Cardiac myosin–binding protein-C (cMyBP-C) is highly phosphorylated under basal conditions. However, its phosphorylation level is decreased in individuals with heart failure. The necessity of cMyBP-C phosphorylation for proper contractile function is well-established, but the physiological and pathological consequences of decreased cMyBP-C phosphorylation in the heart are not clear. Herein, using intact adult cardiomyocytes from mouse models expressing phospho-ablated (AAA) and phosphomimetic (DDD) cMyBP-C as well as controls, we found that cMyBP-C dephosphorylation is sufficient to reduce contractile parameters and calcium kinetics associated with prolonged decay time of the calcium transient and increased diastolic calcium levels. Isoproterenol stimulation reversed the depressive contractile and Ca$^{2+}$-kinetic parameters. Moreover, caffeine-induced calcium release yielded no difference between AAA/DDD and controls in calcium content of the sarcoplasmic reticulum. On the other hand, sodium–calcium exchanger function and phosphorylation levels of calcium-handling proteins were significantly decreased in AAA hearts compared with controls. Stress conditions caused increases in both spontaneous aftercontractions in AAA cardiomyocytes and the incidence of arrhythmias in vivo compared with the controls. Treatment with omecamtiv mecarbil, a positive cardiac inotropic drug, rescued the contractile deficit in AAA cardiomyocytes, but not the calcium-handling abnormalities. These findings indicate a cascade effect whereby cMyBP-C dephosphorylation causes contractile defects, which then lead to calcium-cycling abnormalities, resulting in aftercontractions and increased incidence of cardiac arrhythmias under stress conditions. We conclude that improvement of contractile deficits alone without improving calcium handling may be insufficient for effective management of heart failure.

Despite current diagnostic and treatment approaches, heart failure (HF) remains a major clinical problem with high mortality (1–4). Cardiac arrhythmias, a clinical hallmark of HF, lead to sudden cardiac death and contribute to the onset of HF. Ventricular arrhythmias, including tachycardia, premature contractions, and ventricular fibrillation, are major causes of sudden cardiac death in patients with HF and hypertrophic cardiomyopathy (HCM) by decreasing the efficiency of the whole organ (5). Despite the clear association between HF and arrhythmias, it has proven difficult to develop therapies that can treat both diseases without unacceptable side effects. An essential first step toward identifying novel therapeutic strategies lies in elucidating the cellular and molecular mechanisms that underlie abnormal Ca$^{2+}$ handling in HF. Unraveling these molecular-level pathways could ultimately lead to the development of therapeutic regimens able to reduce both HF-induced arrhythmias and acute HF. The pathophysiological association between HF and arrhythmias strongly suggests an interplay between sarcomere mechanics and calcium cycling (5). Certainly, the absence of current therapies to treat the etiology of HF calls for greater understanding of the cellular and molecular mechanisms underlying the calcium-cycling defects caused by mechanical abnormalities of the sarcomere. Current treatment for HF aims to diminish neurohormonal activation along with fluid volume overload and hemodynamics, but the prognosis remains poor (6–8). Thus, independent of the neurohormonal axis, these alarming statistics require researchers to undertake the development of novel therapies able to improve cardiac performance and prevent, or reverse, the progression of left ventricular dysfunction and remodeling (9–12). HF is associated with attenuation of β-adrenergic responsiveness, cardiac contractility, Ca$^{2+}$ cycling (13, 14), and alterations in the phosphorylation levels of contractile proteins, all leading to cardiac dysfunction (15).

Over 40% of HCM cases are now known to involve mutations in the gene (MYBPC3) encoding cardiac myosin binding protein-C (cMyBP-C) (16–20). cMyBP-C, which contains three key phosphorylation sites at the M domain, is a 140-kDa-thick filament protein involved in regulating sarcomere structure and function (21). Its immunoglobulin or fibronectin-like domains, C0–C10, bind sarcomeric proteins, including titin, myosin, and actin, in a phosphorylation-dependent manner (22–24). cMyBP-C is extensively phosphorylated under basal conditions (25–27), but the level of phosphorylation decreases in diseased animal models (26) and patients with HCM (25) and HF (28) or atrial fibrillation (15). In particular, cMyBP-C phosphorylation is intimately involved in the regulation of myocardial function (26, 29), metabolism (30), and cardioprotection (24, 31), but the precise functional consequences of cMyBP-C phosphorylation have not been fully characterized. The regulation of cMyBP-C phosphorylation involves activating both thin and thick filaments (32) by sensitizing calcium to myofilaments. Decreased cMyBP-C phosphorylation in HF patients causes contractile dysfunction and decreased myofilament calcium sensitivity similar to that seen in the cMyBP-C
cMyBP-C dephosphorylation results in cardiac arrhythmia

knockout animal model (33). Moreover, decreased phosphorylation (i.e. dephosphorylation) in cMyBP-C alters cardiac sarcomere morphology with myoarchitectural disarray, causing contractile impairment (34) and inhomogeneity in excitation-contraction coupling (35), which can, in turn, inhibit actomyosin interactions and reduce contractile force (36). In addition, in vivo studies of haploinsufficiency (37, 38) and knockout (39) mouse models of cMyBP-C show enhanced actomyosin interactions, decreased myofilament calcium sensitivity, and increased rate of force redevelopement (40). However, the factors that underlie contractile dysfunction and the mechanisms associated with altered calcium handling remain to be elucidated.

In previous studies, we used cardiac-specific transgenic mice expressing phospho-ablated cMyBP-C at S273A/S282A/S302A sites (AAA) (33) or phosphomimetic cMyBP-C at S273D/S282D/S302D (DDD) and nontransgenic (NTG) control mice. Accordingly, we were able to define the necessity and sufficiency of cMyBP-C phosphorylation for regulating proper cardiac function (24, 26). The AAA mouse heart showed decreased cardiac contractility and altered sarcomere ultrastructure, along with an increase in transcript levels of hypertrophic markers (26), indicating the necessity of cMyBP-C phosphorylation for proper cardiac function. Using these models, we also showed that β-adrenergic stimulation improves the Frank–Starling regulatory mechanism through cMyBP-C phosphorylation (33). In similar studies, AAA and DDD mouse models were used to define the negative regulation of cMyBP-C, or dephosphorylation, to decrease cardiac function in an age-dependent manner (41–44). Whereas the necessity of cMyBP-C phosphorylation for proper cardiac function is well-established, it is unclear whether decreased cMyBP-C phosphorylation leads to altered calcium cycling, especially at the sarcoplasmic reticulum (SR) level.

In the present study, we hypothesized that mechanical defects caused by the dephosphorylation of cMyBP-C disrupt calcium homeostasis and possibly increase the incidence of arrhythmias under stress. To test this hypothesis, we used AAA, DDD, and NTG mouse models to assess the effects of cMyBP-C phosphorylation status on calcium homeostasis. Our studies showed that dephosphorylation of cMyBP-C not only reduces sarcomere contractility, but also impairs calcium homeostasis in isolated cardiomyocytes, along with impaired sodium–calcium exchanger (NCX) function and decreased phosphorylation of key SR calcium-handling proteins. Furthermore, this abnormal calcium cycling results in spontaneous ex vivo aftercontractions and in vivo arrhythmias under stress conditions. Here, we also pharmacologically demonstrate that a positive cardiac inotropic drug, omecamtiv mecarbil (OM), cannot reverse the altered calcium cycling associated with dephosphorylated cMyBP-C contractile defects. Moreover, although dephosphorylated cMyBP-C showed contractile defects as early as 6 weeks of age, no associated abnormalities in calcium homeostasis and no spontaneous aftercontractions were observed. Taken together, our study demonstrates the important role of cMyBP-C phosphorylation in regulating calcium homeostasis of cardiomyocytes and preventing arrhythmogenesis under stress conditions.

