant difference in HCM regardless of genotype (Figure 1A and 1B). However, SERCA2A (ATP2A2A) was decreased consistently across all samples in both HCM with MYBPC3 and MYH7 mutations (mean reduction, 53%; P<0.001) and sarcomere mutation–negative HCM (mean reduction, 44%; P=0.001). No statistically significant difference was observed among HCM groups.

Consistent with reduced gene expression, a significant reduction was observed in the SERCA2/phospholamban ratio in HCM, regardless of HCM genotype. Total protein abundance for most other calcium regulatory genes was not different in either sarcomere-mutation or sarcomere-negative HCM (Figure 1C). The exception was total CaMKII protein level, which was increased in HCM as a result of MYBPC3 and MYH7 mutations but not in sarcomere-negative HCM (no statistically significant difference between MYBPC3 and MYH7 groups).

Posttranslational CaMKII Pathway Activation in Sarcomere-Mutation HCM

Because autophosphorylation of CaMKII renders the enzyme constitutively active, we also quantified auto-phosphorylated CaMKII (pT287-CaMKII), which was increased only in HCM as a result of MYBPC3 and MYH7 mutations (Figure 2A). The CaMKII phosphorylation target pT17-phospholamban was also found to be significantly increased only in HCM as a result of MYBPC3 and MYH7 mutations (Figure 2A). Because calcineurin is implicated in CAMKII pathway activation, we measured the mRNA transcript level of the calcineurin catalytic subunit beta isoform (PPP3CB), which has been previously demonstrated to be transcriptionally regulated in response to hypertrophic stimuli, and found it to be unaltered. Because direct methods of measuring calcineurin activity in the absence of genetically engineered model systems are limited, we quantified the RCAN1 mRNA transcript level, which has been shown to be a reliable marker of calcineurin activity, and found no statistically significant difference (Figure 1B). Last, we assayed downstream NFAT transcription factor activity and again found no statistically significant difference in HCM regardless of genotype (Figure 2E). We also assessed activation of the CaMKII downstream nuclear phosphorylation target histone deacetylase C4 (HDAC4). pS467-HDAC4 was increased in HCM, but the increase was present across HCM genotypes, seemingly discordant with the evidence for sarcomere-HCM–specific activation of CaMKII (Figure 2A). One possible explanation is that we did not find a significant increase in the nuclear transcript isoform of CAMK2D in any HCM group (Figure 1B), suggesting that increased nuclear HDAC4 phosphorylation may have occurred from an alternative kinase. Because HDAC4 and HDAC5 phosphorylation by CaMKII is known to increase transcriptional activation of MEF2, we also performed a transcription factor assay for MEF2 and found no statistically significant difference in HCM (Figure 2F). MEF2A, MEF2C, and MEF2D mRNA transcript levels were also not different in HCM (Figure 1B). Taken together, these results strongly implicate posttranslational CaMKII pathway activation in sarcomere-mutation HCM but do not support either calcineurin-NFAT signaling as the upstream driver or nuclear CaMKII–driven induction of the HDAC4/5-MEF2 pathway.

