Calcium-Handling Abnormalities Underlying Atrial Arrhythmogenesis and Contractile Dysfunction in Dogs With Congestive Heart Failure

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Background—Congestive heart failure (CHF) is a common cause of atrial fibrillation. Focal sources of unknown mechanism have been described in CHF-related atrial fibrillation. The authors hypothesized that abnormal calcium (Ca$^{2+}$) handling contributes to the CHF-related atrial arrhythmogenic substrate.

Methods and Results—CHF was induced in dogs by ventricular tachypacing (240 bpm ×2 weeks). Cellular Ca$^{2+}$-handling properties and expression/phosphorylation status of key Ca$^{2+}$ handling and myofilament proteins were assessed in control and CHF atria. CHF decreased cell shortening but increased left atrial diastolic intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), transient amplitude, and sarcoplasmic reticulum (SR) Ca$^{2+}$ load (caffeine-induced [Ca$^{2+}$], release). SR Ca$^{2+}$ overload was associated with spontaneous Ca$^{2+}$ transient events and triggered ectopic activity, which was suppressed by the inhibition of SR Ca$^{2+}$ release (ryanodine) or Na$^{+}$/Ca$^{2+}$ exchange. Mechanisms underlying abnormal SR Ca$^{2+}$ handling were then studied. CHF increased atrial action potential duration and action potential voltage clamp showed that CHF-like action potentials enhance Ca$^{2+}$ loading. CHF increased calmodulin-dependent protein kinase II phosphorylation of phospholamban by 120%, potentially enhancing SR Ca$^{2+}$ uptake by reducing phospholamban inhibition of SR Ca$^{2+}$ ATPase, but it did not affect phosphorylation of SR Ca$^{2+}$-release channels (RyR2). Total RyR2 and calsequestrin (main SR Ca$^{2+}$-binding protein) expression were significantly reduced, by 65% and 15%, potentially contributing to SR dysfunction. CHF decreased expression of total and protein kinase A–phosphorylated myosin-binding protein C (a key contractile filament regulator) by 27% and 74%, potentially accounting for decreased contractility despite increased Ca$^{2+}$ transients. Complex phosphorylation changes were explained by enhanced calmodulin-dependent protein kinase IIδ expression and function and type-1 protein-phosphatase activity but downregulated regulatory protein kinase A subunits.

Conclusions—CHF causes profound changes in Ca$^{2+}$-handling and -regulatory proteins that produce atrial fibrillation–promoting atrial cardiomyocyte Ca$^{2+}$-handling abnormalities, arrhythmogenic triggered activity, and contractile dysfunction. (Circ Arrhythmia Electrophysiol. 2008;1:93-102.)

Key Words: atrial fibrillation • congestive heart failure • delayed afterdepolarization • calcium • sarcoplasmic reticulum

Congestive heart failure (CHF) is a common cause of atrial fibrillation (AF). Both reentrant and triggered mechanisms have been implicated in AF. Although CHF induces a substrate for atrial reentry, there is also evidence for a role of focal drivers and triggered activity in CHF-related AF. Dogs with atrial tachycardia remodeling have calcium (Ca$^{2+}$)-handling abnormalities, reduced Ca$^{2+}$ transients, and cardiac ryanodine receptor (RyR2) dysfunction. Ca$^{2+}$-handling abnormalities may play a role in CHF-related AF, but there are no published descriptions of atrial Ca$^{2+}$ handling in patients or clinically relevant animal models with CHF-associated AF substrates. Accordingly, the present study was designed to evaluate atrial cardiomyocyte Ca$^{2+}$ handling, and related protein expression and phosphorylation, in a canine CHF model.

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Methods

A detailed description of materials and methods used in the study is available in the online Data Supplement.

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Animal Model
The animal model was prepared as previously described.9 Forty adult mongrel dogs (22 to 36 kg) were divided into 2 groups: (1) control (n=20) and (2) 2-week ventricular tachypacing–induced CHF (n=20). CHF dogs had unipolar pacing leads inserted fluoroscopically into the right ventricular apex, which were programmed at 240 bpm for 2 weeks.

On the days of study, dogs were anesthetized with morphine (2 mg/kg SC) and α-chloralose (120 mg/kg IV, followed by 29.25 mg/kg per hour) and ventilated mechanically. AF (irregular atrial rhythm >400 bpm) was induced by burst pacing. Mean AF duration was determined on the basis of multiple AF inductions in each dog, as an index of the AF-maintaining substrate (for details see the Methods section in the online Data Supplement). AF duration (mean±SEM) was then calculated for each experimental group as an indicator of the ability of each group to sustain AF. Hemodynamic data were obtained with fluid-filled catheters and transducers.

Cardiomyocyte Isolation
Right atrial (RA) and left atrial (LA) preparations were dissected and coronary perfused at ~10 mL/min for cardiomyocyte isolation as previously described.9 RA and LA cells were stored separately in Tyrode solution with 200 μmol/L Ca2+.