Results

Dephosphorylation of cMyBP-C reduces sarcomere contractility and induces spontaneous aftercontractions

In the present study, we used cardiac-specific transgenic mice expressing cMyBP-C AAA to replace endogenous cMyBP-C at the level of ~40%, which recapitulates the level of cMyBP-C dephosphorylation in HF and HCM (25, 26). NTG mice were used as control mice. To validate the effects of cMyBP-C dephosphorylation on contraction at the cellular level, we first assessed the contractile mechanics in intact ventricular myocytes isolated from AAA, DDD, and NTG hearts. As expected, functional analysis of isolated cells indicated that the amplitude of basal cell contraction (fractional shortening) was significantly depressed in AAA (10.05 ± 0.29%, p < 0.0001) compared with DDD (12.05 ± 3.8%, p < 0.0001) and NTG (12.82 ± 0.37% =cardiomyocytes (Fig. 1A and Table 1). The rate of sarcomere contraction and relaxation in AAA cardiomyocytes was also slower compared with NTG and DDD cardiomyocytes (Fig. 1B, C, E, F, and G) and Table 1). However, β-adrenergic stimulation (isoproterenol (ISO); 100 nM) resulted in enhancement of contractile parameters in NTG, AAA, and DDD cardiomyocytes, and the maximally stimulated parameters were similar among the three groups (Fig. 1 A–C and Table 1). These results are consistent with previous studies showing that dephosphorylation of cMyBP-C results in decreased sarcomere contractility (33). Next, we subjected the cardiomyocytes to stress conditions at 2 Hz field stimulation in the presence of 1 µM ISO to assess the effect of impaired contractility on the propensity to develop aftercontractions. Spontaneous aftercontractions were significantly increased in AAA cardiomyocytes (79.21 ± 0.87%, p < 0.0001), compared with DDD (40.38 ± 0.69%) and NTG (40.15 ± 1.06%) myocytes (Fig. 1D, Fig. S1, and Table S1).

Dephosphorylation of cMyBP-C causes reduced calcium cycling and increased diastolic calcium levels

To examine the effects of altered contractile mechanics on Ca\textsuperscript{2+}-handling properties, owing to cMyBP-C dephosphorylation, we measured the amplitude and kinetics of intracellular Ca\textsuperscript{2+} transients in isolated cardiomyocytes from AAA, DDD, and NTG hearts using the Fura-2 fluorescence indicator. Our results show that Ca\textsuperscript{2+} transient amplitude, as indicated by Fura-2 ratio (340/380 nm), was significantly reduced in AAA cardiomyocytes (0.23 ± 0.01 nm, p < 0.001) when compared with DDD (0.38 ± 0.02 nm) and NTG (0.37 ± 0.02 nm) cardiomyocytes (Fig. 2A and Table 2). Furthermore, the time to 50% decay of calcium peak and the time constant for calcium decay (t) were significantly prolonged in AAA cardiomyocytes (0.21 ± 0.01 s, p < 0.05) compared with DDD (0.17 ± 0.01 s) and NTG (0.18 ± 0.01 s) cardiomyocytes (Fig. 2B, C, E, F, and G) and Table 2). Depressed calcium kinetics were also associated with a significant increase in diastolic Ca\textsuperscript{2+} (Fura-2 ratio (340/380 nm)) in AAA cardiomyocytes (1.28 ± 0.01 nm, p < 0.001) compared with DDD (1.18 ± 0.01 nm) and NTG (1.17 ± 0.01 nm) cardiomyocytes (Fig. 2D and Table 2), suggesting dysfunctional SERCA2a reuptake activity. ISO stimulation enhanced and corrected the Ca\textsuperscript{2+} kinetics in AAA cardiomyocytes, and the maximally stimulated parameters were similar among the
three groups (Fig. 2 (A–C) and Table 2). In addition, diastolic Ca^{2+} was normalized upon ISO stimulation of AAA cardiomyocytes (Fig. 2D and Table 2).

**Dephosphorylation of cMyBP-C exhibits reduced NCX activity but unchanged SR Ca^{2+} content**

The significant effects of dephosphorylated cMyBP-C on calcium transients prompted further study to determine the influence of AAA on SR Ca^{2+} load and calcium extrusion in cardiomyocytes. We measured SR Ca^{2+} content in isolated cardiomyocytes and observed that the caffeine-induced calcium transient peak was similar in all three groups (Fig. 3A, Fig. S2, and Table 3). However, NCX function, assessed as the time constant (τ) of calcium decline during caffeine-induced calcium transient, was significantly decreased in AAA cardiomyocytes (5.28 ± 0.30 s, p < 0.0001) compared with DDD (3.29 ± 0.32) and NTG (3.22 ± 0.34) cardiomyocytes (Fig. 3B and Table 3), indicating prolongation in the extrusion of intercellular calcium in AAA cardiomyocytes. Furthermore, we measured the total diastolic SR Ca^{2+} leak from AAA,
cMyBP-C dephosphorylation results in cardiac arrhythmia

Table 1
Summary of contraction mechanics in cMyBP-C AAA, DDD, and NTG cardiomyocytes

Contractile mechanics were recorded in isolated ventricular cardiomyocytes from phospho-null (AAA), phosphomimetic (DDD), and NTG mice in the absence and presence of 100 nmol/liter ISO at 0.5 Hz. Fractional shortening (FS%), contraction velocity (CV), and relaxation velocity (RV) of sarcomeres from each cardiomyocyte were assessed using a video-based sarcomere length detection system (IonOptix). Data were analyzed by IonOptix LLC software. Data are expressed as mean ± S.E., and statistical analyses were performed in all groups by ordinary two-way ANOVA (see Table 6 for analysis of main factors and interactions), followed by Tukey’s multiple-comparison test. n, number of cells; N, number of animals; *, p < 0.05 versus NTG; #, p < 0.05 versus −ISO; ##, p < 0.001 versus −ISO; ###, p < 0.0001 versus NTG; $$$, p < 0.0001 versus ISO.

|          | AAA (n=45) | DDD (n=45) | NTG (n=45) |
|----------|------------|------------|------------|
| FS (%)   | 10.05 ± 0.29** | 12.05 ± 0.38 | 12.82 ± 0.37 |
| CV (µm/s)| 2.34 ± 0.08** | 2.92 ± 0.09 | 3.05 ± 0.12 |
| RV (µm/s)| 1.96 ± 0.08** | 2.59 ± 0.12 | 2.86 ± 0.11 |

Figure 2. Ca^2+ kinetics and diastolic calcium levels of NTG, AAA, and DDD cardiomyocytes and their response to isoproterenol.