SERCA2 Abundance and Uptake Are Diminished in HCM Regardless of Genotype

Although SERCA2 protein abundance relative to phospholamban was diminished in HCM (Figure 1C), increased phosphorylation of phospholamban at the threonine-17 CaMKII target site (Figure 2A) would be expected to increase SERCA2 calcium uptake activity. We performed a 45Ca uptake assay to determine the net balance of SERCA2 calcium uptake owing to the potential for these opposing effects. We observed an overall decrease in both the velocity and the total capacity of SERCA calcium uptake in HCM, regardless of genotype (Figure 3A and 3C). When normalized to peak calcium uptake for each curve, no shift in the sensitivity of SERCA to calcium was observed, indicating no major net effect on SERCA activity by posttranslational modifications (Figure 3B and 3D). Therefore, the major effect on SERCA uptake in HCM is most likely explained by the reduced ratio of SERCA2/phospholamban. To confirm a lack of a major impact of the phospholamban phosphorylation by CaMKII, we repeated the SERCA2 uptake assay in the absence of phosphatase inhibitor and in the presence of lambda phosphatase to dephosphorylate phospholamban in a subset of samples. A similar reduction in SERCA calcium uptake velocity activity regardless of genotype in HCM was observed (Figure 1A in the online-only Data Supplement). When normalized to maximum uptake, a release of phospholamban phosphorylation would be expected to cause reduced sensitivity of SERCA to calcium and a rightward shift in the normalized uptake velocity curve. Although the required experimental conditions of the SERCA uptake assay do not enable complete dephosphorylation (online-only Data Supplement), no statistically significant difference existed, nor any trend toward rightward shift of SERCA calcium sensitivity, among the HCM groups, indicating a lack of major net effect from the CaMKII phosphorylation of phospholamban.
Figure 1. Transcript levels.

A, mRNA transcript levels of calcium handling genes (control, n=6; sarcomere mutation-positive hypertrophic cardiomyopathy [HCM], n=20; sarcomere-negative HCM, n=10). B, mRNA transcript levels of calcium/calmodulin-dependent protein kinase type II (CaMKII)–calcineurin pathway genes. C, Western blot analysis of calcium handling protein abundance in HCM relative to controls normalized to GAPDH (control, n=6; sarcomere mutation-positive HCM, n=12; sarcomere-negative HCM, n=7). *P<0.05.
Figure 2. Western blot.

A, Western blot quantification of autophosphorylated calcium/calmodulin-dependent protein kinase type II (pT287-CaMKII), histone deacetylase C4 (HDAC4), and the CaMKII phosphorylation targets pS467-HDAC4 and pT17-phospholamban (PLN). Quantification is relative to controls and normalized to GAPDH. For phosphorylation antibodies, quantification is also shown relative to the total antibody quantification. Control, n=6; sarcomere mutation–positive hypertrophic cardiomyopathy (HCM), n=16 (MYBPC3, n=10; MYH7, n=6); sarcomere-negative HCM, n=7. B through D, Representative Western blots for CaMKII, HDAC4, and PLN. E and F, NFATc1 and myocyte enhancer factor 2 (MEF2) transcription factor activity measurement by absorbance at 450-nm wavelength (reference, 655 nm). Control, n=6; sarcomere mutation–positive HCM, n=12 (MYBPC3, n=6; MYH7, n=6); sarcomere-negative HCM n=5. * P<0.05.
Ryanodine Receptors are Normally Regulated in HCM

We hypothesized that ryanodine receptor (RyR) quantity or posttranslational modifications might contribute to abnormal calcium signaling in HCM. We did not find conclusive evidence of lowered RyR abundance in HCM, although there was a trend in the sarcomere-mutation group ($P=0.07$; Figure 4A). We quantified the amount of RyR phosphorylated at both the CaMKII-specific target site (pS2814-RyR2) and the protein kinase A/CaMKII target site (pS2808-RyR2) and found no statistically significant difference in the relative abundance of either (Figure 4B). We then performed $[^3H]$ryanodine binding assays to measure RyR abundance and function in the absence and presence of oxidizing and nitrosylating agents. At baseline, there was again no statistically significant difference in total $[^3H]$ryanodine binding at pCa 5, but there was a trend toward reduced binding in sarcomere-mutation HCM ($P=0.10$; Figure 4C), consistent with the trend toward an absolute reduction in quantity by Western blot. There was no statistically significant difference in response to either oxidation or nitrosylation in HCM, suggesting no baseline difference in

**Figure 3. Sarcoplasmic endoplasmic reticular calcium ATPase (SERCA) uptake velocity.**

A, SERCA uptake velocity measured by $[^{45}]$Ca SERCA uptake assay (control, $n=6$; MYBPC3-mutation hypertrophic cardiomyopathy [HCM], $n=7$; MYH7-mutation HCM, $n=3$; sarcomere-negative HCM, $n=5$). B, Normalized SERCA uptake velocity. C, Total SERCA uptake measured by $[^{45}]$Ca SERCA uptake assay as maximum (saturating) uptake after a 1-hour incubation. D, Normalized total SERCA uptake measured by $[^{45}]$Ca SERCA uptake assay as maximum (saturating) uptake after a 1-hour incubation. *$P<0.05$ for all HCM groups vs control.
these posttranslational modifications (\(P=0.5\) and \(P=0.3\); Figure 4D). These findings reduce the likelihood of significant posttranslational modifications to RyR2 channels as a major mechanism for arrhythmias in HCM.

