The Calcium/Calmodulin/Kinase System and Arrhythmogenic Afterdepolarizations in Bradycardia-Related Acquired Long-QT Syndrome

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Methods and Results—We measured ventricular cardiomyocyte [Ca\(^{2+}\)]\(_i\) (Indo1-AM), L-type Ca\(^{2+}\)-current (I\(_{\text{Cal}}\)) and APs (whole-cell perforated-patch), and Ca\(^{2+}\)-handling protein expression (immunoblot). CAVB increased AP duration, cell shortening, systolic [Ca\(^{2+}\)]\(_i\) transients, and caffeine-induced [Ca\(^{2+}\)]\(_i\) release, and CAVB cells showed spontaneous early afterdepolarizations (EADs). I\(_{\text{Cal}}\) density was unaffected by CAVB, but inactivation was shifted to more positive voltages, increasing the activation-inactivation overlap zone for I\(_{\text{Cal}}\) window current. Ca\(^{2+}\)-calmodulin–dependent protein kinase-II (CaMKII) autophosphorylation was enhanced in CAVB, indicating CaMKII activation. CAVB also enhanced CaMKII-dependent pharmacalbamin-phosphorylation and accelerated [Ca\(^{2+}\)]\(_i\)-transient decay, consistent with phosphorylation-induced reductions in phospholamban inhibition of sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase as a contributor to enhanced SR Ca\(^{2+}\) loading. The CaMKII-inhibitor KN93 reversed CAVB-induced changes in caffeine-releasable [Ca\(^{2+}\)]\(_i\), and I\(_{\text{Cal}}\) inactivation voltage and suppressed CAVB-induced EADs. Similarly, the calmodulin inhibitor W7 suppressed CAVB-induced I\(_{\text{Cal}}\) inactivation voltage shifts and EADs, and a specific CaMKII inhibitory peptide prevented I\(_{\text{Cal}}\) inactivation voltage shifts. The SR Ca\(^{2+}\)-uptake inhibitor thapsigargin and the SR Ca\(^{2+}\) release inhibitor ryanodine also suppressed CAVB-induced EADs, consistent with an important role for SR Ca\(^{2+}\) loading and release in arrhythmogenesis. AP-duration changes reached a maximum after 1 week of bradypacing, but peak alterations in CaMKII and [Ca\(^{2+}\)]\(_i\) required 2 weeks, paralleling the EAD time course.

Conclusions—CAVB-induced remodeling enhances [Ca\(^{2+}\)]\(_i\) load and activates the Ca\(^{2+}\)-calmodulin-CaMKII system, producing [Ca\(^{2+}\)]\(_i\)-handling abnormalities that contribute importantly to CAVB-induced arrhythmogenic afterdepolarizations. (Circ Arrhythmia Electrophysiol. 2009;2:295-304.)

Key Words: long-QT syndrome ▪ remodeling ▪ calcium ▪ ion channels ▪ electrophysiology

Torsade de pointes (TdP) is a polymorphic ventricular tachycardia associated with QT-interval prolongation and sustained bradycardia, potentially leading to sudden cardiac death.\(^1\) Action potential duration (APD) prolongation is ubiquitous in TdP syndromes, and the importance of APD prolongation and transmural APD heterogeneity are well recognized.\(^2\) Rabbits and dogs with sustained bradycardia caused by chronic atrioventricular block (CAVB) are prone to TdP, occurring spontaneously in rabbits\(^3\,4\) and on I\(_{\text{Kr}}\)-blocking challenge in dogs.\(^4\) Recent studies in rabbits with CAVB-related TdP emphasized the importance of downregulation of subunits underlying rapid (I\(_{\text{Kr}}\)) and slow (I\(_{\text{KS}}\)) components of the delayed-rectifier system in APD prolongation and spontaneous TdP generation.\(^3\,5\) Delayed-rectifier K\(^{+}\)-current downregulation is also central in the CAVB dog model.\(^6\) Early afterdepolarizations (EADs) are crucial for the generation of TdP arrhythmias.\(^1\,2\) There is evidence that Ca\(^{2+}\)-handling abnormalities contribute to the generation of
EADs and that Ca²⁺-related signaling promotes EADs in hypertrophic mouse models via Ca²⁺-calmodulin dependent kinase-II (CaMKII). In studies of ventricular cardiomyocytes from CAVB rabbits, we noted that cellular contractility increases, suggesting alterations in cell Ca²⁺ handling. The present study was therefore designed to (1) assess potential Ca²⁺-handling abnormalities in CAVB rabbits; (2) define potential underlying mechanisms; and (3) evaluate a possible role in arrhythmogenic afterdepolarizations.

**Methods**

**CAVB Rabbit Model**

All animal-handling protocols were approved by the Montreal Heart Institute animal research ethics committee. Rabbits were anesthetized with ketamine/xylazine, intubated, and ventilated with room air supplemented with oxygen. CAVB was induced by injecting 37% formalin into the atrioventricular junction. A unipolar pacing lead fixed to the right ventricular free wall was connected to a subcutaneous pacemaker. Ventricular pacing at 110 bpm was instituted immediately after creating CAVB to allow recovery from surgery. One week after surgery, the pacemaker rate was adjusted to 90 bpm for 1 or 2 additional weeks.