A. Ca^2+ transient amplitude as indicated by Fura-2 ratio (340/380 nm) in the absence and presence of 100 nmol ISO at 0.5 Hz with 1.8 mM Ca^2+. B. Time to 95% decay of the calcium transient in the absence and presence of ISO. C. Relaxation time constant (t) of the calcium transient in the absence and presence of ISO. D. Intracellular diastolic calcium levels. Shown are representative Ca^2+ transient tracings of NTG (Δ), AAA (●), and DDD (□) cells before (solid line) and after (dotted line) isoproterenol stimulation. N = 6 hearts (−ISO) for NTG (57 cells) and AAA (59 cells); N = 5 hearts for DDD (47 cells); N = 5 hearts (+ISO) in all groups with NTG (42 cells), AAA (42 cells), and DDD (36 cells). 12-week-old male mice were used. Data are expressed as mean ± S.E. (error bars) on the number of cells, and statistical analyses were performed in all groups by two-way ANOVA (see Table 6 for analysis of main factors and interactions), followed by Tukey’s multiple-comparison test. *, p < 0.05; **, p < 0.001 AAA versus NTG cells. #, p < 0.05 versus −ISO for all groups; ###, p < 0.0001 AAA (−ISO) versus AAA (+ISO).

DDD, and NTG cardiomyocytes using the tetracaine protocol (45) and identified no differences in SR Ca^2+ leak (Fig. 3C and Table 3). The ratio of SR Ca^2+ leak to SR Ca^2+ load was also not altered (Fig. 3D and Table 3) compared with DDD and NTG cardiomyocytes. In addition, elevated diastolic Ca^2+ in AAA cardiomyocytes did not trigger SR Ca^2+ leak, compared with controls. These results suggest that dephosphorylation of cMyBP-C delays calcium extrusion from cardiomyocytes without affecting SR Ca^2+ load.

Dephosphorylation of cMyBP-C reduces the phosphorylation level of SR calcium-handling proteins

To determine the effects of cMyBP-C phosphorylation levels on calcium cycling, we examined the levels of total SERCA2a, phospholamban (PLN), ryanodine receptor (RyR), Ca^2+/calmodulin-dependent kinase II (CaMKII), and NCX under basal and ISO stimulation by Western blotting analyses. We also assessed the levels of PLN, RyR, and CaMKII phosphorylation. Cardiac expression of SERCA2a, NCX, CaMKII, and total PLN was similar in all groups. However, under basal conditions, the phosphorylation levels of PLN Ser-16 (24.28 ± 5.71%, p < 0.0001), PLN Thr-17 (27.48 ± 5.71%, p < 0.0001), RyR p2808 (21.39 ± 4.06%, p < 0.0001), and RyR p2814 (19.85 ± 0.87%, p < 0.0001) were all significantly decreased in AAA hearts compared with DDD and NTG (Fig. 4 (A and B) and Tables S2 and S3). We found no change in the phosphorylation levels of CaMKII (Thr-287). We also examined the expression and phosphorylation levels of cMyBP-C and cardiac troponin I (cTnI) under basal and β-adrenergic stimulation conditions. The phosphorylation levels of Ser-302 in endogenous cMyBP-C were significantly reduced under basal conditions in both AAA (18.8 ± 2.29%, p < 0.0001) and DDD (24.07 ± 2.83%, p < 0.0001) hearts compared with NTG hearts (Fig. 4 (C and D) and Table S4). Cardiac expression and basal phosphorylation levels of cTnI were similar in all groups. ISO treatment induced robust increases in cMyBP-C phosphorylation levels, but only in NTG hearts, and in cTnI of all three groups. ISO treatment was associated with only a small increase in endogenous cMyBP-C phosphorylation levels in transgenic AAA and DDD hearts. These data indicate that the observed decrease in contractile function and associated abnormal calcium cycling may be attributed to the expression of cMyBP-C phospho-null (AAA) in the mouse hearts, compared with the expression of cMyBP-C phosphomimetic (DDD).

AAA mice have increased incidence of arrhythmia upon stress challenge

We next asked whether in vitro aftercontractions and calcium-handling defects might also have an in vivo effect. To
cMyBP-C dephosphorylation results in cardiac arrhythmia

Table 2
Summary of calcium kinetics in cMyBP-C AAA, DDD, and NTG cardiomyocytes

|                    | AAA          | DDD          | NTG          |
|--------------------|--------------|--------------|--------------|
| Peak (340/380 nm)  | 0.23 ± 0.01** | 0.38 ± 0.02  | 0.37 ± 0.02  |
| Time to 50% decay  | 0.27 ± 0.01** | 0.22 ± 0.01  | 0.2 ± 0.01   |
| τ (s)              | 0.21 ± 0.01*  | 0.17 ± 0.01  | 0.18 ± 0.01  |
| Diastolic Ca²⁺ (340/380 nm) | 1.29 ± 0.01** | 1.18 ± 0.02** | 1.17 ± 0.01  |

address this question, we subjected NTG, AAA, and DDD mice to catecholaminergic (caffeine and epinephrine) stress via intraperitoneal injection. Surface electrocardiogram (ECG) was used to measure induced arrhythmias in vivo. ECG tracing data showed no arrhythmic events under basal conditions in AAA (Fig. 5A), NTG, and DDD mice (data not shown). However, catecholaminergic stress did cause arrhythmias in the form of frequent premature ventricular complexes (PVCs) (Fig. 5B) and episodes of nonsustained ventricular tachycardia (NSVT) (Fig. 5C) in AAA mice. Arrhythmias typically began 5 min after intraperitoneal injection and lasted 10 min into recovery. Quantification of ECG tracing shows 6 of 7 AAA mice (85%) with PVCs (Fig. 5D), as compared with 2 of 5 NTG mice (40%) and 1 of 3 DDD mice (33%). Similarly, NSVT quantification shows that 3 of 7 AAA mice (40%) had an incidence of arrhythmias as compared with 1 of 5 NTG mice (20%) and 1 of 3 DDD mice (20%). In addition, the number of arrhythmic irregularities (i.e. PVC together with NSVT) was higher in AAA mice, compared with both DDD and NTG mice (Fig. 5E).

Positive cardiac inotropic drug, OM, reduces spontaneous aftercontractions in dephosphorylated cMyBP-C cardiomyocytes but does not affect calcium handling

To rescue contractile impairment by dephosphorylated cMyBP-C and its effect on abnormal calcium homeostasis, we treated adult cardiomyocytes from the three groups with the positive inotropic agent OM (100 nM), which has shown significant inotropic effects (46). As expected, OM significantly improved the strength of contraction (fractional shortening percentage) and reduced frequency (by 30%) of spontaneous aftercontractions under stress conditions (2 Hz + ISO) (Fig. 6A and B, Fig. S3, and Table 4). Importantly, however, calcium kinetics did not change with OM treatment, as shown by persistent depression of calcium peak and elevated diastolic calcium levels in dephosphorylated AAA cardiomyocytes (Fig. 6C and D and Table 4). These results confirm that OM treatment could improve contractility in cardiomyocytes of 12-week-old AAA mice, but without changing calcium cycling. Furthermore, OM could not fully prevent aftercontractions under stress conditions. Thus, remodeling associated with contractile dysfunction might be responsible for abnormal calcium cycling in AAA cardiomyocytes.