Measurement of Cell Contraction and Ca2+ Fluorescence
Cell-shortening measurements, based on the average of 10 consecutive beats, were obtained from field-stimulated cardiomyocytes with a video edge detector coupled to a charged-coupled device camera. Edge-detection cursors were positioned at both cell ends to measure whole-cell shortening. [Ca2+]i transients were recorded with microfluorimetry.10 Cardiomyocytes were incubated with indo-1 AM (5 μmol/L) for 4 to 5 minutes. The cells were then superfused with Tyrode solution for 10 minutes to allow intracellular de-esterification. Cardiomyocytes were excited with ultraviolet light (340 nm), and emission ratios (R90/900) were measured through a 10-μm aperture focused at the cardiomyocyte center. Ratiometric Ca2+ measurements were converted into intracellular Ca2+ concentration ([Ca2+]i) with the formula [Ca2+]i = Kd×β×(R900/R90)/(R900–R90).10 Experimentally determined R900, R90, and β averaged 0.43, 2.34, and 1.79, respectively. Sarcoplasmic reticulum (SR) Ca2+ content was evaluated with 15-second 10-mmol/L caffeine applications via a rapid-switching perfusion system.

For frequency-response measurements, a minimum of 4 Ca2+ transients (at 0.1 Hz) or a maximum of 20 Ca2+ transients (>1.0 Hz) were time-averaged. The decay time constant (τ) was based on a monoexponential fit to the [Ca2+]i decay curve.

Cellular Electrophysiology
LA cells were used for arrhythmogenic action potential (AP) studies and RA tissue for biochemistry. APs were recorded with whole-cell perforated-patch methods. Borosilicate glass electrodes (1.0 mm outer diameter) had tip resistances between 3 and 5 MΩ. Pipette tips were filled with nystatin-containing (60 μg/mL) intracellular solution. Junction potentials averaged 15.9 mV and were corrected for APs. For solution contents, see the online Data Supplement Methods section. All recordings were obtained at 35±0.5°C.

The AP voltage-clamp (whole-cell perforated patch) technique was used to study AP-dependent effects on [Ca2+]i, transients. [Ca2+]i transients were recorded from LA cardiomyocytes subjected to typical AP waveforms from control and CHF cardiomyocytes at 2 Hz for sequential 2-minute periods (in randomized order).

Western Blot and Phosphatase Activity Measurements
RA tissue homogenates were prepared and protein concentrations determined with Amido-black 10B.11 Proteins were fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Western blotting was performed with primary antibodies as previously described.12 A detailed list of antibodies and sources is presented in the online Data Supplement Methods section. Protein bands were visualized by electrochemoluminescence.

Phosphatase activity was measured in atrial homogenates.13 Oka-daic acid (3 nmol/L) was used to differentiate between PP1 and PP2A activities.13

Table. Hemodynamic Data

<table>
<thead>
<tr>
<th></th>
<th>Control (n=20)</th>
<th>CHF (n=20)</th>
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<tbody>
<tr>
<td>Systolic BP, mm Hg</td>
<td>132±5</td>
<td>114±3*</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>82±4</td>
<td>67±2*</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>131±5</td>
<td>113±4*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>4.9±0.5</td>
<td>15.3±1.0†</td>
</tr>
<tr>
<td>LAP, mm Hg</td>
<td>4.0±0.6</td>
<td>15.4±0.9†</td>
</tr>
<tr>
<td>RAP, mm Hg</td>
<td>2.7±0.5</td>
<td>9.6±0.4†</td>
</tr>
<tr>
<td>DAF, s</td>
<td>26±8</td>
<td>1564±106*</td>
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</tbody>
</table>

*P<0.01, †P<0.001 vs control.
BP indicates blood pressure; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; LAP and RAP, LA and RA mean pressure; and DAF, duration of AF.

Data Analysis
Group data are presented as mean±SEM. Repeated-measures analyses were performed with 2-way analysis of variance (ANOVA), followed by Bonferroni-corrected t tests for statistically significant ANOVAs. Nonpaired t tests were applied for single 2-group comparisons and paired t tests for single repeated measures within one group. Contingency analyses were measured by χ2 test. Two-tailed P<0.05 was considered statistically significant. The authors had full access to the data and take responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

Results
Hemodynamics and AF Duration
Hemodynamic indices and in vivo electrophysiology data are shown in the Table. Arterial pressures were significantly reduced in CHF dogs, whereas left ventricle end-diastolic and LA and RA pressures were increased. CHF significantly prolonged AF duration.

APs, Cell Shortening, and Ca2+ Transients
CHF cardiomyocytes were enlarged, with a mean capacitance of 120±6 pF, versus 91±3 pF for control (n=25/group, P<0.01). APs obtained by averaging all available recordings from each group at 1 Hz are shown in Figure 1A, with mean action potential duration (APD) data provided in the inset. APDs were significantly prolonged by CHF in both LA and RA over a wide range of frequencies (online Data Supplement Figure I). Figure 1B and 1C show recordings of steady-state cell shortening and Ca2+ transients. Cell shortening was significantly decreased in CHF cells (Figure 1D), whereas Ca2+ transients were larger.