**DISCUSSION**

We have investigated calcium mishandling in established human HCM to determine whether ongoing dysregulation could present potential therapeutic targets and whether those targets differ by HCM genotype. We find that the nodal calcium regulatory protein CaMKII is specifically posttranslationally dysregulated and autoactivated in sarcomere-mutation HCM, implicating a genotype-specific pathway for disease pathogenesis. Nonetheless, we do not find evidence linking CaMKII dysregulation to activation of the prohypertrophic/profibrotic calcineurin or MEF2 pathway. In contrast, we find a genotype-independent reduction in SERCA2 activity in HCM that is not directly attributable to CaMKII and may represent a novel target for therapy in HCM.

Calcium mishandling is a common downstream consequence of sarcomere mutations in HCM, demonstrated across a range of experimental systems. The proximal upstream causes are likely diverse, depending on thick versus thin filament involvement, the specific mutated sarcomere protein, the particular domain carrying the mutation within the sarcomere protein, the allosteric effects of the mutation, alteration of the calcium sensitivity of force generation, abnormal calcium buffering, and the effects on sarcomere protein phosphorylation status.\(^3\)\(^–\)\(^5\)\(^,\)\(^15\) The fact that these derangements lead to downstream alterations in calcium handling is highlighted by evidence that calcium channel blockade at least partially prevents adverse remodeling in both the \(MYH7\) R403Q mutant mouse and the \(TNNT2\) I79N mutant mouse.\(^1\)\(^,\)\(^16\) Furthermore, a recent randomized, clinical trial of diltiazem in preclinical HCM demonstrated evidence of attenuated early left ventricular remodeling specifically for \(MYBPC3\) mutation carriers, highlighting the relevance of calcium handling as a disease-altering therapeutic target, at least in the early stage of disease before the development of overt hypertrophy.

The increase in constitutively activated CAMKII specific to sarcomere mutation HCM in this study strongly implicates calcium-sensing posttranslational modifications as a downstream consequence of sarcomere mutations that may be a distinct disease mechanism that is not relevant to sarcomere-negative HCM. CaMKII has been strongly linked to both pathological hypertrophy and dilated cardiomyopathy.\(^17\) Coppini et al\(^1\) have proposed CaMKII as a central modulating disease node specifically in HCM, with postulated adverse downstream effects on both electrophysiological and hypertrophic signaling.

**Figure 4.** Ryanodine receptor (RyR2) abundance.

A, Total RyR2 abundance measured by Western blot (control, \(n=6\); sarcomere-mutation hypertrophic cardiomyopathy [HCM], \(n=16\); sarcomere-negative HCM, \(n=7\)). B, Phosphorylated RyR2 abundance by Western blot for the calcium/calmodulin-dependent protein kinase type II (CaMKII)/protein kinase A phosphorylation site (\(pS2808\)-RyR2) and the CaMKII-specific phosphorylation site (\(pS2814\)-RyR2). C, Total RyR2 binding after the addition of \(^{3}H\)ryanodine to cardiac tissue homogenates. Control, \(n=6\); sarcomere-mutation HCM, \(n=11\) (\(MYBPC3\) mutation, \(n=7\); \(MYH7\) mutation, \(n=4\)); sarcomere-negative HCM, \(n=9\). D, Relative change in RyR2-binding specific counts with preincubation with either \(H_{2}O_{2}\) (oxidation) or spermine NONOate (nitrosyl donor).
found both the total protein level (CaMKIIα) and the auto-phosphorylated (activated) protein level (pT287-CaMKIIα) to be increased in HCM. Increased phosphorylation of phospholamban at the CaMKII-specific site is also consistent with activated CaMKII.

