Ca\(^{2+}\)-Related Signaling and Protein Phosphorylation Abnormalities Play Central Roles in a New Experimental Model of Electrical Storm

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**Background**—Electrical storm (ES), characterized by recurrent ventricular tachycardia/fibrillation, typically occurs in implantable cardioverter-defibrillator patients and adversely affects prognosis. However, the underlying molecular basis is poorly understood. In the present study, we report a new experimental model featuring repetitive episodes of implantable cardioverter-defibrillator firing for recurrent ventricular fibrillation (VF), in which we assessed involvement of Ca\(^{2+}\)-related protein alterations in ES.

**Methods and Results**—We studied 37 rabbits with complete atrioventricular block for \(\approx 80\) days, all with implantable cardioverter-defibrillator implantation. All rabbits showed long-QT and VF episodes. Fifty-three percent of rabbits developed ES (\(\approx 3\) VF episodes per 24-hour period; 103±23 VF episodes per rabbit). Expression/phosphorylation of Ca\(^{2+}\)-handling proteins was assessed in left ventricular tissues from rabbits with the following: ES; VF episodes but not ES (non-ES); and controls. Left ventricular end-diastolic diameter increased comparably in ES and non-ES rabbits, but contractile dysfunction was significantly greater in ES than in non-ES rabbits. ES rabbits showed striking hyperphosphorylation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II, prominent phospholamban dephosphorylation, and increased protein phosphatase 1 and 2A expression versus control and non-ES rabbits. Ryanodine receptors were similarly hyperphosphorylated at Ser2815 in ES and non-ES rabbits, but ryanodine receptor Ser2809 and L-type Ca\(^{2+}\) channel \(\alpha\)-subunit hyperphosphorylation were significantly greater in ES versus non-ES rabbits. To examine direct effects of repeated VF/defibrillation, VF was induced 10 times in control rabbits. Repeated VF tissues showed autophosphorylated Ca\(^{2+}\)/calmodulin-dependent protein kinase II upregulation and phospholamban dephosphorylation like those of ES rabbit hearts. Continuous infusion of a calmodulin antagonist (W-7) to ES rabbits reduced Ca\(^{2+}\)/calmodulin-dependent protein kinase II hyperphosphorylation, suppressed ventricular tachycardia/fibrillation, and rescued left ventricular dysfunction.

**Conclusions**—ES causes Ca\(^{2+}\)/calmodulin-dependent protein kinase II activation and phospholamban dephosphorylation, which can explain the vicious cycle of arrhythmia promotion and mechanical dysfunction that characterizes ES. (Circulation. 2011;123:2192-2203.)

**Key Words:** calcium ■ electrical storm ■ heart failure ■ implantable cardioverter-defibrillator ■ signal transduction

Electrical storm (ES), characterized by repetitive episodes (generally defined as \(\approx 3\) within 24 hours) of ventricular fibrillation (VF) and/or ventricular tachycardia (VT), is an increasing problem among implantable cardioverter-defibrillator (ICD) patients. Recent analyses of large clinical trials indicate a high risk of early mortality in patients who experience ES. Most of this excess risk is attributable to cardiac, nonsudden mechanisms, particularly progressive heart failure (HF). The molecular mechanisms underlying ES and associated mortality are poorly understood.

**Editorial see p 2183**

**Clinical Perspective on p 2203**

Ca\(^{2+}\)-handling protein phosphorylation is a major control mechanism for cardiac contractility and relaxation. Increased activity of protein phosphorylation mediators like cAMP-
dependent protein kinase A (PKA), Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), and protein kinase C (PKC) and resulting hyperphosphorylation of Ca\(^{2+}\)-handling proteins are linked to mechanical dysfunction and arrhythmias.\(^4\)–\(^9\) CaMKII/PKA-mediated phosphorylation of ryanodine receptors (RyR2s) causes diastolic Ca\(^{2+}\) leak from the sarcoplasmic reticulum (SR), causing depletion of SR Ca\(^{2+}\) stores and arrhythmias related to delayed afterdepolarizations.\(^4\)–\(^7\) Hyperphosphorylation of L-type Ca\(^{2+}\) channels (LTCCs) by CaMKII modulates Ca\(^{2+}\) current, predisposing to early afterdepolarizations.\(^8\) PKC activation results in hypophosphorylation of phospholamban, enhancing phospholamban inhibition of SR Ca\(^{2+}\)-ATPase (SERCA2a) and impairing Ca\(^{2+}\) uptake into the SR.\(^9\)\(^,\)\(^10\) These findings, together with the fact that VF is associated with increased [Ca\(^{2+}\)]\(_i\),\(^11\)\(^,\)\(^12\) and that ES is associated with elevated sympathetic tone,\(^1\) led us to hypothesize that Ca\(^{2+}\)-handling alterations caused by changed expression and/or activation of Ca\(^{2+}\) signaling molecules may be responsible for ES-related negative outcomes. In the present study, we report a new experimental model that features repetitive episodes of ICD firing for recurrent VF, in which we tested this hypothesis.

Methods

All animal handling protocols were approved by the Animal Experimentation Ethics Committee of Nagoya University.