Figure 2 shows detailed analyses of LA and RA [Ca2+]i transients. CHF increased diastolic [Ca2+]i, and [Ca2+]i transients at all frequencies from 0.1 to 2 Hz (Figure 2A through 2D). The time to peak and decay time constants of [Ca2+]i,
transient were similar for CHF and control (online Data Supplement Figure IIA through IIB). APD prolongation due to CHF could contribute to \( \text{Ca}^{2+} \) loading, so we performed AP-clamp experiments, imposing the control and CHF waveforms at 2 Hz in control atrial myocytes. \([\text{Ca}^{2+}]_i\), transients obtained in 1 cell with either AP waveform are shown in Figure 1E (left). CHF APs induced a statistically significant increase (\( \approx 18\% \)) in \([\text{Ca}^{2+}]_i\), transient amplitudes (Figure 1E, right).

**SR \( \text{Ca}^{2+} \) Content**

To assess changes in SR \( \text{Ca}^{2+} \) content, we paced cells for 1 minute at 1 Hz and then rapidly applied 10 mmol/L caffeine. Caffeine-induced \( \text{Ca}^{2+} \) transients are illustrated in Figure 2E (LA) and 2F (RA). CHF increased caffeine-induced \( \text{Ca}^{2+} \) transients by \( \approx 85\% \) in LA and \( \approx 50\% \) in RA (Figure 2E and 2F insets). In ventricular cardiomyocytes, \([\text{Ca}^{2+}]_i\), decay depends mainly on \( \text{Ca}^{2+} \) extrusion by NCX.\(^{14}\) The decay time constant of the caffeine-induced transient was comparable for CHF and control in LA and RA (online Data Supplement Figure IIC and IID).

**Spontaneous \( \text{Ca}^{2+} \) Transient Events, Delayed Afterdepolarizations, Triggered APs, and Pharmacological Effects**

The results in Figures 1 and 2 show larger SR \( \text{Ca}^{2+} \) loads and \( \text{Ca}^{2+} \) transients in CHF atria versus control, with LA behaving similarly to RA. We therefore determined whether CHF LA

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**Figure 1.** A, AP waveforms obtained by digitally averaging all AP recordings from LA cardiomyocytes at 1 Hz from control (CTL, left) and CHF (right) dogs. Inset: Mean±SEM results at 1 Hz (n=16, n=37 for CTL and CHF, respectively; \( ***P<0.001 \)). B and C, Recordings of cell shortening (B) and \( \text{Ca}^{2+} \) transients (C) from single control (CTL) and CHF cardiomyocytes. D, Mean±SEM cell shortening as a function of pacing frequency (n=15 and 12 for control and CHF, respectively; \( *P<0.001 \), effect of group: CHF vs control). E, AP-clamp results; left, \( \text{Ca}^{2+} \) transient recordings obtained with CHF and control waveforms in a control LA cell; right, corresponding mean±SEM. \( \text{Ca}^{2+} \) transient amplitudes (n=6 cells, \( **P<0.01 \)).

**Figure 2.** Mean±SEM, \( \text{Ca}^{2+} \) transient indices. A and B, \([\text{Ca}^{2+}]_i\), diastolic levels in left (A) and in right atrial (B) cells. C and D, \( \text{Ca}^{2+} \) transient amplitude in left (C) and right atrial (D) (n=20 to 24 cells/group; \( P<0.05, **P<0.01, ***P<0.001 \) CHF vs control). E and F, Caffeine-induced \( \text{Ca}^{2+} \) transients in control and CHF left (E) and right atrial (F) cardiomyocytes. A 10-mmol/L local caffeine concentration was achieved in <500 ms with a laminar-flow rapid solution-switching system, producing \( \text{Ca}^{2+} \) transients, which are indicated by arrows. Insets: Mean±SEM caffeine-induced \( \text{Ca}^{2+} \) transient amplitudes of LA (E) and RA (F) (n=12/group; \( **P<0.01, ***P<0.001 \)).
cardiomyocytes are predisposed to abnormal Ca$^{2+}$ release–associated arrhythmic events. We first recorded spontaneous Ca$^{2+}$ transients after a 30-second period of pacing at 2 Hz. The cessation of cell stimulation was followed by occasional nonstimulated Ca$^{2+}$ transients in control cells (Figure 3A, top). In CHF (bottom), many more spontaneous Ca$^{2+}$ transients were seen. Nonstimulated Ca$^{2+}$ transients were ≈5-fold more frequent in CHF cells (Figure 3B). Figure 3C shows whole-cell perforated-patch AP recordings immediately after 1-minute stimulation at 2 Hz. In control cells, triggered APs were rare, whereas triggered activity was greatly increased in CHF cells (Figure 3D). Triggered activity was accompanied by prominent diastolic membrane oscillations that sometimes appeared as delayed afterdepolarizations and at other times transitioned smoothly into triggered APs, suggesting abnormal automaticity.