**Cardiomyocyte Isolation**

Rabbits were euthanized by cervical dislocation. Hearts were removed, immersed in normal Tyrode solution, and perfused retrogradely (36°C, 100% O₂-saturated solutions) through the aorta with Tyrode solution containing 200 mM/L CaCl₂ for 3 to 5 minutes, then with Ca²⁺-free Tyrode solution for 5 minutes, followed by Ca²⁺-free Tyrode solution containing collagenase (0.8 mg/mL, CLS II, Worthington Biochemical) for ~40 minutes. The hearts were subsequently washed with 200 mM/L CaCl₂-containing Tyrode solution for 2 to 3 minutes, and left ventricular cells dispersed by trituration. Isolated cardiomyocytes were stored in 200 mM/L Ca²⁺-containing Tyrode solution. Only Ca²⁺-tolerant rod-shaped cells with clear cross-striations and without spontaneous contractions were used for experiments.

**Whole Cell Perforated Patch**

Whole-cell perforated-patch methods were used to record action potentials (APs) in current-clamp and I_CaL in voltage-clamp mode. I_CaL measurements in the presence and absence of the peptide CaMKII inhibitor AC3-I and its inactive congener AC3-C were performed with tight-seal patch-clamp to permit intracellular dialysis. Borosilicate glass electrodes (Suter Instruments) filled with pipette solution were connected to a patch-clamp amplifier (Axopatch 200A, Axon) and had tip resistances of 2 to 4 MΩ. Nystatin-free intracellular solution was back-filled into pipette tips by capillary action (30 seconds), and pipettes were then filled with nystatin-containing (600 µg/mL) internal solution. Cell capacitance and series resistance (Rₛ) were compensated by 75% to 85%. Rₛ was calculated by dividing the capacitive-transient decay time constant by the calculated membrane capacitance. Capacitance was assessed by integrating current elicited by 5-mV, 10-ms hyperpolarizing steps from a holding potential of ~60 mV and dividing by the voltage drop. Before compensation, Rₛ averaged 6.3±0.9 MΩ, and capacitive time-constant values after compensation averaged 1.8±0.3 MΩ and 119±9 µs. Leakage compensation was not used. Currents are expressed as densities (pA/pF). Junction potentials between the bath and pipette solution averaged 15.9 mV and were corrected for APs only. All recordings were performed at 35±0.5°C.

Tyrode solution contained (mmol/L): NaCl 136, CaCl₂ 1.8, KCl 5.4, MgCl₂ 1, Na₂HPO₄ 0.33, dextrose 10, and HEPES 5 (pH 7.4; NaOH). Li⁺ was used to replace Na⁺ for Na⁺-free Tyrode solution. The pipette solution for AP recording contained (mmol/L): GTP 0.1, potassium aspartate 110, KCl 20, MgCl₂ 1, ATP-Mg 5, HEPES 10, Na-phosphocreatine 5, and EGTA 0.05 (pH 7.4; KOH). The extracellular solution for I_CaL measurement contained (mmol/L): n-methyl-d-glucamine 137.0, CaCl₂ 25.0, HEPES 10.0, glucose 10.0, CaCl₂ 1.8, MgCl₂ 0.5 (pH 7.4; HCl). Niflumic acid (50 µmol/L) was added to inhibit Ca²⁺-dependent Cl⁻ current, and 4-aminopyridine (2-mmol/L) was added to suppress transient outward K⁺ current. The pipette solution for I_CaL recording contained (mmol/L): CaCl₂ 120, tetraethylammonium-chloride 20, MgCl₂ 1, EGTA 20 (EGTA 20 for perforated-patch or 0 for tight-seal studies), ATP-Mg 5, HEPES 10, and Li-GTP 0.1 (pH 7.4; CsOH). AC3-I and AC3-C concentrations in pipette solutions were 20 µmol/L.

**Cell Shortening, Ca²⁺ Transients, and AP Clamp**

Isolated cardiomyocytes were field-stimulated by 10 ms 1.5×threshold-old square-wave pulses delivered through 2 platinum electrodes separated by 2 cm in the experimental chamber. Cell shortening was measured relative to diastolic cell length with a video edge detector (Crescent Electronics) coupled to a charged-coupled device camera (digitization at 200 Hz, TL-1/A/D Converter, Axon). Edge-detection cursors were positioned at both cell ends to measure whole-cell shortening.

To record Ca²⁺ transients, ventricular cardiomyocytes were incubated with Indo-1 AM (5 µmol/L, Molecular Probes) in 100 µmol/L pluronic F-127 (Molecular Probes) and 0.5% DMSO (Sigma) for 3 to 5 minutes, then superfused with Tyrode solution at 36±1°C for at least 20 minutes to wash out extracellular dye and allow for deesterification. Ultraviolet light from a 100-W mercury arc lamp passing through a 340-nm interference filter (≥10 nm bandwidth) was reflected through a dichroic mirror into a x40 oil-immersion fluor objective for excitation of intracellular Indo-1 (excitation beam ~15 µm diameter). Exposure to ultraviolet light (5 to 10 seconds of every 30 to 60 seconds) was controlled by an electronic shutter (Optikon, model T132) to minimize photobleaching. Emitted light (<550 nm) was directed into a spectral separator, passed through parallel filters at 400 and 500 nm (≥10 nm), detected by matched photo-multiplier tubes (Hamamatsu R2560 HA) and electronically filtered at 60 Hz. The ratio of fluorescence signals (R400/500) was digitized (1 kHz) and recorded, with subsequent conversion to [Ca²⁺] values as previously described. Before each measurement, background fluorescence was removed by adjusting the 400- and 500-nm channels to zero over an empty field of view near the cell.

