Ca\textsuperscript{2+}/Calmodulin Kinase II–Dependent Phosphorylation of Ryanodine Receptors Suppresses Ca\textsuperscript{2+} Sparks and Ca\textsuperscript{2+} Waves in Cardiac Myocytes


Abstract—The multifunctional Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II \(\delta\textsubscript{C} \) (CaMKII\(\delta\textsubscript{C} \)) is found in the macromolecular complex of type 2 ryanodine receptor (RyR2) Ca\textsuperscript{2+} release channels in the heart. However, the functional role of CaMKII-dependent phosphorylation of RyR2 is highly controversial. To address this issue, we expressed wild-type, constitutively active, or dominant-negative CaMKII\(\delta\textsubscript{C} \) via adenoviral gene transfer in cultured adult rat ventricular myocytes. CaMKII-mediated phosphorylation of RyR2 was reduced, enhanced, or unaltered by dominant-negative, constitutively active, or wild-type CaMKII\(\delta\textsubscript{C} \) expression, whereas phosphorylation of phospholamban at Thr17, an endogenous indicator of CaMKII activity, was at 73%, 161%, or 115% of the control group expressing \(\beta\)-galactosidase (\(\beta\)-gal), respectively. In parallel with the phospholamban phosphorylation, the decay kinetics of global Ca\textsuperscript{2+} transients was slowed, accelerated, or unchanged, whereas spontaneous Ca\textsuperscript{2+} spark activity was hyperactive, depressed, or unchanged in dominant-negative, constitutively active, or wild-type CaMKII\(\delta\textsubscript{C} \) groups, respectively. When challenged by high extracellular Ca\textsuperscript{2+}, both wild-type and constitutively active CaMKII\(\delta\textsubscript{C} \) protected the cells from store overload–induced Ca\textsuperscript{2+} release, manifested by a \(\sim 60\%\) suppression of Ca\textsuperscript{2+} waves (at 2 to 20 mmol/L extracellular Ca\textsuperscript{2+}) in spite of an elevated sarcoplasmic reticulum Ca\textsuperscript{2+} content, whereas dominant-negative CaMKII\(\delta\textsubscript{C} \) promoted Ca\textsuperscript{2+} wave production (at 20 mmol/L Ca\textsuperscript{2+}) with significantly depleted sarcoplasmic reticulum Ca\textsuperscript{2+}. Taken together, our data support the notion that CaMKII\(\delta\textsubscript{C} \) negatively regulates RyR2 activity and spontaneous sarcoplasmic reticulum Ca\textsuperscript{2+} release, thereby affording a negative feedback that stabilizes local and global Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release in the heart. (Circ Res. 2007;100:399-407.)

Key Words: Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II ■ Ryanodine receptor ■ Ca\textsuperscript{2+} sparks ■ Ca\textsuperscript{2+} waves ■ Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release

The type 2 ryanodine receptor (RyR2) Ca\textsuperscript{2+} release channels in the sarcoplasmic reticulum (SR) constitute a key component of excitation–contraction (EC) coupling in cardiac myocytes. Activated by the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mechanism, they are responsible for generating 70% to 90% of the transient increase of intracellular Ca\textsuperscript{2+} that drives the heartbeat.\textsuperscript{1} Under Ca\textsuperscript{2+} overload conditions, RyRs mediate spontaneous Ca\textsuperscript{2+} waves\textsuperscript{2-3} or store overload–induced Ca\textsuperscript{2+} release (SOICR),\textsuperscript{4} which is thought to be arrhythmogenic for disturbance of cardiac electrical activity. Given its central role in cardiac EC coupling and Ca\textsuperscript{2+} signaling, RyR2 activity is under the exquisite control of an array of molecular partners found in the RyR2 macromolecular signaling complex, including protein kinase A (PKA), Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C, and protein phosphatases 1, 2A, and 2B (calcineurin).\textsuperscript{5,6} Among these, CaMKII-dependent phosphorylation of RyR2 is of particular interest, because the waxing and waning of high microdomain Ca\textsuperscript{2+} accompanying the channel gating might activate and deactivate this kinase, affording a local feedback regulation to SR Ca\textsuperscript{2+} release.\textsuperscript{7}