To elucidate the basis of diastolic membrane-potential oscillations and triggered APs occurring after the Ca$^{2+}$ loading by rapid pacing, we studied the response to pharmacological manipulations. Figure 4A (top) shows spontaneous AP generation and diastolic oscillatory activity after 2-Hz pacing in a CHF cardiomyocyte. Ryanodine, an SR Ca$^{2+}$ release channel inhibitor, induced quiescence in this and 5 other similar CHF cells. The Na$^{+}$/Ca$^{2+}$-exchanger (NCX) suppression with Na$^{+}$- and Ca$^{2+}$-free medium produced similar responses (Figure 4B). No change in spontaneous activity was seen on $I_f$ inhibition with 2 mmol/L Cs$^+$ (Figure 4C). Some CHF atrial cardiomyocytes presented spontaneous activity and APs without pacing-induced Ca$^{2+}$ loading (online Data Supplement Figure III). These spontaneous events were completely eliminated by 10 μmol/L ryanodine (n=4 cells), whereas CsCl had no effect (n=4 cells). These results indicate that CHF-induced diastolic membrane potential instability leads to triggered activity via mechanisms involving SR Ca$^{2+}$ release through ryanodine receptors and associated arrhythmogenic Na$^+$– and Ca$^{2+}$-exchange currents.

**Ca$^{2+}$ Handling and Myofilament Proteins**

To gain insights into potential abnormalities of Ca$^{2+}$ handling and contractile protein systems in CHF atria, we performed Western blots with antibodies that recognize total and phosphorylated forms. Protein-band intensities were normalized to those of GAPDH on the same lanes (GAPDH intensities did not differ between CHF and control atria). The expression of total phospholamban was similar in CHF and control atria (Figure 5A). Phospholamban phosphorylation by protein kinase A (PKA) at Ser16 (Ser16-P) and by CaMKII at Thr17 (Thr17-P) functionally enhances SERCA2a Ca$^{2+}$ uptake. Ser16-P-phospholamban expression was unchanged in CHF, but Thr17-P-phospholamban increased by ≈85%. Fractional phospholamban PKA phosphorylation (Ser16-P-phospholamban–to–total phospholamban ratio) was unchanged in CHF,
whereas fractional CaMKII phospholamban phosphorylation increased by ～120%. CHF decreased SERCA2a protein expression (by ～35%).

CHF resulted in a 65% decrease in total RyR2 protein expression (Figure 5B) and a 73% decrease in PKA-phosphorylated RyR2 (Ser2809-P). Fractional RyR2 phosphorylation (ratios of Ser2809-P/RyR2 and Ser2815-P/RyR2 to total RyR2) was not significantly altered by CHF. Calsequestrin, a major SR Ca\textsuperscript{2+} buffer and regulator of RyR2 function,\textsuperscript{15} was reduced ～15% in CHF. No significant changes were noted for NCX1.

Expression values for thin myofilament proteins troponin (Tn)-I and Tn-C are shown in Figure 6A. Total Tn-I expression, PKA-phosphorylated (Ser23/24)–to–total Tn-I ratio, and total Tn-C were unchanged by CHF. However, total thick-myofilament myosin-binding protein C (MyBP-C) and PKA-phosphorylated MyBP-C (Ser282-P) were significantly decreased by ～27% and ～74%, respectively (Figure 6B). The Ser282-P MyBP-C/total MyBP-C ratio was also reduced by 67% in CHF, and phosphorylated myosin light chain-2a protein (MLC2a) was decreased by 46%.

The data in Figures 5 and 6 show potentially important CHF-induced changes in phosphorylation, a critical regulator of protein function,\textsuperscript{8,12,13} for a variety of Ca\textsuperscript{2+} handling and contractile proteins. To assess the underlying mechanisms, we analyzed the expression of key protein kinases and protein phosphatases. CHF increased expression and autophosphorylation of the cytosolic CaMKII isoform by 123% and 114%, respectively (Figure 7A). PKA expression was unchanged for PKAc, whereas PKAc (PKA catalytic subunit) and PKA\textsubscript{c} (PKA regulatory subunit) was decreased by 72% (Figure 7B). Because RyR2 and MyBP-C are dephosphorylated primarily by protein phosphatase (PP)1, their reduced PKA phosphorylation may be caused by the increased PP1 activity. Consistent with this notion, total protein-phosphatase activity was 34% higher in CHF, PP1 activity was increased by 83%, and PP2A activity was unchanged (Figure 7C). Protein expression of PP1 and PP2A catalytic subunits was unchanged in CHF (Figure 7D).