The AP voltage-clamp technique was used to study AP waveform-dependent effects on Ca²⁺ transients. Voltage waveforms recorded at 2 Hz in control and 2-week CAVB rabbits were applied under perforated-patch conditions to control cardiomyocytes for 2 minutes each at 2 Hz from a holding potential of ~80 mV, and Ca²⁺ transients were recorded as described above. Control and CAVB waveforms were applied in randomized order to control for time-related changes.

**Immunoblotting**

Left and right ventricular tissue samples were fast-frozen in liquid nitrogen and stored at ~80°C. Tissue samples were homogenized in RadiolImmuo Precipitation Assay (RIPA) buffer as previously described. The homogenate was centrifuged (15 000 rpm, 20 minutes, 4°C). The supernatant was used for protein concentration measurement by Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as a standard. Protein samples (10, 20, or 40 µg) were denatured with Laemmli buffer and fractionated on 6%, 8%, or 10% SDS-polyacrylamide gels, then transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, 25 mm/L, Tris-base, 192 mm/L glycine, and 20% methanol at 0.3 A for 1 hour or overnight at 4°C. Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) with 5% nonfat dry milk for 1 hour and incubated with primary antibodies (anti-SERCA2a 1:2500, anti-NCX 1:2500, Affinity BioReagents [ABR]; antiphospho-CaMKII, 1:5000, Promega; anti-CaMKII, 1:1000, Santa Cruz; anti-calsequestrin, 1:2500, ABR; antiphospho-phospholamban-thr17, 1:5000, Badrilla; antiphospho-phospholamban-ser16, 1:2000, Badrilla; anti-phospholamban total, 1:10,000, ABR; anti-ryanodine receptor, 1:1000, ABR) overnight at 4°C. After washing and reblooming, membranes were incubated with...
horseradish peroxidase–conjugated goat anti-rabbit or donkey anti-

Data Analysis

APs and Cellular Contractility

CAVB cardiomyocytes were hypertrophied after 2-week (but not 1-week) bradypacing, with a 24% increase in mean cell capacitance (Figure 1A). Figure 1B shows examples of AP recordings. CAVB did not change resting potential (−88 ± 1, −85 ± 4, −86 ± 2 mV for control, 1-week, 2-week CAVB, respectively) or AP amplitude (129 ± 2, 128 ± 2, 126 ± 2 mV respectively) but significantly delayed repolarization (Figure 1C). APD values reached their maximum at 1-week CAVB, remaining significantly increased relative to control at 2-week CAVB. Figure 1D shows recordings of cell shortening at 1 Hz, with corresponding mean data in Figure 1E. Cell shortening did not change appreciably with 1-week CAVB.
but a statistically significant ≈65% increase was seen at 2 weeks.

Changes in Ca²⁺ Transients
Figure 2A through 2C shows examples of steady-state Ca²⁺ transient recordings. CAVB greatly increased Ca²⁺ transient systolic levels after 2-week bradycardia, with much less effect at 1 week. Diastolic [Ca²⁺], transient amplitude changes increased slightly but significantly at 1 week, with striking changes for 2-week CAVB (Figure 2E). The Ca²⁺ transient decay time constants (τ) obtained with monoeponential curve fits were significantly decreased by 2-week CAVB (Figure 2F), indicating accelerated Ca²⁺ transport out of the cytosol.

To obtain a more direct assessment of sarcoplasmic-reticulum (SR) Ca²⁺ load changes, we measured local caffeine (10 mmol/L) puff-induced Ca²⁺ release (caffeine Δ[Ca²⁺]) with the use of a linear-flow rapid delivery system. Figure 3A shows examples of caffeine-induced Ca²⁺ transients in control and CAVB cardiomyocytes. CAVB significantly increased caffeineΔ[Ca²⁺], by >2-fold at 2 weeks (Figure 3B). The decay time constant of the caffeine-induced Ca²⁺ transient, which is dominated by the Na⁺,Ca²⁺-exchanger (NCX), was similar in control and CAVB cells (Figure 3C).