Surgical Technique and Experimental Protocol

Female New Zealand White rabbits (weight, 2.5 to 3.5 kg) were used. We created complete atrioventricular block and implanted ICDs (Medtronic) using methods described previously,\(^13\)\(^,\)\(^14\) with modifications. In brief, 2 unipolar pacing leads (6491, Medtronic) fixed to the right ventricular free wall were connected to an ICD through a Y adaptor (5866–38M, Medtronic) implanted subcutaneously in the back. A custom-made patch electrode was sutured subcutaneously in the chest wall (Figure 1A). Ventricular pacing at 150 bpm (maximum pacing rate available in the device) was instituted immediately after creating atrioventricular block to allow recovery from surgery. Seven days after surgery, the pacing rate was decreased to 90 bpm, and ICD settings were established under ketamine (25 mg/kg)/xylazine (5 mg/kg) anesthesia (day 0). To determine the defibrillation threshold, VF was induced twice by applying 50-Hz burst stimulation via the ICD. The ICD was then programmed to detect and treat VF (see below). Rabbits with complete atrioventricular block (CAVB) equipped with ICDs were subjected to sustained bradycardia with VVI pacing (90 bpm for ~1 week, followed by 60 bpm). CAVB rabbits that survived for ~3 months were euthanized (pentobarbital, 40 mg/kg IV). Left ventricular (LV) free-wall tissue samples were fast-frozen in liquid N\(_2\) and
stored at −80°C. Echocardiograms were obtained at day 0 and end of study.

**Implantable Cardioverter-Defibrillator Settings**
The VF detection interval was set <240 ms (>250 bpm), and the VT detection algorithm was disabled. The number of intervals to detect VF (NID) and to redefect VF (NID-redetect) were programmed to maximum values available (120 of 160 and 30 of 40, respectively) to avoid inappropriate shocks for torsades de pointes (Tdp)-like VTs, which occur frequently in CAVB-rabbits. Up to 6 defibrillation shocks were programmed to treat a detected VF episode. The first therapy was set to at least twice the defibrillation threshold. Figure 1B shows an example of therapy parameters. Mean defibrillation thresholds were not statistically different between groups (Figure 1C).

**Implantable Cardioverter-Defibrillator Interrogation and Real-Time Electrogram Recordings**
Approximately every 10 days, the ICD was interrogated under conscious conditions to assess the number of VF and VT episodes and obtain intracardiac electrograms of VF episodes and percentages of rhythm paced/sensed. Tachyarrhythmias that were ≥5 consecutive beats at >250 bpm (VF detection interval) and terminated spontaneously less than NID, were detected as non-sustained VT-episodes (Figure I in the online-only Data Supplement). ES was defined as ≥3 VF episodes per 24 hours. Real-time electrograms and electrogoms between the active CAN and the patch electrode (CAN-patch electrogram) were recorded through the ICD programmer. Electrogram parameter measurements, including cycle length of escape rhythm and QT interval, were based on averages of 3 consecutive beats in the lead providing the clearest QT-interval definition. QT interval was corrected (QTc) according to the method of Carlson et al15 for rabbits with the following formula: QTc = QT − 0.175 × (RR − 300).

**W-7 Administration**
The calmodulin antagonist W-7 (BIOMOL) was infused continuously in ES rabbits via an osmotic pump (2ML1.1, Alzet) that delivers solutions continuously at 10 μL/h for 7 days. The pump was filled with 2 mL dimethyl sulfoxide/water (50:50 vol/vol) with or without W-7 50 mg. Under ketamine/xylazine anesthesia, 2 pumps without W-7 were implanted subcutaneously into the abdomen for 7-day baseline infusion. W-7 was then administered by 1 pump (W-7 50 mg) for 1 week, followed by 2 pumps (W-7 100 mg) for the subsequent week. During infusion, ICD interrogation was performed daily. The dosages were established on the basis of the study by Mazur et al.16