Early studies have suggested that RyR2 possesses a unique CaMKII phosphorylation site at Ser2809, which is also phosphorylated by PKA to a lesser extent.\textsuperscript{8} In contrast, more recent studies proposed that Ser2809 is a PKA-specific phosphorylation site in RyR2,\textsuperscript{6,9} whereas our own data...
support that Ser2809 is not an exclusive PKA-dependent phosphorylation site, but is also phosphorylated by CaMKII and other kinases. Furthermore, a 4:1 stoichiometry for CaMKII:PKA phosphorylation of RyR2 has been inferred from metabolic labeling. With the recent identification of Ser 2030 as a novel PKA-specific site, 10 Ser2815 has been shown to be phosphorylated by CaMKII. The functional consequences of RyR2 phosphorylation in the healthy and diseased heart are highly controversial, and the conflicting literature falls into 4 categories: enhancement, inhibition, no function, or complicated regulation, with the enhancement view being prevalent. 12 Using canine cardiac functional SR vesicles or partially purified RyR2 fused into planar bilayers, it has been shown that CaMKII phosphorylation activates the channel by increasing open channel probability (P_o). 8,13 However, Hain et al14 found that activation of endogenous CaMKII led to RyR2 channel closure, whereas exogenous CaMKII activated the channel. Using photolytic Ca^{2+} steps, Valdivia et al15 showed that RyR2 response to CaMKII- or PKA-dependent phosphorylation is rather complex, characterized by a higher peak P_o, a markedly accelerated “adaptation,” and a reduced steady-state P_o. The results from studies using swine and rabbit SR vesicles suggested that the intracellular signaling pathway leading to CaMKII activation reduced Ca^{2+} efflux from the SR by inhibiting RyR2 channel activity. 16 By inclusion of constitutive active CaMKII in the patch pipette under whole-cell patchclamp conditions, Anderson and colleagues demonstrated that CaMKII reduced depolarization-triggered Ca^{2+} release from the SR and elevated SR Ca^{2+} content. 17 In a series of studies, Marks and colleagues advocated the idea that PKA phosphorylation at Ser2809 displaces calstabin (FKBP 12/12.6) from the channel complex, that CaMKII:PKA phosphorylation of RyR2 has been inferred from metabolic labeling.11 With the recent identification of Ser2809 as a novel PKA-specific site,10 Ser2815 has been shown to be phosphorylated by CaMKII.

Materials and Methods

Construction of Viral Vectors

Hemagglutinin (HA)-tagged constitutively active CaMKIIδ (CA CaMKII) was generated by replacing the residue Thr287 with aspartic acid (T287D) using the transformer site directed mutagenesis kit (Clontech). Negative control CaMKIIδ (DN CaMKII) was generated by replacing the residue Lys43 with alanine (K43A).26 The generation and amplification of adenoviruses harboring the target gene were performed in HEK293 cells.

Cell Culture and Adenoviral Infection

Single cardiac myocytes were isolated from the hearts of 2- to 3-month-old Sprague–Dawley rats using a standard enzymatic technique, then cultured and infected with adenoviral vectors at a multiplicity of infection (moi) of 100.27 Myocytes were plated at a density of 0.5 to 1×10^5 cells/cm^2 on coverslips or in dishes precoated with 10 μg/mL laminin. The culture medium was medium 199 (Sigma) in the presence of (in mmol/L) creatine 5, L-carnitine 2, taurine 5, insulin/transferrin/selenium-X 0.1%, penicillin and streptomycin 1%, and HEPES 25 (pH=7.4 at 37°C). Experiments were performed in cells cultured for 24 hours following infection. Procedures were performed according to Guiding Principles in the Care and Use of the Animals approved by the Council of the American Physiological Society.

Western Blotting and Immunostaining

To quantify the expression of wild-type (WT) and mutant CaMKIIδ, cell lysates (30–50 μg protein) were loaded in a Ca^{2+}-free loading buffer containing 20 mmol/L EDTA and immunoblotted using anti-HA antibody (1:5000, Berkeley Antibody Co) and horseradish peroxidase–conjugated secondary antibody (Bio-Rad). Other antibodies include the site-specific antibody reacting with PLB phosphorylated at Thr17 (1:10 000, Badrilla, Leeds, UK), anti–total PLB antibody (1:5000, Badrilla), anti-RyR2 antibody (1:5000, ABR), and site-specific antibodies recognizing the phosphorylated RyR2 at Ser2809 (1:5000, Badrilla, UK) and at Ser2030.10 For immunostaining, cells were incubated with anti-HA antibody (1:1000) at 4°C overnight after fixation and blocking and then stained with Cy5-conjugated secondary antibody (1:500, Jackson ImmunoResearch). Confocal images were obtained using a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc, Thornwood, NY) with 633-nm laser line to excite the Cy5 fluorophore.