Young mice are not susceptible to arrhythmias and have normal calcium cycling

To confirm that abnormal calcium cycling in AAA cardiomyocytes results from impaired sarcomere contraction, we examined the contractile mechanics and calcium kinetics in 6-week-old mice. The amplitude of basal cell contraction (fractional shortening percentage) (Fig. 7A and Table 5) was depressed in AAA (11.06 ± 0.42%) compared with DDD (12.41 ± 0.43%) and NTG (13.41 ± 0.44%) cardiomyocytes in these young mice. However, no increases in the frequency of spontaneous aftercontractions were observed in DDD cardiomyocytes (Fig. 7B and Table 5) under stress (2 Hz + ISO) conditions. In addition, the calcium kinetics in the cardiomyocytes of 6-week-old mice showed no alterations, and both the amplitude of Ca²⁺ peak and the diastolic calcium levels were similar among all groups (Fig. 7C and D and Table 5). To confirm the
Table 3
Summary of SR Ca\(^{2+}\) load and SR Ca\(^{2+}\) leak measurement in cMyBP-C AAA, DDD, and NTG cardiomyocytes

|          | AAA n(N) Values  | DDD n(N) Values  | NTG n(N) Values  |
|----------|-------------------|-------------------|-------------------|
| SR Ca load (340/380 nm) | 40(5) 0.46 ± 0.03 | 41(5) 0.39 ± 0.03 | 40(5) 0.39 ± 0.03 |
| Caffeine-\(\tau\) (s) | 40(5) 5.28 ± 0.3** | 41(5) 3.29 ± 0.32 | 40(5) 3.22 ± 0.34 |
| SR Ca leak (340/380 nm) | 41(5) 0.092 ± 0.01 | 39(5) 0.083 ± 0.002 | 40(5) 0.082 ± 0.005 |
| Ca leak/SR load ratio | 41(5) 0.194 ± 0.01 | 39(5) 0.159 ± 0.01 | 40(5) 0.170 ± 0.01 |

**Figure 4. Expression and phosphorylation levels of SR Ca-regulatory proteins and myofilament proteins.** A, representative Western blotting of the expression status of SR Ca-regulatory proteins under pre- and post-ISO stimulation. B, quantification assessment of protein levels and their phosphorylation in NTG, AAA, and DDD mice under basal (top) and ISO stimulation (bottom) conditions. In post-ISO groups, phosphorylation levels were normalized to respective total protein levels and then to the respective pre-ISO NTG controls. Statistical analyses of the quantitations plus or minus ISO are presented in Table S2. All proteins were normalized with CSQ. C, representative Western blotting exhibiting phosphorylation of cMyBP-C (residue Ser-302) and cTnI (residues Ser-22 and Ser-23) using their respective phosphospecific and pan-nonspecific antibodies. Gapdh was probed with a Gapdh antibody as a loading control. D, quantitative assessment of cMyBP-C and cTnI phosphorylation in NTG, AAA, and DDD mice under basal and ISO stimulation conditions. Average phosphorylation of cMyBP-C and cTnI post-ISO was normalized to total cMyBP-C and total cTnI, respectively, and then to NTG baseline (pre-ISO) respective controls. Phosphorylation and total expression of PLN, cMyBP-C, and cTnI proteins were detected by dual-color Western blotting using two IR fluorophores. The intensities of both phosphospecific and pan-nonspecific blot intensities were determined using Image Studio (LI-COR). Proteins were extracted from the whole-heart lysate of 12-week-old male mice. For the ISO group, the hearts were extracted after stimulating 12-week-old male mice with epinephrine and caffeine injection. Data are expressed as mean ± S.E. (error bars), and statistical analyses were performed in all groups by two-way ANOVA (see Table 6 for analysis of main factors and interactions), followed by Tukey’s multiple-comparison test with single pooled variance. n, number of cells; N, number of animals; ***, \(p < 0.0001\) versus NTG.
functional data, we performed immunoblotting to compare the expression and phosphorylation levels of key calcium-handling proteins in all three groups of 6-week-old mice. We found that the phosphorylation levels of PLN (Ser-16 and Thr-17) and RyR (Ser-2808 and Thr-2814) were similar among all 6-week-old mouse groups (Fig. 7(top) and F) and Table S5). No differences were observed in cMyBP-C and cTnI determined between 6 and 12 weeks of age in transgenic mouse hearts, compared with NTG controls (Fig. 7(bottom) and Table S6). These data suggest that compensatory responses in the face of dephosphorylated cMyBP-C elicit Ca$^{2+}$-handling alterations ultimately associated with arrhythmias under stress conditions.

**Discussion**

Our findings clearly showed that contractile dysfunction resulting from cMyBP-C dephosphorylation is sufficient to cause stress-induced arrhythmias at both in vitro and in vivo levels during the development of cardiac dysfunction. These novel findings establish an incontrovertible link between arrhythmogenesis and abnormal calcium homeostasis in the context of depressed contractility caused by cMyBP-C dephosphorylation. Although we can improve contractility by using positive cardiac inotropic agents, such as OM, as demonstrated in this paper, altered calcium handling cannot be fully reversed and needs to be treated separately to improve acute HF. Finally, our studies revealed that posttranslational modification of cMyBP-C can have an early impact on contractile function, which is followed by changes in cardiomyocyte calcium handling over time.

**cMyBP-C phosphorylation and its role in contractility**

Transgenic animal models highlight the functional significance of cMyBP-C phosphorylation during the development of HF (24, 26, 43). Our in vitro results show that dephosphorylation of cMyBP-C contributes to impairment of sarcomere contractility with depressed sarcomere shortening, along with a reduced rate of contraction and relaxation in isolated cardiomyocytes. cMyBP-C is a highly phosphorylated protein under
cMyBP-C dephosphorylation results in cardiac arrhythmia

The purpose of this study was to investigate whether any defects in sarcomere contractility caused by cMyBP-C dephosphorylation could also affect calcium homeostasis, especially under stress conditions (32). Mechanistically, phosphorylated cMyBP-C dynamically relieves the tether-like constraint of myosin heads with an increase in cross-bridge cycling kinetics (35, 48, 49), whereas dephosphorylated cMyBP-C acts as a brake that inhibits actin-myosin interaction to slow cross-bridge cycling (50). Moreover, phosphorylated cMyBP-C binds to the thin filaments and sensitizes calcium by moving tropomyosin away from its blocked position, improving contraction (32, 51). This thin filament activation is absent in dephosphorylated cMyBP-C, leading to decreased calcium sensitivity and defects in sarcomere contractility (Fig. 8, D–F). This line of evidence suggests that cMyBP-C phosphorylation is necessary to regulate proper cardiac contractility.

cMyBP-C dephosphorylation causes impaired calcium cycling

The present follow-up studies have shown that phosphorylated cMyBP-C sarcomeres alter calcium cycling, causing an increase in diastolic calcium levels, depressed transient peak, and slower calcium transient decay, all of which indicate the inability of cardiomyocytes to remove elevated intercellular calcium during relaxation. In the phosphorylated state, cMyBP-C activates thin filament (56, 57) by displacing tropomyosin from the blocked (51) to open position, thus facilitating calcium binding to cardiac troponin C (cTnC), as required for regular contraction. Therefore, although cMyBP-C does not bind to calcium, its phosphorylation indirectly regulates calcium binding to sarcomere via thin filament activation. Conversely, contractile defects in sarcomere were shown to be associated with accelerated Ca\(^{2+}\) dissociation from cTnC during contraction (52, 58–60), leading to elevated systolic calcium during contraction. However, our \textit{in vitro} calcium kinetics data suggest that the contractile defects associated with dephosphorylation of cMyBP-C contribute to calcium-cycling defects affecting the availability of free calcium to myofilaments for thin filament during systolic contraction.