**Western Blots**
Protein extracts were prepared, and immunoblotting was performed as described previously.14 Primary antibodies included the following: anti-calmodulin (Fitzgerald), anti-RyR2 (Affinity BioReagents), antiphospho-Ser286-CaMKII (CaMKII-P, Santa Cruz), anti-CaMKIIβ (Santa Cruz), anti-SERCA (Affinity BioReagents), anti-Ca.1.2 (Alomone), antiphospho-Ser281-RyR2 (RyR2-P2815), a kind gift from Dr. A. Marks, (Columbia University), anti-Ser2809-RyR2 (RyR2-P2809, Badrilla), anti-phospholamban (Affinity BioReagents), antiphospho-Ser16-phospholamban and anti-Thr17-phospho-phospholamban (phospholamban-P16 and phospholamban-P17, both Badrilla), anti-protein phosphatase 1 (PP1) catalytic subunit and anti-protein phosphatase 2A (PP2A) catalytic subunit (both Upstate), anti-PhKα catalytic subunit (Santa Cruz), anti-PKCα (Abcam), antiphospho-Ser677-PKCα (PKCo-P, Upstate), inhibitor-1 (I-1) (Eurogentec), phospho-Thr351-I-1 (I-1 P35, Cell Signaling), phospho-Ser671-I-1 (I-1 P67, Eurogentec), troponin-I (Milipore), phosphorylated Ser23/24-troponin-I (Cell Signaling), total myosin-binding protein C and phosphorylated Ser282-myosin-binding protein C (kind gifts from Dr L. Carrier, University of Hamburg), caldesmotrin (Dianova), phosphorylated myosin light chain-2a (kind gift from Dr T. Eschenhagen, University of Hamburg), and caspase-3 (Santa Cruz). Band densities were quantified by densitometry, standardized to GAPDH (Fitzgerald), and normalized to the control sample. I-1 protein was enriched by an optimized trichloroacetic acid extraction procedure as described previously.10 Phosphorylation analysis for Ca,1.2 was obtained with Pro-Q Diamond stain technology. In brief, proteins electroblotted on polyvinylidene fluoride membranes were fixed and stained with the Pro-Q Diamond Phosphoprotein Blot Stain Kit (P33356, Invitrogen). The membrane was imaged with UV transillumination and ATTO Light Capture molecular imager. Scans were quantified with CS Analyzer (ATTO, Tokyo, Japan).
at day 83. The stored electrogram of the last VF episode showed unsuccessful defibrillation with the sixth therapy after 5 programmed therapies for repeated postshock VF. In contrast, the non-ES rabbit had sporadic single VF episodes. Figure 2C summarizes time-dependent electrogram changes in all rabbits. The RR intervals of escape rhythms were longer in ES rabbits versus non-ES rabbits. In ES rabbits, QTc increased from 183±5 ms on day 10 to near steady state values (229±7 ms) by day 20. Although non-ES rabbits also showed increased QTc (from 187±3 ms to 215±6 ms by day 40), QTc was significantly greater in ES rabbits. The number of VF episodes per rabbit averaged 103±23 for ES rabbits and 3.2±0.9 for non-ES rabbits (P<0.001; Figure 2D) during follow-up periods of 78±8 and 70±8 days, respectively (P=0.53; Figure 1C). The number of self-terminating VT episodes was also substantially greater for ES rabbits (Figure 2D). ES development appeared rate dependent: Clustered VF episodes could be suppressed by increasing the pacing rate and reappeared on returning the rate to 60 bpm (Figure II in the online-only Data Supplement).

ES sometimes occurred early, and only transiently (Figure III in the online-only Data Supplement). Of 17 ES rabbits, 9 died prematurely. Six died during ES, with the last VF episode recording showing VF undersensing or defibrillation failure. Two were euthanized for severe pocket infection, and 1 died of unknown causes. The remaining 8 ES rabbits that survived for 89±7 days were used for immunoblotting studies. ES episodes in 5 rabbits and single VF episodes in 3 rabbits were documented within the 24 hours before euthanasia.

Of 15 non-ES rabbits, 7 surviving for 94±5 days were used for immunoblotting studies. All 7 rabbits had at least 50 self-terminating VT episodes within 24 hours of euthanasia. The remaining 8 died prematurely. Five died suddenly of VF with defibrillation failure, and 3 died of unknown causes.

Echocardiography
Figure 3A shows representative M-mode echocardiograms at day 0 (baseline) and at the end of follow-up. LV end-diastolic diameter increased similarly, by >20%, in non-ES and ES rabbits (Figure 3B). LV fractional shortening decreased from 38±1% at day 0 to 32±1% in non-ES rabbits and more severely to 25±1% in ES rabbits (P=0.006 versus non-ES) by end of study (Figure 3C). There were no significant differences in interventricular septum or posterior LV wall thicknesses among groups (Figure 3D). Pleural effusions were observed in 4 of 7 ES rabbits but not in non-ES rabbits.

Protein Kinases and Phosphatases
Figure 4A shows representative immunoblots for CaMKII-P, CaMKIIδ, and calmodulin on 1 gel each. CaMKII-P expression was significantly increased by ∼500% and ∼165% in ES and non-ES rabbits, respectively, without significant change in total CaMKII expression. Fractional CaMKII autophosphorylation was ∼2 times greater in ES than in non-ES rabbits, suggesting striking CaMKII activation. Calmodulin expression was unaltered. CaMKII activation was also observed in the right ventricle (Figure IV in the online-only Data Supplement) and was qualitatively similar to LV changes. Immunoblots for PKAα catalytic subunit, PKCα-P, and total PKCα are shown in Figure 4B and for phosphatase proteins in Figure 4C. PKAα catalytic subunit expression was reduced similarly by 61% and 55% in ES and non-ES rabbits, respectively, whereas total PKCα and PKCα-P were unchanged (Figure 4D). Expression of PP1 and PP2A catalytic subunits was increased by ∼85% in ES rabbits only (Figure 4E).

Phosphorylation of Ca,1,2, RyR2, and Phospholamban
Figure 5 shows immunoblots for total and phosphorylated forms of key Ca2+-handling proteins. The LTCC α-subunit Ca,1,2 band was observed at the expected molecular mass of
Phosphoprotein bands corresponding to Cav1.2 were stronger in both non-ES and ES rabbits versus controls (Figure 5B), with a larger fractional Cav1.2 phosphorylation (≈500%) in ES versus non-ES rabbits (≈130%).