CaMKII Activity Assay

To measure CaMKII activity, cell lysate (200 μg of protein) was first immunoprecipitated with anti-CaMKII antibody (1:100) in a medium containing (in mmol/L) Tris–HCl 20, NaCl 150, Na_2EDTA 1, EGTA 1, Triton X%, sodium pyrophosphate 2.5, β-glycerophosphate 1, NaVO_3 1, leupeptin 1 μg/mL (pH=7.4). The precipitated proteins were then incubated with a specific peptide substrate (KKALR-RQETVDAL) to evaluate the kinase activity according to the recommendations of the manufacturer (Upstate Biotechnology Inc), as previously described.27

Ca^{2+} Measurements

Myocytes were loaded with the Ca^{2+} indicator fluo-4 acetoxymethyl ester (fluo-4 AM) (20 μmol/L, 30 minutes) (Invitrogen) and Ca^{2+} sparks, Ca^{2+} transients, and Ca^{2+} waves as the physiological readouts in an attempt to further delineate the possible role of CaMKIIδ phosphorylation in regulating RyR function in cardiac myocytes.
applied to the cell locally. For spontaneous contractile wave measurement, the perfusion solution contained (in mmol/L) NaCl 138.2, KCl 4.6, MgCl₂ 1.2, glucose 15, and HEPES (pH 7.4) at various Ca²⁺ concentrations (1, 2, 5, 10, or 20), without readjusting the osmolarity and ionic strength. All measurements were performed at room temperature (22°C to 23°C). Image processing, data analysis, and presentation were performed using IDL software (version 6.1, Research Systems Inc Co).

**Back-Phosphorylation of RyR2**  
Homogenates (500 µg) were suspended in 0.5 mL of buffer (50 mmol/L Tris-HCl, pH 7.4), 0.9% NaCl, 0.5 mmol/L NaF, 0.5 mmol/L Na₂VO₄, 0.25% Triton X-100, and protease inhibitors and immunoprecipitated with anti-RyR2 antibody (1:300). The immunoprecipitated RyR2 was then incubated for 45 minutes at room temperature in 10 µL of preactivated 1× CaMKII reaction buffer (50 U; BioLabs), MgATP (100 µmol/L), and 10% [³²P]ATP for back-phosphorylation assay. The CaMKII inhibitor KN-93 (10 µmol/L) was added to the reaction solution as negative control. After SDS-PAGE, the level of RyR2 protein and the associated radioactivity were determined using an anti-RyR2 antibody (1:5000) and autoradiography, respectively. The densities of total RyR2 and autoradiography were quantified using Multi-Analyst (Bio-Rad).

**Statistics**  
Data were reported as mean±SEM. Student’s t test, paired t test, or Kruskal–Wallis test was applied, when appropriate, to determine statistical significance of the differences. A probability value of less than 0.05 was considered statistically significant.

**Results**

**Manipulation of CaMKIIδc Activity in Cardiac Myocytes**  
To up- and downregulate CaMKIIδc activity, ventricular myocytes were infected with adenoviral vectors carrying the target gene for WT and mutant CaMKIIδc at moi of 100 and cultured for 24 hours. Confocal immunocytochemical imaging (Figure 1A) visualized that the WT and mutant CaMKIIδc were similarly localized to the cytoplasm and the surface membrane including the transverse tubules, consistent with previous reports.²⁸,²⁹ Using Western blotting with an antibody recognizing the HA epitope, we found comparable expression for the mutants (CA and DN CaMKIIδc) and higher expression for CaMKIIδc (Figure 1B). Direct measurement of the Ca²⁺-independent kinase activity was assessed in preactivated form in resting cells at 1 mmol/L extracellular Ca²⁺. In the DN, WT, and CA CaMKIIδc groups, the Ca²⁺-dependent kinase activity relative to the β-gal group was 0.19±0.08 (n=3, P<0.01), 1.8±0.3 (n=3, P<0.05), and 4.0±0.4 (n=4, P<0.01), respectively. PLB, a key physiological regulator of SR Ca²⁺-ATPase (SERCA), bears a PKA-specific phosphorylation site Ser16 and a CaMKII-specific phosphorylation site Thr17. Phosphorylation of either is sufficient to remove PLB inhibition of SERCA, leading to accelerated SR Ca²⁺ recycling and hastened cardiac relaxation.³⁰ Compared with cells infected with adenov-β-gal at the same moi, the expression of CA and DN CaMKIIδc resulted in a 0.6-fold increase and 0.3-fold decrease, respectively, of PLB Thr17 phosphorylation, whereas WT CaMKIIδc expression did not affect PLB phosphorylation (Figure 1C and 1D). Notably, total PLB level was unchanged among the groups, suggesting no compensatory change in PLB expression.