**Discussion**

**Main Findings**

We have shown that atrial Ca\textsuperscript{2+} handling is significantly disturbed in dogs with experimental CHF. Ca\textsuperscript{2+} overload was manifested as increased diastolic Ca\textsuperscript{2+} concentrations, Ca\textsuperscript{2+}...
transient amplitudes, and caffeine-releasable SR Ca$^{2+}$. These alterations resulted in a predisposition to spontaneous Ca$^{2+}$ release, abnormal diastolic membrane potential oscillations, and triggered activity. Despite larger Ca$^{2+}$ transients, CHF cells displayed reduced contractility. These findings were accompanied by altered expression and phosphorylation of key Ca$^{2+}$ handling, myofilament, and contractile proteins, as well as changes in crucially important regulatory kinases and phosphatases. CHF increased atrial APD and enhanced CaMKII phospholamban phosphorylation, both of which likely contributed to the Ca$^{2+}$-loaded state.

**Significance for Clinically Relevant Mechanisms**

AF is very commonly associated with CHF.$^{16}$ CHF-induced atrial fibrosis impairs intra-atrial conduction and favors AF by promoting atrial reentry.$^{2}$ However, focal atrial tachyarrhythmias may also be important in CHF-related AF. Boyden et al.$^{8}$ showed atrial triggered activity due to delayed afterdepolarizations in cardiomyopathic cats. Epicardial mapping suggests focal drivers during AF in CHF dogs.$^{3,5,17}$ and focal-driver ablation can terminate atrial tachyarrhythmias.$^{3}$ In vivo pharmacological responses consistent with atrial Ca$^{2+}$-dependent triggered activity have been noted in animals with CHF.$^{4}$ Although atrial fibrosis favors atrial reentry, reentrant activity is rarely triggered by single atrial extrasystoles, requiring bursts of rapid atrial activation for induction,$^{2}$ a function that could be fulfilled by Ca$^{2+}$-dependent ectopic firing. Triggered activity could thus contribute to AF in 2 ways: (1) by producing atrial tachycardia bursts that trigger reentrant AF in vulnerable substrates and (2) by providing focal drivers that maintain AF.

Our studies have revealed potential mechanisms underlying the Ca$^{2+}$-handling abnormalities that cause atrial-triggered activity in CHF (Figure 8). Changes that we identified in these studies are color coded, with red representing increases, and blue, decreases. The central abnormality

![Figure 6](image-url)  
**Figure 6.** A, Top: Examples of Tn-I, Ser23/24-P-Tn-I, and Tn-C, along with GAPDH bands on the same lanes. Bottom: Mean±SEM protein-band intensities of total Tn-I, Ser23/24-P-Tn-I, and Tn-C normalized to GAPDH, expressed relative to control (n=10 control and 8 CHF atria/analysis). B, Top: Examples of total MyBP-C, Ser262-P-MyBP-C, and phosphorylated MLC2a bands, along with GAPDH on the same lanes. Bottom: Mean±SEM protein-band intensities normalized to GAPDH, expressed relative to control (n=14 control, 10 CHF atria/analysis; *P<0.05 vs control).

![Figure 7](image-url)  
**Figure 7.** A, Top: Examples of total CaMKIIα, Thr287-CaMKII (autophosphorylated), and GAPDH immunoblots. Top bands (58 kDa) represent CaMKIIα and bottom (56 kDa) CaMKIIβ. Bottom: Mean±SEM protein-band intensities normalized to GAPDH, expressed relative to control (n=16 control and 8 CHF atria/analysis; *P<0.05 vs control). B, Top: Examples of PKAα, PKAβ, and GAPDH immunoblots. The PKAα antibody recognized bands at 51 and 54 kDa. Quantification is based on the sum of the bands. Bottom: Mean±SEM protein-band intensities normalized to GAPDH, expressed relative to control (n=10 control and 8 CHF atria/analysis; *P<0.05 vs control). C, D, Serine/threonine protein-phosphatase (PP) activity and corresponding protein expression in control and CHF atria. C, PP activity assessed with phosphorylase-A as substrate, quantified as nanomoles of $^{32}$Pi released per milligram protein per minute (n=10 for control and 11 CHF atria/analysis). D, Representative examples and mean±SEM protein-band intensities of PP1 and PP2A normalized to GAPDH, expressed relative to control (n=14 control and 10 CHF atria/analysis; *P<0.05 vs control).
that we observed is SR Ca\(^{2+}\) overload, likely resulting from 2 primary mechanisms: (1) increased transmembrane Ca\(^{2+}\) entry through \(I_{Ca}\), during the prolonged CHF-induced AP waveform and (2) increased SERCA2a-mediated SR Ca\(^{2+}\) uptake due to the reduced phospholamban inhibition of SERCA2a caused by CaMKII phospholamban hyperphosphorylation. This effect of phospholamban hyperphosphorylation presumably overcomes the CHF downregulation of SERCA2a expression to produce a net increase in SERCA-mediated SR Ca\(^{2+}\) uptake. In addition, decreased ryanodine receptor expression can promote increased SR Ca\(^{2+}\) content by reducing SR Ca\(^{2+}\) release.18 SR Ca\(^{2+}\) overload increases systolic Ca\(^{2+}\) release and also results in spontaneous diastolic Ca\(^{2+}\)-release events. The NCX responds to diastolic [Ca\(^{2+}\)\(_{SR}\)] by exchanging Ca\(^{2+}\) for Na\(^{+}\) in a 1:3 ratio, producing depolarizing inward currents manifesting as delayed afterdepolarizations and abnormal automaticity that cause triggered activity. Triggered activity is a strong candidate to underlie atrial ectopic foci that can initiate or maintain AF.