Potential Mechanisms of CAVB-Induced Ca²⁺ Loading
The results shown in Figures 2 and 3 document increased Ca²⁺ loading in 2-week CAVB rabbit cardiomyocytes. We therefore addressed potential underlying mechanisms. The first mechanism that we considered was enhancement of I_CaL, conductance. Figure 4A shows I_CaL recordings from control and 2-week CAVB. There are no apparent differences, an impression supported by similar mean I_CaL densities over the full test-pulse voltage range, as shown in Figure 4B. Thus, increased CAVB cell Ca²⁺ loading was not due to increased I_CaL conductance. Closer study of I_CaL voltage dependence (Figure 4C) provided evidence for a potentially significant, albeit subtle, change in I_CaL properties with CAVB. I_CaL inactivation voltage dependence was determined with 1000-ms prepulses to voltages between −100 and +30 mV, followed by 300-ms test pulses to 0 mV. Activation voltage dependence was obtained from data obtained as illustrated in Figure 4B, based on the relation I_1=I_max(V−V_r)(G/G_max), where I_1 and G are current and conductance at voltage V; I_max and G_max are maximum current and conductance; and V_r is the reversal potential. V_r was determined from the horizontal axis intercept of the ascending limb of the I_CaL-voltage relation. I_CaL activation voltage was unaffected by CAVB, but I_CaL inactivation V_1/2 was positively shifted in 2-week CAVB cells (Figure 4C). Inactivation V_1/2 averaged −25.5±1.1 mV (n=17) in control versus −21.3±0.9 mV in CAVB (n=11, P<0.05). Consequently, the inactivation-activation overlap “window area” for I_CaL was substantially increased in CAVB cells (checked area under the corresponding curves in Figure 4C) compared with controls (stippled area). The time constants obtained by biexponential curve-fits of I_CaL inactivation were not altered by CAVB (supplemental Figure IA). Time-dependent I_CaL recovery was assessed with a paired-pulse protocol (supplemental Figure IB), providing time constants that were not significantly different between control and CAVB cells (supplemental Figure IC).

We then turned our attention to the potential impact of APD prolongation on Ca²⁺ homeostasis in CAVB rabbits. Figure 5A shows Ca²⁺ transients from control rabbit cardiomyocytes that were exposed alternately and in randomized order to a series of 2-Hz AP-clamp waveforms recorded from control and 2-week CAVB cardiomyocytes at the same frequency (inset). Application of the CAVB waveform led to significantly increased Ca²⁺ transients, as indicated in Figure 5B, without affecting diastolic [Ca²⁺], or the time constant of [Ca²⁺]i decay. These results suggest that CAVB-induced increases in Ca²⁺ entry related to APD prolongation probably contribute to the Ca²⁺-loaded state of CAVB cardiomyocytes.
We then considered alterations in the expression and/or phosphorylation of key Ca<sup>2+</sup>-handling proteins as explanations for the Ca<sup>2+</sup>-loaded state of CAVB cardiomyocytes. Figure 6A shows immunoblots for a number of important proteins that regulate cellular Ca<sup>2+</sup> handling, separated according to left ventricular and right ventricular findings. Corresponding mean data for 5 individual hearts per group are shown in Figure 6B. CAVB hearts showed significantly increased CaMKII autophosphorylation, without any significant change in total CaMKII expression. Consistent with the increased CaMKII activation indicated by enhanced CaMKII autophosphorylation, phosphorylation of phospholamban at the CaMKII-specific threonine-17 site was significantly increased in CAVB hearts. Statistically significant changes in CaMKII autophosphorylation and threonine-17 phospholamban phosphorylation occurred at 1-week CAVB, with maximum changes at 2 weeks. We also observed significant serine-16 phospholamban hyperphosphorylation (mediated principally by protein-kinase A, PKA), with 1- and 2-week CAVB. Overall phospholamban expression was not altered. Similarly, SERCA2a, NCX, calsequestrin, and ryanodine receptor type-2 expression were unchanged by CAVB (supplemental Figure II).

**Arrhythmogenic Role of Altered Ca<sup>2+</sup> Handling**

Mechanistic studies of acquired TdP have tended to focus on APD prolongation and underlying ionic current alterations, particularly K<sup>+</sup>-current modifications.2–6 To assess the potential importance of changes in the Ca<sup>2+</sup>-handling system, particularly those associated with sarcoplasmic-reticulum (SR) Ca<sup>2+</sup>-loading, in arrhythmogenesis produced by CAVB, we studied afterdepolarizations observed during AP recording with the perforated patch method to minimize alteration of the intracellular macromolecular milieu.

Figure 7A shows spontaneous EADs in CAVB cardiomyocytes paced at 0.1 Hz. Spontaneous EADs were seen in 62 of 92 CAVB cardiomyocytes. A, I<sub>CaL</sub> recordings at 0.1 Hz in control (CTL) and 2-week CAVB cardiomyocytes. B, Mean±SEM I<sub>CaL</sub> density (n=29 and 18 cells in CTL and CAVB groups, respectively). C, Voltage dependence of I<sub>CaL</sub> inactivation and activation. Curves are Boltzmann fits to mean data (n=11 and 29 cells for CTL and CAVB, respectively). Dotted, checked areas indicate window current range for CTL and CAVB, respectively. D, Mean±SEM V1/2 of the I<sub>CaL</sub> inactivation curve before versus after KN-93 and W-7 (n=11 and 12 cells in CTL and CAVB, respectively, for KN-93 and 9 and 9 cells, respectively, for W-7; *P<0.05, **P<0.01, ***P<0.001). E, Mean±SEM I<sub>CaL</sub> inactivation V1/2 in CAVB cells without versus with dialysis of AC3-I and AC3-C (n=10 and 12 cells in parallel CAVB groups without and 10 and 12 cells with peptide dialysis. **P<0.001).
92 2-week CAVB cardiomyocytes (67%) from 20 rabbits, compared with none of 34 cardiomyocytes (0%, \( P \leq 0.001 \) versus CAVB) from 4 control rabbits (supplemental Figure IIIA) and 3 of 28 1-week CAVB cardiomyocytes (11%, \( P = \text{NS} \) versus control) from 5 rabbits. When cardiomyocytes were paced at 2 Hz, they rarely showed spontaneous afterdepolarizations. However, isoproterenol commonly induced EADs in both 1-week and 2-week CAVB (but not control) cardiomyocytes lacking them under baseline conditions (Figure 7B). Isoproterenol also induced delayed afterdepolarizations (DADs) in all CAVB-cardiomyocytes (supplemental Figure IV). Isoproterenol-induced afterdepolarizations were rare in control rabbit cardiomyocytes (supplemental Figure IIIIB), occurring in only 3 of 34 (8.4%, \( P < 0.01 \) versus CAVB) and consisting in all cases of DADs. Isoproterenol commonly induced spontaneous \( \text{Ca}^{2+} \) transient events in CAVB cells after a 1-minute period of 3-Hz pacing to cause further \( \text{Ca}^{2+} \) loading, but this was rarely seen in control cells (supplemental Figure V).