**Effect of CaMKIIδc on the Decay of Ca²⁺ Transients**  
Using the aforementioned cell models, we examined the overall effects of CaMKIIδc activation on intracellular Ca²⁺ transients and contraction. Figure 2A shows typical examples of fluo-4 line-scan images and their corresponding time courses from cells expressing WT, CA, and DN CaMKIIδc. CA CaMKIIδc expression induced a profound positive relaxant effect at the single-cell level (Figure 2A and 2D). The T50 was reduced by ≈15% compared with WT CaMKIIδc or β-gal. Conversely, DN CaMKIIδc expression prolonged the T50 by ≈15% (Figure 2A and 2D). Enhanced CaMKII activity accelerates cardiac relaxation and reduced CaMKII activity slows the decay of Ca²⁺ transients. These changes occurred in parallel with the changes in the status of PLB Thr17 phosphorylation (Figure 1C and 1D), in agreement with previous reports.³¹,³²

Interestingly, we found no significant effects of CaMKIIδc (WT, CA, or DN) on the amplitudes of the Ca²⁺ transients.
(Figure 2B) or contraction (Figure 2C). Because the CaMKII-mediated enhancement of L-type Ca\(^{2+}\) currents (Figure I in the online data supplement, available at http://circres.ahajournals.org)\(^{28,32}\) and SR Ca\(^{2+}\) recycling\(^{30,31,33}\) (Figure 2D) would predict a positive (or negative) inotropic effect in response to enhanced (or reduced) CaMKII activity, this result provided the first clue that CaMKII may also negatively regulate some key component(s) of EC coupling, giving rise to a net null effect on cardiac inotropy.

**Phosphorylation of RyR2 by CaMKII\(_C\)**

We measured the status of RyR2 phosphorylation in response to the expression of WT and mutant CaMKII\(_C\) by using the back-phosphorylation assay. Figure 3A shows a representative autoradiograph of \(^{32}\)P-ATP incorporation into RyR2 after CaMKII phosphorylation in vitro and Western blotting analysis of the corresponding RyR2 protein. As a negative control, we showed that inhibition of CaMKII by KN-93 completely blocked back-phosphorylation of RyR2 (Figure 3A and 3B). We found no difference in the \(^{32}\)P-RyR/total RyR densitometric ratio between \(\beta\)-gal control (0.653±0.046, n=6) and WT CaMKII\(_C\) (0.630±0.078, n=6) but a significant decrease in the CA CaMKII\(_C\) group (0.159±0.017, n=6, P<0.001) and an increase in the DN CaMKII\(_C\) group (0.857±0.054, n=6, P<0.05), respectively. Hence, RyR2 was hyperphosphorylated in the CA CaMKII\(_C\) group and hypophosphorylated in the DN CaMKII\(_C\) group compared with WT CaMKII\(_C\) and \(\beta\)-gal.

To test the possibility that RyR2 phosphorylation by PKA might also be altered via crosstalk between CaMKII and PKA pathways, we used a site-specific antibody reacting with phospho-Ser2030, a PKA-specific site in cardiac myocytes.\(^{10}\) Western blotting clearly showed that there is no detectable signal in all 4 groups (Figure 3B, bottom). In the positive control group, the same antibody detected PKA phosphorylation of purified RyR2 expressed in HEK 293 cells.
Using a phospho-Ser2809–specific antibody, we found significant basal phosphorylation of RyR2 in the β-gal group, whereas no significant changes were found among the WT and DN CaMKII ΔC and β-gal groups (Figure 3B), except for a slight hyperphosphorylation in the CA CaMKII ΔC group (2.18 ± 0.16 versus 1.55 ± 0.09 of β-gal control; n = 5 for both groups, P < 0.05). This observation supports the notion that Ser2809 retains some sensitivity to CaMKII. Unlike the CaMKII ΔC transgenic animal model,21 acute CaMKII manipulation did not alter the RyR protein level (Figure 3A and 3B).