In contrast with genotype-specific activation of CaMKII, we found reduced SERCA2 activity levels in all HCM samples relative to controls, correlating with reduced transcription and protein levels across all samples, suggesting a common mechanism of calcium mishandling in HCM (see online-only Data Supplement Table I for per-subject summary). Despite increased CaMKII phosphorylation of phospholamban, the net balance of SERCA2/phospholamban function appears to be driven more by the reduced abundance of SERCA2 because we found reduced total SERCA uptake velocity but no alteration in the calcium sensitivity of SERCA and no statistically significant difference in SERCA activity when pretreated with phosphatase. SERCA2 abundance was also noted to be reduced in human HCM samples by Coppini and colleagues.9 Reduction in SERCA2 activity is not unique to HCM. Loss of SERCA2 abundance and activity has been well established in heart failure, and clinical trials of gene therapy to replace SERCA2 function are ongoing.18,19 Whether intracellular calcium levels or hypertrophic remodeling plateaus. In the R403Q MYH7 mutant mouse model, increased MEF2 expression was shown only in focal areas of fibrosis, so it is possible that our assay of human surgical samples may not have detected this type of focal nonmyocyte involvement.21

Calcineurin, a calcium-sensitive regulator of cardiac growth and hypertrophy, has been implicated in pathologic cardiac hypertrophy.22 However, the calcineurin inhibitor cyclosporine paradoxically was found to worsen the disease phenotype in the R403Q MYH7 mutant mouse through unclear mechanisms.1 We find that the hypertrophy-regulating12 calcineurin catalytic subunit (PPP3CB) transcript level is not different in HCM compared with control hearts. Because calcineurin activity is not able to be robustly quantified in tissue samples, we measured the RCAN1 mRNA level, which has been shown to strongly correlate with calcineurin activity,13 and found no statistically significant difference, nor did we observe a difference in NFATc1 transcription factor activity. These findings counter the hypothesis that calcineurin is an active upstream driver of hypertrophy in HCM.

Approximately 50% of familial HCM is the result of mutations in sarcomere genes. A very small proportion of the remaining genetic causes (<2%) have been explained. Thus, the remaining 50% likely are associated with heterogeneous causes that may or may not be directly associated with sarcomere function. Sarcomere mutations either directly or indirectly influence intracellular calcium, potentially explaining altered CaMKII activation. Of note, Coppini et al9 found a similar increase in action potential duration in HCM, regardless of genotype, in their study of human myectomy samples (n=6 for sarcomere-negative HCM, n=14 for sarcomere-mutation HCM), but they did not specify mutational status in their other assays. Although CaMKII activity may be one driver for prolonged action potential duration, other mechanisms common to both sarcomere-mutation HCM and sarcomere-negative HCM could also induce a prolonged action potential.

This study has several limitations. First, we investigated human surgical samples, which represent a mature form of the disease. Advanced remodeling may have distinct pathways from disease-originating pathways. In addition, in this cross-sectional study of human samples, causality of abnormally regulated pathways cannot be proven. In addition, human sample tissue quantity was limited and variable, depending on the extent of surgical myectomy; therefore, not all assays could be performed for all subjects. Last, we limited our study of sarcomere-
mutation HCM to samples with MYBPC3 and MYH7 mutations because of the lesser availability of surgical samples with thin filament mutations.

CONCLUSIONS

Protein abundance and activity of CaMKII are increased in sarcomere-mutation HCM, highlighting the possibility that CaMKII could be an important genotype-specific therapeutic target. Although evidence of persistent CaMKII-induced hypertrophic signaling through MEF2 was not present in the human HCM cardiac tissue, further study will be required to determine whether aberrant CaMKII signaling could link abnormal calcium handling with early development of hypertrophy. Last, altered SERCA2 activity in HCM is likely driven by a mechanism that is independent of CaMKII signaling, highlighting the likelihood of multiple disease pathways that may be independent targets for therapy in HCM.