RyR2 has multiple phosphorylation sites including RyR2-Ser2815, phosphorylated by CaMKII,17 and RyR2-Ser2809, which can be phosphorylated by CaMKII and/or PKA.5,18 Figure 5A (bottom) shows representative immunoblots of total RyR2, CaMKII-phosphorylated RyR2-P2815, and PKA/CaMKII-phosphorylated RyR2-P2809. Fractional RyR2-P2815 phosphorylation was increased significantly in both ES and non-ES rabbits, but that of RyR2-P2809 was significantly increased, by ≈70%, in ES rabbits only (Figure 5C). Total RyR2 and calsequestrin expression were unaltered in either group.

Phospholamban is an endogenous inhibitor of SERCA2a function. Phospholamban phosphorylation causes phospholamban dissociation from SERCA2a and removes inhibition. SERCA2a activity and SR Ca²⁺ uptake are enhanced on phospholamban phosphorylation. Phospholamban is phosphorylated at Ser16 by PKA and at Thr17 by CaMKII.19 Figure 5D shows representative immunoblots for SERCA2a, phospholamban-P16, phospholamban-P17, and total phospholamban. Fractional phospholamban phosphorylation was greatly reduced in ES rabbits, by 96% at Ser16 and 89% at Thr17 (Figure 5E), whereas there was no statistically significant change in phospholamban phosphorylation in non-ES rabbits. SERCA2a and total phospholamban expression were unchanged (Figure 5E).

Data for expression/phosphorylation of myofibrillar proteins are shown in Figure V in the online-only Data Supplement. PKA-phosphorylated myosin-binding protein C and troponin-I were reduced by ≈75% and ≈50% in ES and by ≈66% and ≈47% in non-ES groups, respectively, which was compatible with similar reductions in PKA⁵ catalytic subunit expression in both groups.

Protein Phosphatase I-1 Phosphorylation
PP1 activity is modulated by protein phosphatase I-1, which is phosphorylated at Thr35 by PKA.⁹,¹⁰ PKA-phosphorylated I-1 inhibits PP1, augmenting the phospholamban phosphorylation resulting from direct PKA action. PKCα phosphorylates I-1 at a different site, Ser67, suppressing I-1 inhibition of PP1 and thereby reducing phospholamban phosphorylation.⁹,¹⁰ Figure VI in the online-only Data Supplement shows representative blots for total I-1, PKA-phosphorylated I-1 (I-1-P35), and PKCα-phosphorylated I-1 (I-1-P67). Bands were observed at the expected molecular mass.²⁰ There were no significant differences among groups, suggesting that phospholamban dephosphorylation in ES rabbits is due to enhancement of protein phosphatase activity unrelated to changes in PKA or PKCα phosphorylation of I-1.

Direct Effects of VF/Defibrillation on CaMKII and Phospholamban Phosphorylation
CaMKII hyperphosphorylation is arrhythmogenic, and phospholamban dephosphorylation negatively affects SR Ca²⁺ stores and contractility. We considered the possibility that repeated VF and defibrillation can cause these changes, initiating a vicious cycle of self-reinforcing contractile failure and arrhythmogenesis. To examine the effect of repeated VF/defibrillation on protein phosphorylation, we obtained LV tissue samples from day 0 rabbits subjected to repeated VF
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phosphorylated protein kinase C

phosphatases 1 and 2A (PP1 and PP2A).

differences were decreased significantly, by 11% and 19%, respectively, in Figure 7D. LV end-diastolic and end-systolic diameters were decreased significantly, by 11% and 19%, respectively, and LV fractional shortening was increased to 30%

induction and termination. Under ketamine/xylazine anesthesia, repetitive VF induction by 50-Hz burst stimulation was performed 10 times over 1 hour (each VF episode was ICD terminated like spontaneous VF), and then rabbits were euthanized with pentobarbital (40 mg/kg IV). LV free-wall tissue was removed, fast-frozen in liquid N₂, and stored at −80°C. Figure 6A and 6B show representative immunoblots for CaMKII and phospholamban phosphorylation and corresponding mean data. CaMKII-P expression and fractional CaMKII phosphorylation were increased by ~400% and ≈700%, respectively. Phospholamban phosphorylation was reduced at Ser16 and Thr17 by ~80% and ~50%, respectively, which was qualitatively similar to results in ES rabbit hearts.

We then tested whether repeated electrical shocks per se contribute to altered protein phosphorylation, using LV tissue samples from additional control rabbits that received ten 5-J shock pulses without VF induction over 1 hour. Unlike repeated VF/defibrillation cycles, electrical shocks alone did not reproduce the ES-associated Ca²⁺-handling protein changes (compare Figure 6C and 6D with Figures 4A, 5D, 6A, and 6B). These findings suggest that VF/defibrillation and electrical shocks differentially modulate Ca²⁺ handling and that alterations associated with ES derive from repeated VF/defibrillation cycles rather than electrical shocks alone.