CaMKII ΔC Modulation of Ca2+ Sparks

Characteristics of Ca2+ sparks are informative in characterizing CICR sensitivity, RyR gating, and SR Ca2+ leakage in intact cells. The frequency of spontaneous Ca2+ sparks was diminished from 2.75 ± 0.30 (n = 35 cells from 6 hearts) in the β-gal group to 1.64 ± 0.17 Hz/100 μm (n = 50, P < 0.05) in CA CaMKII ΔC group (Figure 4B). In contrast, DN CaMKII ΔC induced hyperactive Ca2+ sparks, increasing the spark frequency to 4.21 ± 0.45 Hz/100 μm (n = 56, P < 0.01 versus β-gal) (Figure 4B). In WT CaMKII ΔC, there was a trend of decreasing spark frequency (2.33 ± 0.28, n = 60), which did not reach statistical significance. Properties of individual Ca2+ sparks were largely unchanged among groups, with 2 exceptions (Figure 4C and supplemental Figures II and III).

The spark rising time, which reflects the termination kinetics of the release channel opening, was significantly prolonged in the DN CaMKII ΔC compared with the WT group (21.12 ± 0.44 ms, n = 611, versus 19.44 ± 0.60 ms, n = 274, P < 0.01) (supplemental Figure II). DN CaMKII ΔC also slightly reduced spark amplitude (Figure 4C) despite the longer rising time and unchanged SR Ca2+ content (Figure 4D), suggesting smaller Ca2+ release fluxes in Ca2+ sparks on CaMKII inhibition. Taken together, CaMKII inhibition and RyR hypophosphorylation cause a “leaky” SR, whereas CaMKII activation and RyR hyperphosphorylation suppress spontaneous SR Ca2+ release.

Acute manipulation of CaMKII activity in cultured cells did not affect the overall Ca2+ homeostasis in quiescent cells at 1 mmol/L extracellular Ca2+. Ratiometric Ca2+ measurements with indo-1 as the Ca2+ indicator revealed no significant change in either the resting Ca2+ level (r410/490) (Figure 5B) or the caffeine-labile SR Ca2+ content (Figure 5C) among the groups (see below for results at 20 mmol/L). Moreover, the half-decay time of caffeine-induced Ca2+ transients, an index of Ca2+ clearance by Na+/Ca2+ exchange, showed no detectable difference either (Figure 5D).

CaMKII ΔC Suppresses Ca2+ Waves

The above results suggest that CaMKII stabilizes, rather than destabilizes, Ca2+ signaling. If such stabilization were the case, it might be expected that CaMKII activation would reduce the occurrence of spontaneous Ca2+ waves or SOICR.35 To test this possibility, we challenged the cells with increasing concentrations of extracellular Ca2+ to create Ca2+-overload conditions. Resting cells expressing β-gal exhibited a low frequency of Ca2+ waves at 1 mmol/L Ca2+ (0.19 ± 0.07 min−1, n = 42 cells from 3 hearts). By raising extracellular Ca2+ concentrations gradually to 2, 5, 10, and 20 mmol/L, the wave frequency was increased progressively and reversibly, up to 11.8 ± 1.1 min−1 (Figure 6A and 6B). CA CaMKII ΔC markedly reduced the genesis of Ca2+ waves at all Ca2+ concentrations higher than 2 mmol/L (Figure 6B). Interestingly, in the WT CaMKII ΔC group, the Ca2+ wave frequency was suppressed to ~30% of that in β-gal, as if WT

Figure 4. CaMKII modulation of Ca2+ sparks. A, Confocal line-scan images showing spontaneous Ca2+ sparks in cells expressing WT, CA, or DN CaMKII ΔC. B and C, Statistics of spark frequency (n = 35 to 60 cells from 6 hearts for each group; *P < 0.05 and **P < 0.01 vs β-gal or WT CaMKII ΔC) (B) and spark amplitude (F/F0) (n = 250 to 800 sparks for each group; ***P < 0.001 vs β-gal, WT, and CA CaMKII ΔC) (C).
CaMKIIΔC became activated at high extracellular Ca²⁺. DN CaMKIIΔC exerted an opposite effect on Ca²⁺ stability, evidenced by a 37% increase in Ca²⁺ wave frequency at 20 mmol/L Ca²⁺ (Figure 6A and 6B). Nevertheless, the propagation velocity and amplitude of Ca²⁺ waves were almost identical in all groups (supplemental Figure IV).