Figure 6. Determination of total and phosphorylated form expression for CaMKII and phospholamban (PLB) in control, 1-week, and 2-week CAVB rabbit left (LV) and right (RV) ventricles. A, Representative examples of bands corresponding to phosphorylated CaMKII (CaMKII-P), total CaMKII (CaMKII-T), Thr17-phosphorylated PLB (PLB-P-17), Ser16-phosphorylated PLB (PLB-P-16), total PLB (T-PLB), and GAPDH. B, Mean±SEM protein band intensities normalized to GAPDH (5 separate control and 5 separate CAVB blots, each from 1 heart, for each analysis, *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) versus control; #\( P < 0.05 \) versus 1-week CAVB).

Figure 7. A, Representative example of spontaneous EADs at 0.1 Hz in CAVB myocytes (left) and percentage of cells showing EADs (right). B, Isoproterenol (Iso)-induced EADs and DADs in CAVB cardiomyocytes without spontaneous afterdepolarizations before Iso (left) and percentage of cells showing isoproterenol-induced EADs (right).
Figure 8A illustrates the effect of thapsigargin (1 μmol/L), which suppresses SERCA function, on spontaneous EADs in CAVB rabbits. In this and 6 other cells studied in a similar fashion, thapsigargin fully suppressed EAD generation. Figure 8B shows that preventing SR Ca\textsuperscript{2+} release by adding ryanodine (10 μmol/L) to the superfusate similarly suppressed CAVB-associated EADs in all 7 cells tested. Suppression of NCX function by changing the bath solution to a modified Tyrode solution lacking Na\textsuperscript{+} (Li\textsuperscript{+} substitution) and with Ca\textsuperscript{2+} removed suppressed EADs in 5 cells (eg, see supplemental Figure VI). These results implicate SR Ca\textsuperscript{2+} stores, SR Ca\textsuperscript{2+} release through ryanodine-receptors, and NCX function as essential contributors to EAD generation in CAVB cardiomyocytes.

Evidence for Involvement of the Ca\textsuperscript{2+}-Calmodulin/CaMKII System

Our results are consistent with a central role for Ca\textsuperscript{2+}/calmodulin-activated CaMKII in CAVB-related cellular arrhythmogenesis. We sought to explore this role further with the use of the calmodulin antagonist W-7, the CaMK-II inhibitor KN-93, and the CaMK-II inhibitory peptide AC3-I. First, we assessed whether suppressing CaMKII-phosphorylation could reduce SR Ca\textsuperscript{2+} loading in CAVB cells, which would be the case if CaMKII phosphorylation of phospholamban is important in enhancing Ca\textsuperscript{2+} stores. Figure 3D shows that exposure of cells to 1 μmol/L KN-93 significantly reduced the caffeine-induced Ca\textsuperscript{2+}-transient in CAVB cardiomyocytes without affecting its decay time constant (Figure 3E). We then examined the positive shift in \( I_{\text{CaL}} \) inactivation observed in CAVB cells. Both KN-93 and W-7 (1 μmol/L) significantly shifted the \( I_{\text{CaL}} \) inactivation voltages of CAVB cells in a hyperpolarizing direction, restoring them to values not significantly different from control (Figure 4D). In control cells, neither KN-93 nor W-7 significantly affected inactivation voltage, arguing against a nonspecific effect. Intracellular dialysis with the CaMKII inhibitory peptide AC3-I also reversed the inactivation voltage shift in CAVB cells, whereas the inactive analogue AC3-C had no effect (Figure 4E). Finally, we examined the ability of W-7 and KN-93 to suppress EADs in CAVB cardiomyocytes. The examples shown in Figure 8C and 8D indicate the ability of these agents to suppress CAVB-associated afterdepolarizations. Similar results were seen in 9 cells with W-7 and 7 cells with KN-93. In contrast, the inactive congener of KN-93, KN-92 (1 μmol/L), had no effect on CAVB-related EADs in 5 cells (Figure 8E).