Responses of ES Rabbits to Calmodulin Inhibition

CaMKII activation results from Ca²⁺/calmodulin binding. Anderson and colleagues have demonstrated in rabbit models of drug-induced long-QT syndrome that the CaMKII inhibitor KN-93 suppresses early afterdepolarizations and that the calmodulin antagonist W-7 prevents torsades de pointes, suggesting that CaMKII is a proarrhythmic signal. In the present study, to assess the potential central role of Ca²⁺/calmodulin/CaMKII signaling in ES, we administered W-7 to ES rabbits. Figure 7A illustrates the response in 1 ES rabbit. Multiple VF and VT episodes were documented almost every day during baseline infusion of dimethyl sulfoxide. Both VF and VT episodes decreased transiently after initiating 50-mg W-7 infusion and disappeared completely with 100-mg W-7 infusion. After discontinuation of W-7, arrhythmias eventually recurred. One-week infusion of W-7 to 5 rabbits caused dose-dependent reductions in VF episodes (from 25 ± 7 during dimethyl sulfoxide infusion to 13 ± 4 with the lower dose and 2.0 ± 0.9 with the higher dose), self-terminating VT episodes (from 2022 ± 579 to 711 ± 178 and 160 ± 25, respectively), and ES days (from 3.8 ± 0.7 to 1.5 ± 0.6 and 0.3 ± 0.2, respectively) over 1-week observation periods (Figure 7B). W-7 did not significantly alter RR interval or QTc (Figure VII in the online-only Data Supplement).

Five ES rabbits were added to study whether W-7 rescues LV dysfunction. We obtained LV tissue samples from ES rabbits at the end of W-7 infusion for molecular studies. Figure 7C shows representative M-mode echocardiograms in 1 ES rabbit before and after 2-week W-7 infusion. Mean echocardiographic parameters in 5 ES rabbits are summarized in Figure 7D. LV end-diastolic and end-systolic diameters were decreased significantly, by 11% and 19%, respectively, and LV fractional shortening was increased to 30 ± 2% from 23 ± 2% before infusion (P = 0.047). Representative immunoblots show that CaMKII-P bands were attenuated by W-7 in ES rabbits (Figure 8A). Overall, W-7 significantly reduced CaMKII-P expression and fractional CaMKII phosphorylation (Figure 8B). Because CaMKII promotes apoptosis and

Figure 4. A, Top, Representative immunoblots of autophosphorylated Ca²⁺/calmodulin-dependent protein kinase II (CaMKII-P), CaMKIIΔ, calmodulin (CaM), and GAPDH in left ventricular tissue samples from 4 control (CTL), 4 non–electrical storm (ES), and 4 ES rabbits. Bottom, Mean ± SEM band intensities in control (n = 6), non-ES (n = 7), and ES (n = 8) rabbits. B and C, Immunoblots of protein kinase A (PKAα-catalytic subunit, autophosphorylated protein kinase CaMKIIα) catalytic subunit, autophosphorylated protein kinase CaMKIIα-catalytic subunit, total PKCα, protein phosphatases 1 and 2A (PP1 and PP2A). D and E, Mean ± SEM band intensities corresponding to B and C in control (n = 6 to 8), non-ES (n = 5 to 7), and ES (n = 6 to 8) groups. *P < 0.05, **P < 0.01 vs control; ††P < 0.01 vs non-ES group.
stimulates hypertrophic transcriptional programs, we examined whether the cardiomyopathic effects of CaMKII were inhibited by W-7. Compared with controls, ES rabbits showed significant increases in caspase-3 subunits (Figure 8C and 8D) and atrial natriuretic factor and brain natriuretic peptide mRNA expression (Figure 8E), which were not suppressed by W-7 treatment. These findings indicate that the beneficial effects of W-7 are not associated with suppression of biomarkers of apoptosis or cardiac hypertrophy.

**Discussion**

ES is a significant clinical problem with a substantial mortality rate, particularly in the ICD population. In this article, we report findings in an animal model of ES based on an arrhythmic substrate induced by severe bradycardia due to CAVB. ES, manifested as frequently recurrent VT/VF episodes, was observed in approximately half of CAVB rabbits equipped with ICDs and allowed us to assess associated changes in CaMKII and phospholamban, suggesting that they may result from ES rather than simply being an associated finding. Calmodulin inhibition with W-7 suppressed VT/VF episodes and rescued LV dysfunction in association with a significant reduction in CaMKII hyperphosphorylation.

**Relationship to Previous Studies in Related Arrhythmogenic Animal Models**

The cellular and molecular bases of arrhythmogenic ventricular remodeling in CAVB rabbits have been characterized previously. CAVB rabbit cardiomyocytes show action potential duration (APD) prolongation and increases in cell shortening, systolic [Ca^{2+}] transients, and SR Ca^{2+} content. Downregulation of subunits encoding rapid and slow delayed-rectifier K^-current components underlies APD prolongation. CaMKII activation and Ca^{2+}-handling alterations enhance contractile function but at the same time promote arrhythmogenic afterdepolarizations. SR Ca^{2+} load is increased by APD prolongation and enhanced SR Ca^{2+} uptake due to phospholamban hyperphosphorylation, leading to increased systolic Ca^{2+} release.

The findings in the present study of ES rabbits show some similarities and differences relative to previous work in...
shorter-term CAVB rabbits. QT prolongation and CaMKII hyperphosphorylation are prominent in both. However, phospholamban was dephosphorylated in ES rabbits, in contrast to phospholamban hyperphosphorylation in rabbits with 2-week CAVB. Perhaps as a result, contractility was reduced in ES rabbits, contrasting with hypercontractility in shorter-term CAVB. The discrepancies may be partly due to the effects of repeated VF/defibrillation, which directly induces phospholamban dephosphorylation (Figure 6B).