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DISCLOSURES

None.

AFFILIATIONS

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FOOTNOTES

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Expanded Methods

Transcript and Protein Analysis

Isolation of mRNA was performed using the Rneasy kit (Qiagen) and reverse transcription was performed with Omniscript (Qiagen). Quantitative RT-PCR was performed using validated Taqman® (Life Technologies) assays, except for the nuclear isoform transcript of CAMK2D (custom Taqman® assay) and RCAN 1.2 (SYBR® Green assay) (see Supplemental Table 1). On target PCR amplification of the PCR product was validated for both the latter assays using gel electrophoresis, DNA extraction, and Sanger sequencing.

Protein immunoblotting was performed using standard methods. All blots were blocked with 5% milk and incubated overnight unless otherwise stated. The antibodies and specific conditions are as follows: SERCA2 (Thermo Pierce MA3-919, 1:1000), PLN (Millipore 05-205, 1:1000), Thr17-phospho-PLN (Badrilla, UK A010-13, 1:5000), Ser16-phospho-phospholamban (Badrilla, UK A010-12, 1:5000), Ca,v,1.2 (Alomone Labs, Israel ACC-003, 1:500, blocked with 5% nonfat milk in PBS, 0.3% Tween, 0.05% NaN3), NCX1 (Thermo Pierce MA3-926, 1:1000), and CSQ2 (Thermo Pierce PA1-913, 1:1000).

Primary antibodies were visualized with a secondary goat anti-rabbit antibody or goat anti-mouse antibody (Licor) diluted 1:5000 for 45 minutes. Imaging and densitometry
analyses were performed using the LI-COR5 Odyssey laser scanner and band intensity was normalized to GAPDH as a protein loading control.

**SERCA Uptake Assay**

SERCA Ca\(^{2+}\) uptake was measured as previously described.\(^1\) In brief, 50 mg of frozen human heart tissue was homogenized in 750 µl of buffer containing 50 mM KH\(_2\)PO\(_4\), 10 mM NaF, 1 mM EDTA, 300 mM sucrose, 0.3 mM phenylmethysulfonyl fluoride, and 0.5 mM dithiothreitol (pH 7.0). Additional buffer was added to achieve a uniform protein concentration of 5µg/µl. Sodium azide inhibited Ca\(^{2+}\) uptake by the mitochondria, whereas ruthenium red and procaine inhibited Ca\(^{2+}\) release from the SR. Ca\(^{2+}\) uptake was measured over a range of pCa values from 8 to 5 in uptake buffers containing potassium oxalate as a quantitative marker for SR vesicles. After a 2-min pre-incubation of the reaction mixture containing 150 µCi/ml \(^{45}\)Ca at 37°C, 75 µg of ventricular homogenate were added. At exactly 2 min at 37°C, the reaction was stopped by filtration through a 45-µm Millipore filter and washed with a cold buffer containing 20 mM Tris and 2 mM EGTA (pH 7.0). The SERCA Ca\(^{2+}\) uptake velocity was calculated by determining the amount of \(^{45}\)Ca bound to the Millipore filters divided by 2 min. Total SERCA uptake capacity at each pCa was also determined by allowing reactions to continue for 1 hour at 37°C before filtering. Each measurement was performed in triplicate. Reactions were repeated for a subset of samples without phenylmethysulfonyl fluoride and with 2.7 units/ul lambda phosphatase (NEB). Phosphatase was incubated with samples on ice for 15 minutes since incubation at
room temperature or at 37 C, as recommended by the manufacturer to achieve complete dephosphorylation, substantially reduces signal from the assay.