In addition to the increased SR Ca\(^{2+}\) loading, we found a significant reduction in calsequestrin expression. Calsequestrin function–impairing mutations are associated with catecholaminergic polymorphic ventricular tachycardia and increased Ca\(^{2+}\)-spark and -wave generation.19–21 Calsequestrin knockout mice show spontaneous Ca\(^{2+}\)-release events that are enhanced by isoproterenol.15 Even moderate downregulation of calsequestrin can increase SR Ca\(^{2+}\) leak and arrhythmia susceptibility.22 Therefore, the reduced calsequestrin expression that we observed may contribute to spontaneous SR Ca\(^{2+}\)-release events independently of absolute Ca\(^{2+}\) levels.23

In recent years, there has been a concerted effort to develop novel, mechanistically based approaches to treating AF that avoid the risks of traditional ion channel targets. The identification of Ca\(^{2+}\)-handling abnormalities as an important participant in CHF-related AF opens up interesting possibilities for novel small molecule – and gene transfer–based therapeutics.24,25

Impaired atrial contractility predisposes to atrial thromboembolism, one of the most significant complications of AF. Our study is the first to analyze atrial myocyte contractility changes and related mechanisms in the CHF setting. Prominent decreases in atrial cardiomyocyte contractility despite enlarged systolic Ca\(^{2+}\) transients imply atrial contractile protein dysfunction. We identified dramatically reduced PKA-phosphorylation of MyBP-C at Ser-282 as a potential underlying factor. MyBP-C PKA-phosphorylation is important for maximum force development, stretch-dependent augmentation of force generation, normal cross-bridge cycling kinetics, and diastolic relaxation.26–29 Reduced phosphorylation of MLC2a, another important regulator of myofilament function,30 may also contribute to contractile dysfunction. Anticoagulant therapy has been the traditional mainstay of AF-related thromboembolism prophylaxis but is complex to maintain and may cause bleeding complications. A better recognition of the molecular basis of atrial hypocontractility could lead to new and potentially safer approaches to combating thromboembolic risk.

**Comparison With Previous Studies of Ca\(^{2+}\) Handling in Failing Ventricles and AF**

Early studies of Ca\(^{2+}\) handling in ventricular cardiomyocytes from humans31 and rats32 with CHF showed increased diastolic [Ca\(^{2+}\)], reduced Ca\(^{2+}\) transients, and slowed Ca\(^{2+}\) transient decay. Subsequent work consistently showed reduced and slowed Ca\(^{2+}\) transients in failing ventricular cardiomyocytes, with reduced SR Ca\(^{2+}\) stores and abnormal Ca\(^{2+}\) uptake and release mechanisms.14,33–35 The molecular basis of these ventricular Ca\(^{2+}\)-handling abnormalities has been studied extensively.33 Decreased SERCA2a expression tends to reduce SR Ca\(^{2+}\) stores and slow Ca\(^{2+}\) transient decay.34–36 NCX upregulation attenuates diastolic Ca\(^{2+}\) accumulations for novel small molecule – and gene transfer–based therapeutics.24,25

Impaired atrial contractility predisposes to atrial thromboembolism, one of the most significant complications of AF. Our study is the first to analyze atrial myocyte contractility changes and related mechanisms in the CHF setting. Prominent decreases in atrial cardiomyocyte contractility despite enlarged systolic Ca\(^{2+}\) transients imply atrial contractile protein dysfunction. We identified dramatically reduced PKA-phosphorylation of MyBP-C at Ser-282 as a potential underlying factor. MyBP-C PKA-phosphorylation is important for maximum force development, stretch-dependent augmentation of force generation, normal cross-bridge cycling kinetics, and diastolic relaxation.26–29 Reduced phosphorylation of MLC2a, another important regulator of myofilament function,30 may also contribute to contractile dysfunction. Anticoagulant therapy has been the traditional mainstay of AF-related thromboembolism prophylaxis but is complex to maintain and may cause bleeding complications. A better recognition of the molecular basis of atrial hypocontractility could lead to new and potentially safer approaches to combating thromboembolic risk.
mulation due to the reduced SR Ca\(^{2+}\) uptake by SERCA2a and minimizes diastolic dysfunction,\(^{36}\) but it further decreases SR Ca\(^{2+}\) stores.\(^{33,34}\) Ca\(^{2+}\) release channel dysfunction caused by CaMKII (Ser-2815) and/or PKA (Ser-2809) RyR2 hyperphosphorylation contributes to SR Ca\(^{2+}\) depletion and arrhythmogenesis by causing diastolic Ca\(^{2+}\) leak.\(^{35,37,38}\)