Contractile deficits in AAA cardiomyocytes cause dephosphorylation of SR proteome while maintaining SR Ca content

To dissect the role of calcium-handling proteins in arrhythmogenesis of AAA cardiomyocytes, we next measured cytosolic Ca\(^{2+}\) removal by SERCA2a and NCX. We found that NCX calcium efflux was significantly decreased, whereas total SR Ca\(^{2+}\) load did not change, contributing to increased Ca\(^{2+}\) levels during relaxation. Electrogenic NCX works in the forward mode extruding intracellular Ca\(^{2+}\), but during elevated Ca\(^{2+}\), it reverses its role, resulting in more Ca\(^{2+}\) influx (61). In reversal mode, NCX changes the resting membrane potential of cardiomyocytes, which are susceptible to “spontaneous” contraction under stress. Furthermore, although total protein expression of SERCA2a, PLN, CaMKII, RyR, and NCX remained the same at basal level, a significant decrease in the phosphorylation levels of PLN (Ser-16 and Thr-17), RyR (Ser-2808 and Ser-2814), and CaMKII (Thr-287) was observed. In the phosphorylated state, PLN strongly inhibits the ability of SERCA2a to reuptake calcium during diastole, causing abnormal calcium cycling and putting a strain on NCX efflux (Fig. 8E) (62). Interestingly, SR calcium load remained unaltered, which may reflect a balance between reduced SR calcium uptake and attenuated calcium release from dephosphorylated RyR. The complex role of calcium release from RyR and its role in SR Ca\(^{2+}\) leak has been the subject of several investigations (62–66). In addition, our results show no change in the phosphorylation levels of CaMKII (Thr-287), but other posttranslational modifications, including oxidation, S-nitrosylation, and O-GlcNAcylation, which all regulate CaMKII activity (67), may also contribute to CaMKII status quo, even under elevated intracellular calcium levels in AAA cardiomyocytes. Additional experiments are required to address the “cross-talk” and the role of cMyBP-C dephosphorylation in regulating posttranslational modification of other cardiac proteins (68).

cMyBP-C dephosphorylation and arrhythmias

In this study, we observed that AAA cardiomyocytes had a higher frequency of spontaneous aftercontractions, indicating a
Figure 7. Contractility mechanics and calcium kinetics of NTG, AAA, and DDD in early-age mice. A, fractional shortening (%) in 6-week-old male mice at 0.5 Hz. B, percentage of cells that developed aftercontractions under stress conditions (2 Hz and 1 μM ISO). C, Ca$^{2+}$ transient amplitude as indicated by Fura-2 ratio (340/380 nm) in 6-week-old male mice at 0.5 Hz with 1.8 mM Ca$^{2+}$. D, intracellular diastolic calcium levels. N = 4 hearts (40 cells)/group. E, representative Western blotting of the expression status of SR Ca-regulatory proteins and cardiac myofilament protein in the 6- and 12-week-old male mice. F, quantification assessment of protein levels and their phosphorylation in AAA and DDD mice in the 6- and 12-week-old male mice relative to NTG basal levels. Average protein phosphorylation was normalized to respective total protein. Phosphorylation and total expression of PLN, cMyBP-C, and cTnI proteins were detected by dual-color Western blotting using two IR fluorophores. Both phosphospecific and pan-nonspecific blot intensities were determined using Image Studio (LI-COR). Proteins were extracted from the whole-heart lysate of 6- and 12-week-old male mice. Immunoblotting data are expressed as mean ± S.E. (error bars), and statistical analyses were performed in all groups by two-way ANOVA (see Table 6 for analysis of main factors and interactions), followed by Tukey’s multiple-comparison test to compare mice at 6 and 12 weeks of age; n = 3-4 hearts/group. Six-week functional data are expressed as mean ± S.E. on the number of cells, and statistical analyses were performed between groups by one-way ANOVA (see Table 7 for analysis of main factors and interactions), followed by Tukey’s multiple-comparison test with single pooled variance.**, p ≤ 0.001 AAA versus NTG cells; ***, p ≤ 0.0001 AAA versus NTG hearts.
cMyBP-C dephosphorylation results in cardiac arrhythmia

Table 5
Summary of contraction mechanics and calcium kinetics in cMyBP-C AAA, DDD, and NTG cardiomyocytes from 6-week-old mice

Contractile mechanics and calcium kinetics were recorded in isolated ventricular cardiomyocytes from phospho-null (AAA), phosphomimetic (DDD), and NTG mice at 0.5 Hz. Fractional shortening (FS%), from each cardiomyocyte was assessed using a video-based sarcomere length detection system (IonOptix). The percentage of spontaneous aftercontractions was measured within 7 s of stopping electrical stimulation in the presence of 1 μmol/liter ISO at 2 Hz. Fura-2 fluorescence from each cardiomyocyte was excited at 340 and 380 nm and acquired at an emission wavelength of 515 ± 10 nm using a spectrophotometer (IonOptix) to measure calcium transient peak (340/380 nm) of the calcium transient and diastolic Ca²⁺ levels. Data were analyzed by IonOptix LLC analyzing software. Data are expressed as mean ± S.E., and statistical analyses were performed in all groups by one-way ANOVA (see Table 7 for analysis of main factors and interactions), followed by Tukey’s multiple-comparison test with single pooled variance. n, number of cells; N, number of animals; *, p < 0.01 versus NTG.

|          | n(N) | AAA | DDD | NTG |
|----------|------|-----|-----|-----|
| FS (%)   | 40(4) 11.06 ± 0.42** 12.42 ± 0.44 13.41 ± 0.44 |
| % AC     | 4 24.07 ± 1.07 23.15 ± 0.47 24.2 ± 0.37 |
| Peak (340/380 nm) | 40(4) 0.43 ± 0.06 0.40 ± 0.02 0.38 ± 0.03 |
| Diastolic Ca²⁺ (340/380 nm) | 40(4) 1.18 ± 0.01 1.17 ± 0.01 1.17 ± 0.01 |

predisposition toward arrhythmias under stress, which is also confirmed by our electrocardiography data. Even though transgenic AAA mouse hearts show normal cardiac function and dimension, as assessed by echocardiography under baseline conditions, they show increased abnormalities like fibrosis, myocyte disarray, and increased cardiac hypertrophic markers, such as β-myosin heavy chain and atrial natriuretic factor, compared with NTG controls (26). Moreover, under dobutamine stimulation, AAA mice showed decreased contraction and relaxation with invasive hemodynamic load catheterization (26). Therefore, dissecting these effects to define the impact of cMyBP-C dephosphorylation is further warranted. β-Adrenergic stimulation results in spontaneous rises in cytoplasmic Ca²⁺ concentration, activating forward NCX action (64). During relaxation, the defective calcium handling in AAA cardiomyocytes puts stress on NCX forward activity, sufficiently depolarizing cells to trigger a new action potential, allowing action potential to self-perpetuate as ventricular tachycardia (Fig. 8F) (36, 64). Taken together, then, our studies suggest that cMyBP-C dephosphorylation contributes to abnormal calcium homeostasis affecting NCX forward function, causing arrhythmias under stress.