[^3H]Ryanodine Binding

[^3H]Ryanodine binding to cell lysates was performed as previously described. To maximize signal from limited quantity of surgical myectomy tissue, total binding was determined at the optimal calcium concentration (pCa 5) to reflect maximal ryanodine activity. A binding mixture of 300 µl contained 30 µl of lysate (125 µg protein), plus a standard mixture of 200 mM KCl, 25 mM Tris/50 mM Hepes (pH 7.4), 3 mM MgATP, 1 mM EGTA, 5 nM[^3H]ryanodine (68.4 Ci·mmol⁻¹, Dupont NEN, Wilmington, DE, USA), and protease inhibitors. The reaction was incubated for 2 hr at 36°C, filtered on Whatman GF/B glass filters (Whatman, Clifton, NJ, USA) presoaked with 1% polyethylenimine and washed twice with 5 mL of distilled water using a Brandel M24-R cell harvester (Gaithersburg, MD, USA). Non-specific binding was determined in the presence of 20 µM unlabelled ryanodine and was subtracted from each sample. Ryanodine modifications by nitrosylation and oxidation were determined by addition of 500 mM spermine NONOate or 5 mM H₂O₂, respectively, to the binding buffer prior to incubation. Each measurement was performed in triplicate.

Transcription Factor Studies

Nuclear protein extraction was performed as previously described with modifications. To prepare nuclei, frozen heart tissue was pulverized in liquid N₂, then resuspended in NB1 (10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100,
0.1 M sucrose). The tissue was rotor-homogenized with 3 pulses of 15 seconds each on ice, gently mixed with NB2 (NB1 with added 0.25 M sucrose), then passed through a 40 µm filter. NB3 (10 mM Tris, pH 8.0, 5 mM MgCl2, 0.5 mM DTT, 0.33 M sucrose) was layered under the suspension, and the nuclei were pelleted at 1000 g for 5 min. Isolated nuclei were re-suspended in 25 mM HEPES, pH 7.8, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, and protease inhibitors, and slowly rotated at 4 °C for 30 min. Samples were then centrifuged at 4 °C in a tabletop centrifuge at maximum speed. The supernatant was transferred to a clean tube to be used for the transcription factor assays.

Activation of MEF2 was assessed by an ELISA-based method per manufacturer’s recommended conditions (Active Motif, Carlsbad, CA). 10 µg of nuclear extract was added to wells coated with oligonucleotides containing the transcription factor consensus binding site and incubated for one hour on a plate shaker. Wells were treated with primary antibody (MEF2) for one hour, followed by an HRP-conjugated secondary antibody. Quantification was performed using a microplate reader at 450 nm with a reference wavelength of 655 nm. C2C12 nuclear extract was used as a positive control. Activation of NFATc1 was assessed by a similar ELISA-based method (Active Motif, Carlsbad, CA). Nuclear extract from Jurkat T-cells served as a positive control.
Supplemental Figure 1.

A. SERCA uptake velocity measured by $^{45}$Ca SERCA uptake assay following pre-treatment with lambda phosphatase and in the absence of phosphatase inhibitor (control n=3, MYBPC3-mutation HCM n=3, sarcomere-negative HCM n=3). B. Normalized SERCA uptake velocity following phosphatase pre-treatment. *p<0.05 for controls vs. all HCM using the Mann-Whitney test with exact p-value calculation for small sample sizes.
# Supplemental Table 1

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MWT = maximal wall thickness. EF = left ventricular ejection fraction. Rest Grad = resting left ventricular outflow tract gradient (mm Hg). CCB = calcium channel blocker prescription. BB = beta blocker prescription. NYHA Class = New York Heart Association congestive heart failure symptom class. Septal anatomy = morphology of left ventricular septal hypertrophy. Reverse = reverse curvature. NSVT = nonsustained ventricular tachycardia. *CaMKII activation defined as at least 1.5-fold upregulation of pT287-CaMKII ("Borderline" if at least 1.3-fold upregulation). ^SERCA2 reduced defined as a reduction in SERCA2 mRNA or protein abundance by at least 20% compared to average of controls. +SERCA uptake reduced defined as a reduction in SERCA uptake velocity at pCa=5 of at least 20% of average of controls.
### Supplemental Table 2.

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