The Ca\(^{2+}\)-handling abnormalities we identified in atrial cardiomyocytes from CHF dogs differ from previously reported CHF-induced ventricular changes.\(^{14,33-35,37}\) Instead of reduced Ca\(^{2+}\) transients and SR Ca\(^{2+}\) stores, atrial cells show increases in both. We were concerned by the discrepancies between our atrial Ca\(^{2+}\)-handling changes and previous reports in ventricular cardiomyocytes. We therefore subjected paired ventricular and atrial cardiomyocytes from 2 dogs to Ca\(^{2+}\) transient measurements. As shown in online Data Supplement Figure IV, CHF-induced ventricular Ca\(^{2+}\)-handling changes resembled those in previous reports and differed clearly from Ca\(^{2+}\)-handling changes in the atria.

Several studies have examined the functional abnormalities in Ca\(^{2+}\) handling of AF patients, generally without overt CHF. Quantal Ca\(^{2+}\)-release events and Ca\(^{2+}\) waves are more frequent in AF atria.\(^{39}\) PKA hyperphosphorylation of RyR2 promotes FKBP12.6 unbinding and Ca\(^{2+}\)-release channel opening.\(^{8}\) We observed no significant changes in fractional RyR phosphorylation. This discrepancy may relate to the specific pathophysiological properties of our model, which involved recent-onset CHF, as opposed to clinical series including patients with long-standing AF, a variety of underlying heart diseases, and various cardioactive medications.

Studies of atrial Ca\(^{2+}\)-handling protein biochemistry in AF patients have provided widely varying results, possibly because of variations in patient populations.\(^{40}\) Ca\(^{2+}\)-handling protein abnormalities in AF subjects reflect the effects of both underlying cardiac diseases and changes induced by the arrhythmia itself. Atrial tachycardia alters atrial cardiomyocyte Ca\(^{2+}\) handling, prominently decreasing Ca\(^{2+}\) transients.\(^{7,41}\) Mice with tumor necrosis factor-\(\alpha\) overexpression develop a cardiomyopathic phenotype with atrial fibrosis and atrial reentrant arrhythmias.\(^{42}\) They also display reduced Ca\(^{2+}\) transients and caffeine-releasable Ca\(^{2+}\) stores in contrast to the increased Ca\(^{2+}\) loading and Ca\(^{2+}\) release in our canine model. Further studies in well-defined animal models and clinical populations are needed to clarify the factors contributing to abnormal Ca\(^{2+}\) handling in AF.

**Potential Limitations**

Some of the changes we observed in protein expression and steady-state phosphorylation appear to contradict our functional observations. Decreased SERCA2a protein expression should reduce SR Ca\(^{2+}\) loading, yet we noted increased SR Ca\(^{2+}\) loads. Increased Ca\(^{2+}\) influx due to prolonged atrial APDs, decreased SR Ca\(^{2+}\) discharge due to reduced RyR2 expression, and SERCA function enhancement via phospholamban hyperphosphorylation may be sufficient to offset decreased SERCA expression and increase SR Ca\(^{2+}\) content. We observed statistically significant \(\approx15\)% decreases in the principal SR Ca\(^{2+}\)-binding protein calsequestrin, which would be expected to reduce SR Ca\(^{2+}\) storage. However, calsequestrin-knockout mice maintain normal SR Ca\(^{2+}\) stores, apparently by increasing SR volume.\(^{15}\) The decreased RyR2 expression that we observed might be expected to reduce [Ca\(^{2+}\)], transient amplitude by decreasing SR Ca\(^{2+}\) release. Previous studies have shown that decreased RyR2 function initially does decrease [Ca\(^{2+}\)], transients but results in SR Ca\(^{2+}\) accumulation that eventually restores Ca\(^{2+}\) release.\(^{18}\)