Discussion

Previous studies of the rabbit CAVB model of spontaneous TdP have appropriately emphasized ionic current remodeling, particularly changes in K\textsuperscript{+}-channel expression, that underlie pathological QT prolongation. In this report, we provide evidence for CAVB-induced alterations in cellular Ca\textsuperscript{2+} handling, along with their underlying molecular basis, that appear central to the generation of arrhythmogenic afterdepolarizations.

Mechanisms of Cellular Ca\textsuperscript{2+} Handling Changes and Role in Early Afterdepolarizations

We identified 2 abnormalities in Ca\textsuperscript{2+}-handling systems that could contribute to arrhythmogenic EADs. The most prominent is an increased SR Ca\textsuperscript{2+} load caused by APD prolongation and phospholamban hyperphosphorylation. Phospholamban hyperphosphorylation disinhibits SERCA and enhances SR Ca\textsuperscript{2+} uptake, leading to enhanced Ca\textsuperscript{2+} loading and
accelerated \([\text{Ca}^{2+}]\) transient decay. A second is a depolarizing shift in \(I_{\text{cal}}\) inactivation, which increases the calculated \(I_{\text{cal}}\) window current conductance and voltage range. Evidence for an important role of CaMKII activation in CAVB derives from the demonstration of enhanced CaMKII auto-phosphorylation (indicating CaMKII activation) and phospholamban hyperphosphorylation at the CaMKII-specific threonine-17 site, along with the ability of the CaMKII inhibitor KN-93 to normalize the caffeine-induced \(\text{Ca}^{2+}\) transient and \(I_{\text{cal}}\) inactivation voltage dependence. Support for the role of CaMKII in arrhythmogenic EADs derives from the ability of KN-93, and not its inactive congener KN-92, to suppress EADs in CAVB cells.

To our knowledge, our study is the first to indicate a role for \(\text{Ca}^{2+}\) handling abnormalities and the CaMKII system in the CAVB rabbit model of spontaneous TdP and the first to detail the molecular basis of CAVB induced \(\text{Ca}^{2+}\) handling disturbances. In the CAVB dog model of drug-induced TdP, altered \(\text{Ca}^{2+}\) handling has been implicated but underlying molecular mechanisms have not been defined. Antoonst et al11 found changes in \(I_{\text{cal}}\) properties of CAVB dogs similar to those in our CAVB rabbits (unchanged overall \(I_{\text{cal}}\) density-voltage relations but a depolarizing shift in \(I_{\text{cal}}\) inactivation) and implicated enhanced \(I_{\text{cal}}\) window current in isoproterenol-induced EADs. CaMKII activation is known to be able to shift \(I_{\text{cal}}\) inactivation voltage.12 Our observations (reversal of CAVB-induced \(I_{\text{cal}}\)-inactivation shifts with the CaMKII blocker KN-93, the inhibitory peptide AC3-I, and the calmodulin inhibitor W-7) implicate \(\text{Ca}^{2+}\)/calmodulin-dependent CaMKII activation in CAVB-induced \(I_{\text{cal}}\) window current enhancement. Sipido et al13 reported increased SR \(\text{Ca}^{2+}\) loading, enhanced NCX function, and \(\text{Ca}^{2+}\) transients in CAVB dogs. We did not directly examine NCX function, but unchanged NCX protein expression and caffeine-induced \(\text{Ca}^{2+}\) transient decay kinetics argue against intrinsic NCX changes. Nevertheless, the ability of Na+/free, \(\text{Ca}^{2+}\)-free solution to suppress EADs indicates that intact NCX function is required for EAD generation in CVB rabbits. Ryanodine and flunarizine suppress drug-induced TdP in CAVB dogs,14 consistent with the role for SR-Ca2+ handling abnormalities in APD prolongation.

We previously showed a role for combined downregulation of \(I_{\text{Ks}}\) and \(I_{\text{Kr}}\)-encoding subunits (\(\text{KCNH}2\), \(\text{KCQ}1\), and \(\text{KCNel}\)) in spontaneous TdP of CAVB rabbits.5 In the present study, we demonstrate that CaMKII activation is required for EAD generation. Our time-course data showed that APD was maximally increased at 1-week CAVB but that cellular hypertrophy, \(\text{Ca}^{2+}\) loading, CaMKII activation, and EAD generation were maximal at 2-weeks. Along with the W-7 and KN-93 data in Figure 8, these results suggest that APD prolongation is a necessary but not sufficient condition for spontaneous EADs. Although APD prolongation may provide an early signal for CAVB-related arrhythmogenic remodeling, it appears insufficient to generate EADs on its own. The ability of isoproterenol to unmask latent EAD predisposition in 1-week CAVB rabbit cells (Figure 7B) indicates that \(\text{Ca}^{2+}\) loading and/or CaMKII activation can dynamically trigger EAD generation in the presence of APD prolongation.

Our experiments with thapsigargin and Ryanodine implicated SR \(\text{Ca}^{2+}\) loading and release in EAD generation, and our Na+/\(\text{Ca}^{2+}\) replacement studies indicate NCX involvement. Forward-mode NCX generates inward currents that contribute to APD prolongation and EAD generation in CAVB dogs.13 In our CAVB rabbits, SR \(\text{Ca}^{2+}\) loading resulting from APD prolongation and enhanced SR \(\text{Ca}^{2+}\) uptake caused by phospholamban hyperphosphorylation increased systolic \(\text{Ca}^{2+}\) release. Subsequent removal of the cytoplasmic \(\text{Ca}^{2+}\) load by NCX generates enhanced inward currents, promoting EAD generation.