Figure 5. CaMKII modulation of resting Ca²⁺ homeostasis. A, Typical fluo-4 confocal image and its time course of caffeine-elicited Ca²⁺ transient in a cell expressing DN CaMKIIΔC. B, Resting cytosolic Ca²⁺ level, indexed by indo-1 fluorescent ratio r₄₁₀/₄₉₀ (n=20 to 37 cells from 2 hearts). C, The amplitude of caffeine-elicited Ca²⁺ transients, indexed by the difference between peak and basal indo-1 fluorescent ratio (Δr₄₁₀/₄₉₀). D, The 50% decay time (T₅₀) of caffeine-elicited Ca²⁺ transients in fluo-4–loaded cells (n=34 to 40 cells from 5 to 6 hearts).

Figure 6. CaMKII modulation of Ca²⁺ waves. A, Typical confocal line-scan images of Ca²⁺ waves at 20 mmol/L extracellular Ca²⁺. B, Ca²⁺ wave frequency in response to elevated extracellular Ca²⁺ in dye-free cells. (#P<0.01 vs β-gal control; &P<0.001 of both WT and CA CaMKIIΔC vs β-gal and DN CaMKIIΔC; n=42 to 47 from 3 to 4 hearts for each data point.) C, SR Ca²⁺ content (at 20 mmol/L extracellular Ca²⁺) measured by caffeine-induced (20 mmol/L) Ca²⁺ transient (n=30 cells from 3 hearts for each group; *P<0.05, **P<0.01, ***P<0.001 vs β-gal control).
Although no significant changes in the SR Ca\textsuperscript{2+} content were observed at 1 mmol/L Ca\textsuperscript{2+} (Figure 5C), high Ca\textsuperscript{2+} challenge did bring out the differences: increasing the SR content by WT and CA CaMKII\delta\textsubscript{c} and depleting it by DN CaMKII\delta\textsubscript{c}, as measured at 20 mmol/L Ca\textsuperscript{2+} (Figure 6C). The reciprocal regulation of Ca\textsuperscript{2+} wave frequency and SR Ca\textsuperscript{2+} content suggests that CaMKII phosphorylation of RyR2 raises the threshold of SR luminal Ca\textsuperscript{2+} concentration at which SOICR occurs.

Discussion
In the present study, we have provided experimental evidence pointing to the unexpected finding that phosphorylation of RyR2 by CaMKII negatively regulates Ca\textsuperscript{2+} spark and Ca\textsuperscript{2+} wave activities, thus serving as a stabilizing factor for SR Ca\textsuperscript{2+} release in intact cardiac myocytes. This result is in sharp contrast to the prevalent view that RyR2 phosphorylation by CaMKII enhances RyR2 Ca\textsuperscript{2+} sensitivity and causes leaky SR and Ca\textsuperscript{2+} instability.\textsuperscript{24,33}

Models of Altered CaMKII Activity
The controversy of CaMKII modulation on RyR function may arise in part from methodological limitations. Results from studies using cell-free system may not be directly relevant to intact cells, and experiments using intracellular CaMKII dialysis may not be readily reproducible because of technical difficulties. The transgenic animal approach also has its own limitations as a result of plasticity, adaptation, and maladaptation typical of any biological systems. Indeed, transgenic CaMKII\delta\textsubscript{c} overexpression is accompanied by a substantial remodeling of the Ca\textsuperscript{2+} signaling system, characterized by remarkable downregulation of RyR2, PLB, and SERCA and substantial upregulation of Na\textsuperscript{+/Ca\textsuperscript{2+}} exchange.\textsuperscript{24} In this study, we have established 3 cell models expressing WT, CA, and DN CaMKII\delta\textsubscript{c}, respectively. These ex vivo cellular models allow for bidirectional genetic manipulation of CaMKII activity, with minimal compensatory changes in the other components of the Ca\textsuperscript{2+} signaling system. Similar efforts have also been made previously to express WT CaMKII\delta\textsubscript{c} in cultured rabbit ventricular myocytes,\textsuperscript{33} although an opposite conclusion on CaMKII modulation of RyR function has been drawn (see below).