The striking CaMKII hyperphosphorylation (indicating CaMKII autophosphorylation and activation) and phospholamban dephosphorylation observed in the present study are likely key findings. CaMKII-P expression was increased 5.7-fold in ES rabbits, a level exceeding changes seen previously in other arrhythmogenic animal models, including CaMKIIδ-overexpressing mice (≈3-fold wild-type values),7 CaMKIV-overexpressing mice (CaMKII-P data not shown, but CaMKII activity increased ≈1.5-fold),8 rabbits with HF after aortic banding/regurgitation (≈1.5-fold),24 and 2-week CAVB rabbits (≈2.5-fold),23 whereas changes in non-ES rabbits remained comparable to previous reports. CaMKII activation causes RyR2 hyperphosphorylation, producing RyR diastolic Ca\(^{2+}\) leak that promotes both HF (because of loss of SR Ca\(^{2+}\)) and arrhythmogenesis (via arrhythmogenic afterdepolarizations).22,25,26 CaMKII activation also increases LTCC phosphorylation, as observed in the present study, leading to arrhythmogenic increases in Ca\(^{2+}\) window current.23,25 Phospholamban phosphorylation removes phospholamban-induced SERCA inhibition, enhancing SR Ca\(^{2+}\) uptake and improving cardiac relaxation and contraction, whereas phospholamban dephosphorylation has opposite effects, impairing diastolic and systolic function.10,22 Thus, both CaMKII hyperphosphorylation and phospholamban hypophosphorylation are likely to be important contributors to the contractile failure we observed in ES rabbits.

Extensive work implicates CaMKII hyperphosphorylation of RyR2 in arrhythmogenesis and defective Ca\(^{2+}\) han-
In a rabbit HF model, Ai et al recently showed CaMKII activation and RyR hyperphosphorylation at both Ser2809 (PKA/CaMKII) and Ser2815 (CaMKII) sites. HF cardiomyocytes had enhanced SR Ca\(^{2+}\)/H\(^{+}\) leak and reduced SR Ca\(^{2+}\)/H\(^{+}\) load, which improved with CaMKII inhibition. CaMKII overexpression in rabbit cardiomyocytes also causes RyR2 hyperphosphorylation at both sites, as well as increased diastolic Ca\(^{2+}\)/H\(^{+}\) spark frequency. There have been disagreements about which RyR2 phosphorylation site(s) (e.g., Ser2809, Ser2815, and/or others) induces diastolic SR Ca\(^{2+}\) leak. In the present study, ES rabbits showed RyR2 hyperphosphorylation at both sites, whereas non-ES rabbits demonstrated Ser2815 hyperphosphorylation alone, potentially contributing to the arrhythmic phenotype and LV dysfunction in ES rabbits.

The effect of CaMKII on LTCCs has also been implicated in arrhythmogenesis. Recently, Koval et al provided direct evidence that Ca\(_{\text{v}1.2}\) \(\beta\)-subunits Thr498 and Leu493 are responsible for CaMKII actions to promote a high-activity Ca\(^{2+}\) channel gating mode (mode 2), Ca\(_{\text{v}1.2}\) current facilitation, and early afterdepolarizations that CaMKII actions at the \(\beta\)-subunit are sufficient to induce early afterdepolarizations in the absence of SR Ca\(^{2+}\) release. These findings, together with our results showing CaMKII activation and Ca\(_{\text{v}1.2}\) hyperphosphorylation, suggest that LTCC reactivation might be a more central mechanism than RyR2 Ca\(^{2+}\) leak for ES in this model.

**Mechanism of Postshock VF Reinitiation**

Few studies have addressed mechanisms of postshock VF reinitiation. Zaugg et al showed in Langendorff-perfused rat hearts that VF-induced [Ca\(^{2+}\)]\(_{\text{i}}\) overload causes failure of electric defibrillation and postshock VF reinitiation. Successful defibrillation led to a reduction in [Ca\(^{2+}\)]\(_{\text{i}}\) load. Incomplete reversal of [Ca\(^{2+}\)]\(_{\text{i}}\) overload after defibrillation was followed by spontaneous [Ca\(^{2+}\)]\(_{\text{i}}\) oscillations and VF reinitiation. Impaired SERCA function caused by phospholamban dephosphorylation impairs diastolic Ca\(^{2+}\) clearance from the cytosol and, along with diastolic Ca\(^{2+}\) releases from hyperphosphorylated RyRs, promotes diastolic [Ca\(^{2+}\)]\(_{\text{i}}\) overload.

Another mechanism proposed by Ogawa et al is that postshock APD abbreviation associated with unaltered [Ca\(^{2+}\)]\(_{\text{i}}\) transient duration enhances Na\(^{+}\)-Ca\(^{2+}\) exchanger currents, increasing the likelihood of afterdepolarizations that
reinduce VF. Recently, Wagner et al. demonstrated that CaMKII overexpression in a transgenic mouse model prolonged APD by downregulating Kv4.2/KChIP2 and Kir2.1 and accelerating I_o, recovery in rabbit cardiomyocytes.