There is evidence for participation of \(I_{\text{cal}}\) in arrhythmogenic EADs associated with drug-induced APD prolongation,23,24 primarily through increased \(I_{\text{cal}}\) window current.25 Mathematical modeling suggests that APD prolongation associated with K+ current downregulation plays a particularly important role in generating the \(I_{\text{cal}}\)-mediated component of EADs and in determining their properties.26

Adrenergic stimulation may also contribute to arrhythmia generation in the CAVB rabbit model. In addition to phospholamban phosphorylation at the threonine-17 CaMKII site, we also found substantial hyperphosphorylation at the PKA-related27 serine-16 site. PKA phosphorylation is primarily

**What Activates CaMKII in This Model?**

Our results show that CaMKII is required for the generation of EADs in the CAVB rabbit TdP model. The effectiveness of W-7 in suppressing arrhythmogenesis in a \(\text{Ca}^{2+}\)-loaded context points to \(\text{Ca}^{2+}\)/calmodulin activation as the signal for CaMKII activation. CAVB waveforms increase cell \(\text{Ca}^{2+}\) loading (Figure 5), suggesting that APD prolongation resulting from \(\text{K}^{+}\)-current downregulation in CAVB rabbits5 may initiate \(\text{Ca}^{2+}\)/calmodulin activation and CaMKII stimulation as previously described.15 However, other components of the adaptive response to CAVB could also be involved. The volume-overloaded and/or neurohumoral state resulting from CAVB may promote CaMKII activation, for example, through \(\beta\)-adrenergic pathways.20,21 Tissue oxidation also promotes CaMKII activation.22 Further work is needed to define specific contributors to CAVB-induced CaMKII activation.
driven by β-adrenergic stimulation and synergistically enhances the effects of CaMKII phosphorylation on phospholamban function. A recent mathematical modeling study suggested that spontaneous SR Ca$^{2+}$ release prolongs APD but only generates EADs when K$^+$-current density is decreased and that reentry reinitiation by EADs requires enhancement of $I_{\text{CaL}}$ density and SR Ca$^{2+}$ cycling to simulate adrenergic stimulation effects.28

**Novel Findings and Potential Significance**

Acquired long-QT syndrome is a significant clinical problem, and bradyarrhythmias are an important precipitator of TdP. Our results provide direct evidence for a significant role of Ca$^{2+}$ homeostasis alterations, along with a delineation of underlying molecular mechanisms, in a bradycardia-related animal model of spontaneous TdP. Work in this area traditionally focused on ion-channel alterations and associated APD prolongation, which are undoubtedly very important. The present study adds to growing evidence indicating that APD prolonging ion-channel dysfunction is only part of the pathophysiology of TdP and that Ca$^{2+}$ handling alterations and CaMKII activation play important roles.8,31 Improved understanding of the interplay between various pathophysiological contributors to TdP-related arrhythmogenic mechanisms in different clinically relevant paradigms should lead to new mechanistic insights and improved clinical management.

**Potential Limitations**

We used a pharmacological approach to inhibit KN-93 for AP studies, and all pharmacological probes have potential non-specific actions. We selected a concentration of KN-93 that is the lowest concentration producing substantial CaMKII inhibition to minimize nonspecific actions. We tried to repeat AP studies with cell dialysis of AC3-I.18 However, APD and EADs decreased rapidly with tight-seal patch-clamp, in contrast to the recording stability seen with perforated-patch methods (supplemental Figure VII). We did succeed in recording stable $I_{\text{CaL}}$ with tight-seal patch-clamp and found that AC3-I-dialysis reversed $I_{\text{CaL}}$ inactivation shifts, confirming the role of CaMKII.

We used right ventricular bradypacing, which can induce ventricular activation dyssynergy that may contribute to ventricular remodeling over and above the effect of bradycardia per se.29 There are regional differences in AP and ionic-current properties, with left ventricular APD being more prolonged than right ventricular in CAVB dogs.8 The present study focused on left ventricular cardiomyocytes: Further work on left-right and regional differences would be of interest.

We found APD to be more prolonged at 1- versus 2-week CAVB, and it is unclear why this should be the case. One possible explanation relates to the high sudden death mortality rate (≈50%) that occurred in the last week before intended euthanasia in 2-week CAVB rabbits. Animals with longer APDs may have been more likely to have malignant TdP and die, leaving a population with shorter mean APDs (albeit still much greater than control) because of data dropout.

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**Disclosures**

None.