Validating criteria of the current models include CaMKII expression and localization, direct measurement of the kinase activity, and CaMKII phosphorylation of PLB and RyR2. Physiological readouts, including the decay time of action potential–elicited Ca\textsuperscript{2+} transients (Figure 2A and 2D) demonstrate either an enhanced or depressed SR Ca\textsuperscript{2+} recycling on CaMKII activation or inhibition, as expected. As an advantage of acute genetic manipulation, we detected little unwanted alterations in the Ca\textsuperscript{2+} signaling system: the expression levels of PLB and RyR2 remain unchanged and the Na\textsuperscript{+/Ca\textsuperscript{2+}} exchange activity indexed by the decay kinetics of caffeine-induced Ca\textsuperscript{2+} transients remains intact in all groups. A combination of multiple models further permitted us to detect reciprocal changes in response to up- or down-manipulation of CaMKII activity. Hence, the present cell models should be useful for investigation of direct CaMKII actions with minimal secondary long-term effects caused by remodeling of the Ca\textsuperscript{2+} signaling system.

Negative Regulation of RyR2 Activity by CaMKII\delta\textsubscript{c}
One of the main findings of the present study is that enhanced CaMKII activation reduces the rate of occurrence of spontaneous Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} waves, whereas inhibition of CaMKII activity exerts an opposite effect. Examination of spark properties has led to the insight that CaMKII modulation of sparks is predominantly via the frequency-dependent modulation mechanism. At the cellular level, the CaMKII-mediated inhibitory effect on SR Ca\textsuperscript{2+} release should negate the CaMKII effects on enhancing L-type Ca\textsuperscript{2+} channel current\textsuperscript{28,32} and SR Ca\textsuperscript{2+} recycling,\textsuperscript{30,31,33} explaining the lack of change in the peak of Ca\textsuperscript{2+} transients elicited by action potentials.

In cultured rabbit ventricular myocytes expressing WT CaMKII\delta\textsubscript{c}, however, Kohlhaas et al\textsuperscript{33} detected a large reduction of the SR Ca\textsuperscript{2+} content and, after “normalizing” the spark frequency by the corresponding depleted SR Ca\textsuperscript{2+} content, an 88% increase in spark production, reaching a conclusion that is opposite from ours. The discrepancies between the rat and rabbit results may reflect genuine species-dependent differences. To this end, it is well known that cardiac Ca\textsuperscript{2+} transients and contraction display postrest potentiation in the rat and postrest depression in the rabbit. Nevertheless, the normalization procedure used needs to be justified because our previous studies in the rat have shown that unloading the SR Ca\textsuperscript{2+} below its physiological level does not affect Ca\textsuperscript{2+} spark production.\textsuperscript{37}

The most compelling evidence for CaMKII-mediated negative regulation of spontaneous SR Ca\textsuperscript{2+} release came from investigating global Ca\textsuperscript{2+} release. We found that CaMKII activation protects myocytes from arrhythmogenic Ca\textsuperscript{2+} waves under Ca\textsuperscript{2+}-overload conditions, and the opposite is true for CaMKII inhibition. The protective effect is so powerful that the Ca\textsuperscript{2+} wave frequency is reduced by more than 50% over a wide range of extracellular Ca\textsuperscript{2+} (2 to 20 mmol/L) in cells expressing CA or WT CaMKII. Because the wave frequency reduction occurs in spite of an elevated SR Ca\textsuperscript{2+} load, CaMKII activation apparently increases the threshold of SOICR. Thus, based on both Ca\textsuperscript{2+} and Ca\textsuperscript{2+} wave measurements, we conclude that CaMKII negatively regulates the SR Ca\textsuperscript{2+} release. This conclusion is in general agreement with the observation that intracellular dialysis of constitutively active CaMKII suppresses SR Ca\textsuperscript{2+} release\textsuperscript{17} and that protein phosphatases PP1 and PP2A enhance spontaneous Ca\textsuperscript{2+} sparks in chemically permeabilized myocytes.\textsuperscript{38}