We observed complex changes in protein phosphorylation, the functional result of which is difficult to predict. Phosphorylating enzymes showed varying changes: unchanged or decreased expression of PKA subunits and unchanged or increased expression of CaMKII components. Dephosphorylating enzyme changes also varied, including unchanged PP2A and enhanced PP1 activity. Thus, for each Ca\(^{2+}\)-handling protein, alterations at specific phosphorylation sites will depend on the net changes in phosphorylating and dephosphorylating enzymes acting on that site. Previous studies have reported varying results with regard to phosphorylation-dependent regulation of RyR2 in CHF, with increased or decreased ventricular RyR2 Ser-2809 phosphorylation having been observed (for review, see George et al\(^{43}\)). Ser-2809 is 75% maximally phosphorylated at rest, producing a minimum basal activity level of RyR2 channels.\(^{44}\) Either increased or reduced RyR2 phosphorylation enhances RyR2 channel activity.\(^{44}\) We observed a decrease in total RyR2 phosphorylation but no significant changes in fractional RyR2 phosphorylation because decreased phosphorylated RyR2 expression paralleled decreased total RyR2 levels. Changes in expression and activity of ventricular kinases and phosphatases within local macromolecular complexes do not necessarily follow global changes in these enzymes,\(^{23,37}\) suggesting localized regulation of kinase or phosphatase activity in cellular microdomains. Thus, local changes in atrial macromolecular complexes containing RyR2, phospholamban, MyBP-C, and/or MLC2a may also have contributed to CHF-induced alterations.

We found similar Ca\(^{2+}\)-handling changes in LA and RA free-wall cardiomyocytes, but we cannot be sure that our results extend to other atrial sites. Having shown the similarity of LA and RA Ca\(^{2+}\)-handling effects of CHF, we optimized the efficiency of dog usage by studying cellular arrhythmias and their response in LA cells and analyzing biochemical changes in RA tissue. We did not perform detailed complementary studies to assess arrhythmogenic changes in RA cells and biochemistry in LA tissue. We estimate that such studies would have required us to euthanize at least 20 additional dogs (10 dogs per group), which we felt was not justified because of very similar Ca\(^{2+}\)-handling changes with CHF in LA versus RA. We did have frozen LA tissue samples from 6 additional dogs/group, which we subjected to biochemical analyses. The results, shown in Data Supplement Figures V through VIII, indicate that Ca\(^{2+}\)-handling protein changes in LA tissue were qualitatively quite similar to those in RA (which are reproduced beside the LA data for comparison).

**Conclusions**

Dogs with ventricular tachypacing–induced dilated cardiomyopathy display important abnormalities in atrial Ca\(^{2+}\) handling and in the expression, phosphorylation, and/or
activity of key atrial Ca\textsuperscript{2+}-handling, contractile, and regulatory proteins. They show signs of cellular Ca\textsuperscript{2+} overload, which results in a predisposition to spontaneous diastolic Ca\textsuperscript{2+} release, arrhythmogenic diastolic membrane potential oscillations, and triggered activity. These abnormalities in Ca\textsuperscript{2+} homeostasis likely account for focal atrial tachyarrhythmias that contribute to atrial arrhythmogenesis in CHF.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Congestive heart failure (CHF) is a common cause of atrial fibrillation (AF). The therapeutic tools available for AF management are suboptimal, and new insights into fundamental mechanisms underlying AF may permit the development of new treatment approaches. The basis of spontaneous atrial ectopic activity that can trigger or drive AF is poorly understood. This study focused on the potential role of Ca²⁺-handling abnormalities in CHF-related atrial ectopic activity. CHF was induced in dogs by ventricular tachypacing (240 bpm × 2 weeks). Cellular Ca²⁺-handling properties and expression/phosphorylation status of key Ca²⁺-handling and myofilament proteins were assessed. CHF increased atrial cell Ca²⁺ loading, leading to spontaneous sarcoplasmic reticulum Ca²⁺ release (especially under Ca²⁺ entry–enhancing conditions like tachycardia), which resulted in diastolic atrial membrane potential oscillation and ectopic firing. Ca²⁺ overload was due to multiple factors: (1) increased action potential duration, which enhanced Ca²⁺ entry during the action potential plateau; (2) increased phosphorylation of phospholamban by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), which reduces the phospholamban-dependent suppression of sarcoplasmic reticulum Ca²⁺ uptake through the sarcoplasmic reticulum Ca²⁺ transporter (SERCA); and (3) decreased ryanodine receptor expression, which reduces sarcoplasmic reticulum Ca²⁺ egress through Ca²⁺-release channels. Despite increased sarcoplasmic reticulum Ca²⁺ loading, atrial contractility was reduced in CHF, likely because of decreased phosphorylation of 2 important contraction-regulating proteins, myosin-binding protein C and myosin light chain-2a. Phosphorylation abnormalities in CHF were related to altered expression and/or activity of phosphorylation-enhancing (CaMKII expression/activity upregulated) and phosphorylation-suppressing (protein phosphatase-1 activity upregulated) proteins. Thus, CHF causes profound changes in phosphorylation and expression of key Ca²⁺-handling and myofilament regulatory proteins that cause atrial cardiomyocyte Ca²⁺ homeostasis and contractile abnormalities, which in turn contribute to AF-promoting triggered activity and thrombosis-promoting contractile dysfunction.
Calcium-Handling Abnormalities Underlying Atrial Arrhythmogenesis and Contractile Dysfunction in Dogs With Congestive Heart Failure

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