**References**


CLINICAL PERSPECTIVE

The association between bradycardia, particularly severe bradycardias occurring with atrioventricular block (AVB), and clinical long-QT syndrome is well recognized. In rabbits, experimental AVB causes ventricular remodeling and acquired long-QT syndrome with spontaneous torsades de pointes (TdP). Combined decreases in slow (IKs) and rapid (IKr) current, rate dependence, and potassium channel downregulation increased Ca2+-handling. We therefore measured single-cell Ca2+-concentrations with a Ca2+-sensitive dye and found that heart cells from AVB rabbits have increased Ca2+ stores. Looking for causes, we found that prolonged action potentials caused by K+ channel downregulation increased Ca2+ entry during the plateau phase. Increased cell Ca2+-activated the Ca2+-sensitive phosphorylating enzyme Ca2+/calmodulin-dependent protein kinase-II (CaMKII), as shown by CaMKII autophosphorylation and enhanced phosphorylation of the sarcoplasmic-reticulum (SR)-associated protein phospholamban. Phospholamban phosphorylation enhances Ca2+ uptake into SR stores, aggravating cell Ca2+ loading. In addition, we found functional evidence of CaMKII Ca2+ channel phosphorylation, which causes increased inward Ca2+ current during the action potential plateau and early phase-3. Cellular Ca2+ overloading enhances Na+-Ca2+ exchange (NCX) activity in an attempt to eliminate the extra Ca2+, but enhanced NCX activity generates depolarizing inward currents. We found that arrhythmogenic early afterdepolarizations were much more common in AVB cells than in control cells and that early afterdepolarizations could be prevented by interventions that suppress SR Ca2+ loading, SR Ca2+ release, NCX function, or activation of CaMKII, despite the persistence of repolarization abnormalities. These results indicate that changes in cell Ca2+ signaling are important for AVB-induced long-QT/TdP arrhythmogenesis and suggest that targeting Ca2+-dependent abnormalities caused by repolarization deficiency could be a useful approach to treating acquired TdP.
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SUPPLEMENTAL MATERIAL
### On-line Figure I

**Figure A:**
- Graph showing inactivation time constant (ms) against TP (mV)
- Curves for different conditions: CTL, 1 week AVB, 2 weeks AVB

**Figure B:**
- Graph showing I/I₁ against P₁P₂ (ms)
- Y-axis: I/I₁ ratio ranging from 0.00 to 1.25
- X-axis: Time (ms) from 0 to 500
- Voltage conditions: 0 mV, -80 mV

**Figure C:**
- Bar chart showing recovery time constant (ms)
- Conditions: CTL, CAVB 1 wk, CAVB 2 wk
On-line Figure IV

A  Control cell

B  CAVB cell

Pre-1 μM iso  Post-1 μM iso
After-transients (Events/run)

1 µM ISO 3 Hz 1 min

Post-pacing follow-up 1 min
On-line Figure VI

Pre- Na\(^+\)-free, Ca\(^{2+}\)-free

Post- Na\(^+\)-free, Ca\(^{2+}\)-free

0.1 Hz  2 min

100 mV
On-line Figure Legends

On-line Figure I. **A**, $I_{\text{CaL}}$ inactivation time-constants (n=11~29 cells in CTL, 1 and 2 wk CAVB respectively, $P=\text{NS}$); **B**, $I_{\text{CaL}}$ time-dependent recovery kinetics, obtained by applying 2 identical test pulses ($P_1, P_2$) with varying $P_1P_2$ intervals and relating the relative current of the test pulse ($I_2$) to the $P_1P_2$ interval. Monoexponential fits to mean data are shown Mean±S.E.M (n=10~15 cells in CTL, 1 and 2-week CAVB group respectively, $P=\text{NS}$). **C**, Mean±S.E.M time-dependent recovery time constants (n=10~15 cells in CTL, 1 and 2-week CAVB group respectively, $P=\text{NS}$).

On-line Figure II. Total RyR2, SERCA2α, NCX, CSQ in control, 1- and 2-wk CAVB-rabbit ventricular tissue. **A**, Representative examples of Total RyR2, SERCA2α, NCX, CSQ, and GAPDH immunoblots. **B**, Mean±S.E.M. protein-band intensities normalized to GAPDH (n=5 control, 1- and 2-week AVB hearts for each analysis).

On-line Figure III. **A**, An example of lack of afterdepolarizations at 0.1 Hz 10 in control cells. **B**, An example of lack of afterdepolarizations upon perfusion with 1-μmol/L isoproterenol (Iso) during 2-Hz pacing for 1 min following a 1-min quiescent period.

On-line Figure IV. **A**, Representative examples showing lack of DADs at 2 Hz following perfusion with 1-μmol/L isoproterenol (iso) in CTL ventricular myocytes. **B**, Iso-induced DADs at 2 Hz in CAVB ventricular myocytes.

On-line figure V. After-transients recorded upon perfusion with 1-μmol/L isoproterenol (Iso) during and following 3-Hz pacing for 1 min. **A**, Example in a control rabbit
ventricular cardiomyocyte.  

**Inset,** Mean±S.E.M. after-transient events during 1-min post-pacing period (n=26 cells/group, *P<0.05).

**On-line Figure VI.** Representative examples of spontaneous EADs at 0.1 Hz in CAVB ventricular myocytes, before (top) and after (bottom) Na\(^+\) and Ca\(^{2+}\) removal from external solution, with Na\(^+\) replaced by equimolar Li\(^+\) in same cells.

**On-line Figure VII.**  

A, B, AP recordings from CAVB ventricular myocytes obtained with tight-seal dialysis-patch.  

C, D, AP recordings from CAVB ventricular myocytes obtained with perforated patch.