cMyBP-C dephosphorylation triggers cardiac remodeling affecting calcium cycling

Recent studies have established that the positive cardiac inotropic agent OM can enhance contractility without affecting calcium cycling in animals (69) and humans (70). To test this finding, we employed OM to analyze the distinct role of sarcomere impairment and abnormal calcium homeostasis in arrhythmias. Specifically, we treated AAA cardiomyocytes with OM, and we found that the incidence of aftercontractions was reduced, but not eliminated, upon stress challenge, confirming its role in improving contraction, but not changing calcium cycling. OM results imply that the dephosphorylation of cMyBP-C triggers a cascade whereby decreasing actomyosin interactions, inhibiting thin filament orientation, and decreasing myofilament calcium sensitivity finally lead to cardiac dysfunction, which is hard to reverse using only myosin activators. AAA mice also show an increase in transcript levels of sensitive molecular markers, such as atrial natriuretic factor and β-myosin heavy chain, suggesting that cardiac stress and initial remodeling at the molecular level (26), along with altered sarcomere structure, trigger changes in cardiac myoarchitecture causing ventricular dysfunction (34, 71). Consequently, we further investigated this cascade effect by comparing 6-week-old mice with 12-week-old mice. Our functional and biochemical data show that 6-week-old AAA mice have cardiac dysfunction similar to that of adult mice yet maintain regular calcium cycling and normal phosphorylation of SR proteome. Also, dephosphorylation of cMyBP-C destabilizes the ultrastructure of sarcromere morphology by changing the thin filament orientations (34) as a result of altering interactions between cMyBP-C and both thick and thin filament proteins. Functional studies of 6-week-old mice confirm that the cascade of pathophysiological effects during mechanical remodeling in AAA hearts may originate from sarcomeric triggering of the pan-dephosphorylation of calcium-handling proteins, especially PLN and RyR, along with decreased NCX activity, gradually causing abnormal calcium homeostasis. Future preclinical studies treating mice with OM will validate these findings. We propose that preventing cardiac dysfunction in AAA mice with OM at an earlier time point would be cardioprotective with normal calcium handling and contractility.

However, several potential pitfalls with this idea can occur when extrapolating the current findings to humans. For example, the NCX contribution to Ca²⁺ removal is lower in mice (~7%), thus poorly mimicking the contribution of NCX to cellular Ca²⁺ flux in humans (~30%) (72). Moreover, both time and lack of proper infrastructure during our preliminary studies made it difficult to confirm the susceptibility of female mice to arrhythmias; however, in future studies, we will include both male and female mice to determine sex differences in cMyBP-C dephosphorylation in regulating calcium homeostasis.

In conclusion, dephosphorylation of cMyBP-C impairs sarcomeric contractility, causing mechanical remodeling and altered calcium handling leading to ventricular dysfunction. Our study determined that reduced cardiac function from dephosphorylated cMyBP-C results in pan-dephosphorylation of SR proteome, decreasing SERCA2a reuptake with an increase in diastolic calcium. The gradual increase in diastolic calcium affects NCX forward function, which, in turn, triggers “spontaneous” action potential, finally increasing the propensity toward ventricular arrhythmias under stress. As an essential lynchpin in this cascade of pathophysiological events, the present study has elucidated the link between impairment in sarcomere contractility and abnormal calcium cycling, thus presenting an opportunity to study sarcomeric contractility and calcium cycling as one functional (or dysfunctional) system, suggesting the potential benefit of a unitary therapeutic target for the dual treatment of patients with HCM and HF.

Experimental procedures

Mouse models

Cardiac-specific phosho-ablated and phosphomimetic mouse models of cMyBP-C, using α-myosin heavy chain promoter, have been described previously (24, 26, 33, 73). In brief,
cMyBP-C dephosphorylation results in cardiac arrhythmia

**NTG (Phosphorylated cMyBP-C)**

**A** Normal contraction

**B** Normal relaxation

**C** No electrical defects
- Low “spontaneous” after-contractions
- Low incidences of PVC & NSVT

**AAA (Dephosphorylated cMyBP-C)**

**D** Contraction defects
- Thick and thin filament inactivation

**E** Relaxation defects
- Decrease NCX function
- Decrease PLN & RyR phosphorylation
- Decrease CaMKII activation
- Increase diastolic Ca2+
- Remodeling (ANF, β-MHC)

**F** Electrical defects
- High after-contractions
- High incidences of PVC & NSVT
cMyBP-C dephosphorylation results in cardiac arrhythmia

in the phoso-ablated (AAA) and phosphomimetic (DDD) cMyBP-C mouse models, three key phosphorylation sites (Ser-273, Ser-282, and Ser-302) of cMyBP-C were mutated to alanine and aspartic residues, respectively. NTG mice were used as controls. All experiments were conducted under institutional guidelines and approved by the University of Cincinnati Animal Care and Use Committee for Dr. Sadayappan and Dr. Kranias. Adult mice (12-16 weeks of age, male) and young mice (6 weeks of age, male) were used in this study.

Mouse cardiomyocyte isolation

Isolation of mouse ventricular cardiomyocytes was carried out as described previously (74). Briefly, mice were injected intraperitoneally with 0.15 ml of heparin (1000 units/ml) and anesthetized with euthasol (200 mg/kg intraperitoneally) (Virbac AH, Inc.). Hearts were excised and mounted on a Langendorff system for retrograde perfusion through the aorta with a calcium-free perfusion solution consisting of NaCl (120 mM), KCl (5.4 mM), MgSO4 (1.4 mM), NaH2PO4 (12 mM), NaHCO3 (20 mM), 2,3-butanediene monoxime (10 mM), tauarine (5 mM), and glucose (5.6 mM) at 37 °C for 3 min. Perfusion was then switched to the perfusion solution containing liberase enzyme (0.25 mg/ml; Roche Applied Science) for 8–15 min until the heart became flaccid. The ventricular tissue was excised, minced, and pipette-dissociated, and cardiomyocytes were filtered through a 200-μm screen. Cardiomyocytes were resuspended in perfusion solution containing 0.5% BSA. The cell suspension was subjected to sequential extracellular Ca2+ restoration by 25 μM, 100 μM, 200 μM, and 1 mM calcium solution. This cell suspension was used for contractility. For calcium kinetics, the cells were resuspended in 1.8 mM Ca2+ Tyrode’s solution containing NaCl (140 mM), KCl (4 mM), MgCl2 (1 mM), glucose (10 mM), and HEPES (5 mM), pH 7.4, at room temperature.