**Figure 8.** A, Immunoblots of autophosphorylated Ca^2+/-calmodulin-dependent protein kinase II (CaMKII-P) and CaMKIIβ in left ventricular tissue samples from 1 control (CTL), 3 electrical storm (ES) rabbits, and 5 ES rabbits treated with W-7 (ES+W-7). B, Mean±SEM band intensities in control (n=6), ES (n=6), and ES+W-7 (n=5) rabbits. C and D, Immunoblots of procaspase-3 and caspase-3 subunits (activated forms) and mean data in control (n=6), ES (n=6), and ES+W-7 (n=5) rabbits. E, Mean±SEM mRNA expression of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) in control (n=5), ES (n=5), and ES+W-7 (n=5) rabbits. *P<0.05, **P<0.01 vs control; ††P<0.01 vs ES rabbits.

**Novel Findings and Potential Significance**

To our knowledge, this study is the first to report the molecular basis of ES in a clinically relevant chronic animal model. Many studies have described CaMKII activation as a signal for proarrhythmia and myocardial dysfunction, but ours is the first to implicate Ca^2+/-calmodulin/CaMKII signaling in ES pathophysiology. Not only was CaMKII hyperphosphorylation observed at functionally important CaMKII autophosphorylation, RyR2, and LTCC sites, but a therapeutic intervention targeting Ca^2+/-calmodulin interaction demonstrated ES suppression and LV dysfunction improvement along with a reduction in CaMKII hyperphosphorylation. However, the phosphorylation abnormalities associated with ES were more complex than would be expected from CaMKII activation alone. The catalytic subunit of PKA was downregulated, and protein phosphatases (PP1 and PP2A) were upregulated, accounting for profound hypophosphorylation of phospholamban. The phosphorylation state of phospholamban determines its association with and inhibition of SERCA2a. Phospholamban dephosphorylation reduces SERCA2a function and, in combination with diastolic SR Ca^2+ leak due to RyR2 hyperphosphorylation, would be expected to reduce SR Ca^2+ stores and impair contractility, potentially accounting for the LV function impairment that we observed in ES rabbits. These observations may account for the tendency of ES to induce myocardial pump failure, which is the principal cause of excess mortality in ES patients.

Our results provide potential insights into the pathophysiology of ES. The initiating factors for ES are often cryptic, although psychological stress, acute myocardial ischemia, and electrolyte imbalances have all been implicated. We noted that repeated cycles of VF/defibrillation by themselves cause CaMKII hyperphosphorylation, a central component of CaMKII overactivity that favors arrhythmia occurrence and myocardial dysfunction. It is very possible that repeated VF/defibrillation cycles induce a positive-feedback cycle that enhances the probability of recurrent VF episodes and exacerbates contractile failure. This notion is supported by successful therapeutic effects of W-7.

Our findings also have potential implications for the clinical management of ES. Drug therapy of ES is problematic, with conventional antiarrhythmic agents providing little benefit and β-blockers and amiodarone seeming to be of the greatest value. Adrenergic receptor stimulation activates CaMKII; thus, antagonism of adrenergic activation of CaMKII may account for the beneficial actions of β-blockers and amiodarone. Our results also point to potential new approaches to treating and/or preventing ES. A Ca^2+/-calmodulin interaction inhibitor (W-7) was highly effective in suppressing ES in our study. Interventions targeting the Ca^2+/-calmodulin/CaMKII system may prove highly beneficial in preventing both further arrhythmic episodes and hemodynamic deterioration in ES patients.

**Limitations**

We focused on molecular changes underlying ES, performing detailed immunoblotting studies of Ca^2+/-handling proteins in ES and non-ES rabbits. Because of the long preparation time and high mortality rates in this model, the number of available ES and non-ES rabbits was limited, and therefore we were unable to perform extensive functional studies of the
cellular mechanisms predicted to result from protein phosphorylation disturbances. The precise mechanisms underlying the prominent phospholamban dephosphorylation observed in ES rabbits remain to be determined. Further studies on subcellular distribution of PP1 are needed to understand why phospholamban was dephosphorylated, a change opposite that seen for RyR2 and LTCC, which were hyperphosphorylated. Specific macromolecular compartmentalization of kinases, phosphatases, and target proteins is likely involved.