Measurements of cardiomyocyte contraction mechanics and Ca2+ kinetics

Cell contractility and Ca2+ transients were measured simultaneously at room temperature (22–23 °C) as described previously (37, 45, 74, 75). Briefly, under basal conditions, cardiomyocytes were incubated with 1.0 μM Ca2+-sensitive Fura-2 dye (Invitrogen) at room temperature for 15 min and subsequently washed with Tyrode’s solution for 10 min. The cells were then placed in a perfusion chamber (at the stage of an inverted Nikon eclipse TE2000-U fluorescence microscope) and were superfused (Warner Instruments, 6-channel valve controller) with Tyrode’s buffer at room temperature. Sarcomere shortening was assessed using a video-based sarcomere length detection system (IonOptix, Milton, MA, USA). To measure intraacellular Ca2+ transients, Fura-2 fluorescence was excited at 340 and 380 nm and acquired at an emission wavelength of 515 ± 10 nm using a spectrophotometer (IonOptix), and the emission field was restricted to a single cell with the aid of an adjustable window. Simultaneous measurements of mechanics and Ca2+ kinetics were also performed in the presence of 100 nmol/liter OM at 0.5 Hz. Similarly, contractile mechanics and calcium kinetics were measured in isolated cardiomyocytes in the absence and presence of 100 nmol/liter OM. Data were analyzed by IonOptix LLC analyzing software. OM was purchased from Selleck (catalog no. CK-1827452), dissolved in DMSO, and diluted in a Tyrode’s buffer to achieve final concentrations of 100 nm OM at room temperature as described earlier (46).

Spontaneous aftercontractions

Isolated mouse cardiomyocytes were paced at 2 Hz in the presence of 1 μmol/liter ISO. After regular trains of stimulation, the pacing was stopped, and spontaneous contractions within 7 s were recorded. Similarly, spontaneous aftercontractions were measured after treatment of 100 nmol/liter OM in the presence of 1 μmol/liter ISO and 2-Hz pacing (76).

SR Ca2+ load and SR Ca2+ leak measurement

Cardiomyocytes were superfused with Tyrode’s solution and were stimulated at 0.5 Hz until the twitch characteristics were stable before applying caffeine. After cessation of pacing cardiomyocytes, 10 mM caffeine solution was quickly introduced into the chamber and was present during the kinetics study (30 s) of [Ca2+]i, decline (76). SR Ca2+ content was measured as the amplitude of the caffeine-induced Ca2+ transients. NCX activity could be attributed to the decline of [Ca2+]i, during a caffeine-induced Ca2+ transient in normal Tyrode’s solution. Data were analyzed by IonOptix LLC analyzing software. Diastolic SR Ca2+ leak was measured using the modified protocol described previously (76–78). Fura-2–loaded cardiomyocytes
were field-stimulated at 0.5 Hz until they reached a steady-state Ca\(^{2+}\) transient height. After cessation of cardiomyocyte pacing, superfusion buffer was immediately switched for Tyrode’s buffer containing LiCl (144 mM), MgCl\(_2\) (1 mM), glucose (10 mM), HEPES (5 mM), KCl (5 mM), and EGTA (10 mM) (pH adjusted up to 7.4 with lithium hydroxide) with 0 Na\(^{+}/0\) Ca\(^{2+}\) during ~30 s to prevent Ca\(^{2+}\) fluxes through the sarcolemma, resulting in a new steady-state [Ca\(^{2+}\)]. Then RyR2 channels were inhibited by 1 mmol/liter tetracaine (Sigma–Aldrich, catalog no. T7383) in Tyrode’s solution for 60 s. Under this condition, Ca\(^{2+}\) shifted from the cytosol into the SR to reduce cytosolic [Ca\(^{2+}\)]. Then the cardiomyocytes were exposed for 4 s to tetracaine-free Tyrode’s buffer before assessing SR Ca\(^{2+}\) content by a 10 mm caffeine challenge and 20 mmol/liter 2,3-butanedione monoxime (to prevent myocyte hypercontracture). The amplitude of the caffeine-induced Ca\(^{2+}\) transient was used as an estimate of the total [Ca\(^{2+}\)], which included Ca\(^{2+}\) leak. SR Ca\(^{2+}\) leak was determined as a tetracaine-sensitive drop in Fura-2 ratio. Data were analyzed by IonOptix LLC analyzing software.

**Quantification of protein expression and phosphorylation**

Proteins were extracted from 6- and 12-week-old male mouse whole-heart lysates. Snap-frozen hearts were homogenized in ice-cold lysis buffer (Cell Signaling #98035) with protease and phosphatase inhibitors (Sigma mixture #PPC1010) and sampled for protein concentration by Bradford assay (Thermo Scientific #23226). For the ISO group, hearts were extracted after stimulating 12-week-old male mice with epinephrine and caffeine injection (2 mg/kg body weight; Sigma #E4250) and caffeine (120 mg/kg body weight; Sigma #C0750) injection. 10–100 µg of protein samples were separated by SDS-PAGE using 6–12% polyacrylamide gels using Precision Plus Protein™ (Bio-Rad, catalog no. 1610375, 10\(^{ – 0.45-0.7} \)) for 4 h at room temperature. Immunoblot membranes were incubated in Odyssey TBS blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. Immunoblot analysis was performed with primary antibodies diluted in Odyssey TBS blocking buffer and probed overnight at 4°C. The following commercially available primary antibodies were used: cTnI (Abcam, #ab47003), phosphoserine 22/23 cTnI (Cell Signaling Technology, #4004S), the cardiac isoform of sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a) (Santa Cruz Biotechnology, #sc-8095), PLN (Pierce, Thermo Scientific™, #MA3-922), phosphoserine 16 PLN (Badrilla, #A010-12), phosphothreonine 17 PLN (Badrilla, #A010-13), RyR (Thermo Scientific, #MA3-916), RyR phosphoserine 2808 (RyR p2808) and phosphoserine 2814 (RyR p2814) (Badrilla, NCX (Swant, #R3F1), CaMKIIß (Abcam, #181052), phosphothreonine 287 CaMKII βThr (Abcam, #182647), monoclonal anti-cMyBP-C antibody to recognize total protein (Santa Cruz Biotechnology, #sc-137237), and anti-cMyBPc antibodies, as reported previously (50). Anti-calsequestrin (CSQ) antibody (Thermo Scientific, catalog no. PA1-913) was used as a control for SR proteins, and anti-glyceraldehyde-3-phosphate dehydrogenase (Gapdh) antibody was used as a control (Fitzgerald, catalog no. 10R-G109a). LI-COR fluorescent mouse- or rabbit-specific secondary antibodies (1:10,000, both from LI-COR Biosciences) diluted in Odyssey TBS Blocking Buffer were used and incubated for 1 h at room temperature. Immunoblot membranes were washed with TBS and imaged using the Odyssey FC imaging system (LI-COR Biosciences). Phosphorylation and total expression of PLN, cMyBP-C, and cTnI proteins were detected by dual-color Western blotting using two IR fluorophores. All protein levels were analyzed and quantified using Image Studio version 3.1 (LI-COR Biosciences). Phosphorylated PLN was expressed as a ratio of pSer-16/PLN or pThr-17/PLN, phosphorylated CaMKII was expressed as p287/CaMKII, phosphorylated RyR was expressed as RyR p2808/RyR or RyR p2814/RyR, phosphorylated cTnI was expressed as pSer22-23/cTnI, and phosphorylated cMyBP

| Table 6 | Two-way ANOVA |
| Figure | Analysis | F(DFn, DFd) | p |
| A | Factor A: –ISO versus +ISO | F(1, 303) = 91.91 | <0.0001 |
| A | Factor B: Transgenic groups | F(2, 303) = 10.72 | <0.0001 |
| A | A*B: Interaction | F(2, 303) = 5.888 | 0.0031 |
| B | Factor A: –ISO versus +ISO | F(1, 303) = 51.72 | <0.0001 |
| B | Factor B: Transgenic groups | F(2, 303) = 6.330 | 0.0020 |
| B | A*B: Interaction | F(2, 303) = 1.363 | 0.2573 |
| C | Factor A: –ISO versus +ISO | F(1, 303) = 83.52 | <0.0001 |
| C | Factor B: Transgenic groups | F(2, 303) = 12.92 | <0.0001 |
| C | A*B: Interaction | F(2, 303) = 2.874 | 0.0580 |
| D | Factor A: –ISO versus +ISO | F(1, 303) = 56.55 | <0.0001 |
| D | Factor B: Transgenic groups | F(2, 303) = 18.24 | <0.0001 |
| D | A*B: Interaction | F(2, 303) = 5.735 | 0.0036 |
| E | Factor A: –ISO versus +ISO | F(1, 303) = 53.72 | <0.0001 |
| E | Factor B: Transgenic groups | F(2, 303) = 13.91 | <0.0001 |
| E | A*B: Interaction | F(2, 303) = 0.5153 | 0.5979 |
| F | Factor A: –ISO versus +ISO | F(1, 277) = 101.8 | <0.0001 |
| F | Factor B: Transgenic groups | F(2, 277) = 25.10 | <0.0001 |
| F | A*B: Interaction | F(2, 277) = 3.956 | 0.0287 |
| G | Factor A: –ISO versus +ISO | F(1, 277) = 1.415 | 0.2352 |
| G | Factor B: Transgenic groups | F(2, 277) = 7.831 | 0.0005 |
| G | A*B: Interaction | F(2, 277) = 17.41 | <0.0001 |
| H | Factor A: –ISO versus +ISO | F(1, 160) = 96.00 | <0.0001 |
| H | Factor B: Transgenic groups | F(2, 160) = 103.1 | <0.0001 |
| H | A*B: Interaction | F(14, 160) = 16.04 | <0.0001 |
| I | Factor A: –ISO versus +ISO | F(3, 96) = 414.1 | <0.0001 |
| I | Factor B: Transgenic groups | F(2, 96) = 224.3 | <0.0001 |
| I | A*B: Interaction | F(9, 96) = 78.45 | <0.0001 |
| J | Factor A: –OM versus +OM | F(2, 217) = 78.39 | <0.0001 |
| J | Factor B: Transgenic groups | F(2, 217) = 4.790 | 0.0092 |
| J | A*B: Interaction | F(2, 217) = 8.896 | 0.0002 |
| K | Factor A: –OM versus +OM | F(1, 18) = 90.32 | <0.0001 |
| K | Factor B: Transgenic groups | F(2, 18) = 267.0 | <0.0001 |
| K | A*B: Interaction | F(2, 18) = 19.25 | <0.0001 |
| L | Factor A: –OM versus +OM | F(2, 218) = 0.09143 | 0.7627 |
| L | Factor B: Transgenic groups | F(2, 218) = 17.56 | 0.0001 |
| L | A*B: Interaction | F(2, 218) = 0.3358 | 0.7151 |
| M | Factor A: –OM versus +OM | F(1, 215) = 0.007893 | 0.9293 |
| M | Factor B: Transgenic groups | F(2, 215) = 12.97 | <0.0001 |
| M | A*B: Interaction | F(2, 215) = 0.1882 | 0.8286 |
| N | Factor A: 6 weeks versus 12 weeks | F(9, 126) = 11.46 | <0.0001 |
| N | Factor B: Transgenic groups | F(2, 126) = 72.50 | <0.0001 |
| N | A*B: Interaction | F(18, 126) = 12.56 | <0.0001 |
cMyBP-C dephosphorylation results in cardiac arrhythmia

Table 7
One-way ANOVA

| Figure | F (DFn, DFd) | p       |
|--------|-------------|---------|
| Fig. 1D | F(2, 15) = 533.6 | <0.0001 |
| Fig. 3A | F(2, 118) = 1.676 | 0.1916  |
| Fig. 3B | F(2, 118) = 12.90  | <0.0001 |
| Fig. 3C | F(2, 117) = 2.758  | 0.0676  |
| Fig. 3D | F(2, 115) = 3.995  | 0.0210  |
| Fig. 7A | F(2, 117) = 7.248  | 0.0011  |
| Fig. 7B | F(2, 9) = 0.4931   | 0.6263  |
| Fig. 7C | F(2, 119) = 0.1549 | 0.8567  |
| Fig. 7D | F(2, 119) = 0.2931 | 0.7465  |

was expressed as pSer282/cMyBP-C. For each protein in NTG controls, arbitrary values were converted to 100%, and values from the other group of protein samples were normalized as a percentage of NTG controls.

In vivo stress-induced cardiac arrhythmias

AAA, DDD, and NTG mice were anesthetized using 2.5% Avertin, while maintaining body temperature using a heating pad. As described previously (76), we performed an equivalent of lead I ECG recording using PowerLab (AD Instruments). We first recorded a control ECG for 5 min. We then injected caffeine (120 mg/kg body weight; Sigma) and epinephrine (2 mg/kg body weight; Sigma) intraperitoneally and recorded an ECG for 10–20 min. ECG tracings were analyzed for stress-induced arrhythmias.

Statistical analysis

Data were expressed as mean ± S.E. and were obtained using Prism 8.0. For isolated cardiomyocyte contractility studies, 10–12 cardiomyocytes were analyzed for each heart, with each heart counting as a single n (n = 6 hearts). For multiple-group comparisons, one-way ANOVA was performed, followed by Tukey’s multiple-comparison test with single pooled variance. All of the protein levels were quantified with LI-COR Image Studio. The densitometric values from each protein were normalized with the loading control. The phosphorylation levels were further normalized with their specific total proteins. The NTG control values were arbitrarily converted to 100%, and the other group’s values were normalized accordingly and expressed as a percentage of NTG. For SR proteins, CSQ was expressed as a percentage of NTG controls. For CSQ, arbitrary values were converted to 100%, and values from the other group of protein samples were normalized as a percentage of NTG.

Data availability

All relevant data are contained within the article.

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Conflict of interest—S. S. provides consulting and collaborative research studies to the Leducq Foundation (CURE-PLAN), Red Saree Inc., Greater Cincinnati Tamil Sangam, AstraZeneca, Myo-Kardia, Merck, and Amgen, but such work is unrelated to the content of this article.

Abbreviations—The abbreviations used are: HF, heart failure; AAA, cardiac-specific transgenic mice, expressing cMyBP-C with phospho-ablation at Ser-273, Ser-282, and Ser-302 by mutation to Ala; DDD, cardiac-specific transgenic mice, expressing cMyBP-C with phospho-mimetic at Ser-273, Ser-282, and Ser-302 by mutation to Asp; ECG, electrocardiogram; NTG nontransgenic WT mouse; cMyBP-C, cardiac myosin binding protein-C; cTnC, cardiac troponin C; cTnl, cardiac troponin I; CaMKII, calmodulin-dependent kinase II; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HCM, hypertrophic cardiomyopathy; ISO, isoproterenol; NCX, sodium–calcium exchanger; NSVT, nonsustained ventricular tachycardia; OM, omecamtiv mecarbil; PLN, phospholamban; PVC, premature ventricular complex; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SERCA2a, sarco/endoplasmic reticulum Ca2+-ATPase; ANOVA, analysis of variance; CSQ calsequestrin.
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