Long-term CAVB rabbits studied here had lower LV fractional shortening than at baseline, which is in contrast to a dog model of CAVB in which contractile function remains preserved. The ventricular rate decreases comparably, from \( \approx 280 \) bpm in sinus rhythm to 90 to 115 bpm in escape rhythm in rabbits versus from \( \approx 115 \) to 40 to 50 bpm in dogs. The discrepancy in cardiac function response may be due to different species-dependent adaptation mechanisms or to greater spontaneous VT/VF occurrence in rabbits. The detailed electrophysiological mechanisms underlying VF storm, particularly related to the relative roles of afterdepolarization-related triggered activity versus reentry, remain to be defined. These issues go beyond the scope of the present study. We corrected QT intervals by the Carlson formula for rabbits, the validity of which was not tested under the bradycardic conditions of this study. We primarily examined LV tissue and function because LV function appears to be the principal factor associated with the occurrence and consequences of ES. We found that CaMKII activation, the central change involved in ES pathophysiology, occurs in the right ventricle as well as the LV, but we did not otherwise examine the right ventricle in detail.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Electrical storm (ES), characterized by recurrent ventricular tachycardia/fibrillation, is an increasing problem among implantable cardioverter-defibrillator patients. Despite acute cessation of ES with medical therapy and/or catheter ablation, the early mortality (within a few months of ES) is high and often nonsudden, involving progressive heart failure in particular. Underlying mechanisms are unknown. Defective Ca\(^{2+}\) handling is central to the pathogenesis of heart failure. Changes in phosphorylation of L-type Ca\(^{2+}\) channels, sarcoplasmic reticulum Ca\(^{2+}\)-release channel ryanodine receptors, and the sarcoplasmic reticulum Ca\(^{2+}\) uptake regulator protein phospholamban are linked to heart failure–related mechanical dysfunction and arrhythmogenic afterdepolarizations. To study the molecular basis of ES-related cardiac deterioration, we studied rabbits with chronic complete atrioventricular block equipped with implantable cardioverter-defibrillators. They developed QT prolongation and implantable cardioverter-defibrillator–detected ventricular fibrillation episodes, with ES (defined as clustered, frequently recurrent ventricular fibrillation episodes) occurring in \(\approx\)50%. ES rabbits showed left ventricular function deterioration, along with striking activation of the Ca\(^{2+}\)-sensitive phosphorylating enzyme Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and enhanced protein phosphatase expression. These alterations produced important changes in phosphorylation patterns, notably hyperphosphorylation of L-type Ca\(^{2+}\) channels and ryanodine receptors and dephosphorylation of phospholamban, which could explain arrhythmias and impaired contractility. Repeated ventricular fibrillation induction/defibrillation with implantable cardioverter-defibrillator shocks in control rabbits reproduced ES-related changes in CaMKII and phospholamban phosphorylation. Infusion of the calmodulin antagonist W-7 suppressed ventricular tachycardia/ventricular fibrillation episodes and rescued left ventricular dysfunction in ES rabbits, indicating a central pathophysiological role of CaMKII activation. These results strongly support the notion that CaMKII activation and Ca\(^{2+}\)-handling abnormalities resulting from ES events might be responsible for negative outcomes and suggest that interventions targeting the Ca\(^{2+}\)/calmodulin/CaMKII system might provide benefits in ES patients.
Ca\(^{2+}\)-Related Signaling and Protein Phosphorylation Abnormalities Play Central Roles in a New Experimental Model of Electrical Storm

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SUPPLEMENTAL MATERIAL
Supplemental Figure Legends

**Supplemental Figure 1.** Representative 2 episodes of non-sustained ventricular tachyarrhythmias (NSVT) detected and stored in ICDs. TdP-like VTs were detected as NSVTs.

**Supplemental Figure 2.** VF episodes were almost completely suppressed while the pacing rate was increased to 110 bpm. Multiple VF episodes developed again 1 day after the pacing rate back to 60 bpm at Day 48. This rabbit died at Day 50, with the last VF episode report showing VF-undersensing following 4 programmed therapies for repeated post-shock VF initiation.

**Supplemental Figure 3.** The daily number of VF-episodes in 2 ES-rabbits that had multiple VF-episodes early and transiently. VF-episodes occurred in a cluster in rabbit 2. Both rabbit 1 and 2 died of defibrillation failure during ES on Day 23 and Day 46, respectively.

**Supplemental Figure 4.** Top: Representative western blots of CaMKII-P and CaMKIIδ in RV tissue samples from 3 CTL, 3 non-ES and 3 ES-rabbits. Bottom: Mean±SEM band intensities in CTL (n=7), non-ES (n=5) and ES (n=5) rabbits. **P<0.01 versus CTL, †P<0.05 versus non-ES.

**Supplemental Figure 5.** A, Representative western blots of MyBP-C total, phospho-MyBP-C Ser282, Tn-I total, phospho-Tn-I Ser23/24, and phosphorylated MLC-2a. B, Mean±SEM band intensities and phosphorylation-fractions in CTL (n=5), non-ES (n=5) and ES (n=6) rabbits. *P<0.05 versus CTL.
**Supplemental Figure 6.** Top: Representative western blots for I-1, I-1 P67 and I-1 P35. Bottom: Mean±SEM I-1, I-1 P67 and I-1 P35 protein expression and phosphorylation-fraction in CTL (n=6), non-ES (n=5) and ES (n=6) rabbits.

**Supplemental Figure 7.** QTc (circles) and RR intervals (squares) during infusion in 5 ES-rabbits.
Supplemental Figure 2

The number of VF episodes per day vs. day for VVI pacing rate (bpm).
Supplemental Figure 7

The figure shows a graph with two sets of data: "RR" and "QTc". The x-axis represents different treatment periods: DMSO, W-7 50 mg, W-7 100 mg, and No treatment. The y-axis represents time in milliseconds (ms) ranging from 100 to 700 ms.

The graph indicates changes in RR and QTc over time with error bars representing variability. The data points suggest a trend in the treatment effects over the 30-day period.