How can the vast discrepancies across different studies (see the introduction) reconciled? One possibility is that CaMKII phosphorylation and functional modulation of RyR2 might be more complex than had been thought previously. If there were 8 CaMKII phosphorylation sites per monomer as suggested by metabolic labeling,\textsuperscript{11} there would be 9 (0 to 8) phosphorylation levels and 256 combinations of phosphorylation patterns. The possibilities are expanded to 33 (0 to 32) phosphorylation levels and \(\approx 10^{10}\) patterns of phosphorylation for a tetrameric RyR and are increased astronomically for...
some 50 to 200 RyRs assembled in a Ca\(^{2+}\) release unit.\(^{39}\) Added to the complexity, the functionality of CaMKII phosphorylation might be context sensitive, depending on PKA phosphorylation of the channel, binding of calstabin and other regulatory proteins, and the species of animals. The distinct phenotypes in transgenic animal models and ex vivo cell models reported here testify that it is also necessary to isolate direct CaMKII effects from those that are convoluted with changes of the Ca\(^{2+}\) signaling system. In perspective, there is still much to be learned about the intricacy and exquisiteness of CaMKII regulation of cardiac RyR2 function.

**Possible Physiological Significance**

The CICR, with its inherent positive-feedback nature, provides the speed, the sensitivity, and the high-gain amplification that are much needed for cardiac Ca\(^{2+}\) signaling. Equally important, it is necessary to enforce signaling stability for local Ca\(^{2+}\) release events (spark termination, refractory to reactivation) and global Ca\(^{2+}\) transients (suppression of unwanted Ca\(^{2+}\) waves). In this scenario, our data indicate that CaMKII affords a molecular mechanism that negates the positive feedback of the CICR. Activated by high local Ca\(^{2+}\), CaMKII phosphorylates RyR2 and consequentially reduces the Ca\(^{2+}\) sensitivity of the channel and increases the threshold for SOICR, imposing a frequency-dependent modulation of local and global spontaneous SR Ca\(^{2+}\) release.

The above finding shows that CaMKII-dependent RyR2 phosphorylation might not be the underlying cause for the dysregulation and instability of Ca\(^{2+}\) signaling common to many types of heart failure. Nevertheless, caution should be excised when extrapolating the cell results to situations in failing hearts, where extensive functional and structural remodeling may undermine Ca\(^{2+}\) stability by mechanisms other than RyR2 phosphorylation.\(^{40,41}\) It is also noteworthy that CaMKII is multifunctional, and its cellular phenotypes would be context dependent. A comprehensive understanding of CaMKII in cardiac Ca\(^{2+}\) physiology and pathophysiology awaits future investigation using diverse cellular and animal models.

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**Disclosures**

None.

**References**


Ca\(^{2+}\)/Calmodulin Kinase II-Dependent Phosphorylation of Ryanodine Receptors Suppresses Ca\(^{2+}\) Sparks and Ca\(^{2+}\) Waves in Cardiac Myocytes

Dongmei Yang, Wei-Zhong Zhu, Bailong Xiao, Didier X.P. Brochet, S.R. Wayne Chen, Edward G. Lakatta, Rui-Ping Xiao and Heping Cheng

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Materials and Methods

Measurement of L-type $Ca^{2+}$ currents

An Axopatch 200B patch-clamp amplifier (Axon Instruments) was used for the recording of L-type $Ca^{2+}$ currents ($I_{Ca}$). The pipette (~2.5 MΩ) filling solution contains (in mmol/L) EGTA 4, CsCl 120, Hepes 20, MgCl₂ 1.3, MgATP 5, tetraethylammonium chloride 10, CaCl₂ 2 (pH=7.2). The extracellular solution contains (in mmol/L) N-methyl-D-glucamine (NMDG) 130, MgCl₂ 1.2, CsCl 10, glucose 10, tetrodotoxin 0.01, CaCl₂ 1 and Hepes 20 (pH=7.4). $I_{Ca}$ was activated by 200ms-depolarizations from holding potential of −50mV to test potentials ranging from -40 to +60mV. Access resistance was compensated by 70-75%. The magnitude of $I_{Ca}$ was indexed by the difference between the peak inward current and the baseline. The capacitive current in response to a hyper-polarization pulse of 10 mV was used for calculating cell capacitance. Steady state inactivation of $I_{Ca}$ was achieved by a double-pulse protocol. All measurements were performed at room temperature (23-25°C).

Figure Legends

Supplement Fig 1. Characteristics of L-type $Ca^{2+}$ currents ($I_{Ca}$) in the presence of CA-CaMKIIδC. A. Typical traces of $I_{Ca}$ at 0mV depolarization from holding potential -50mV, with β-gal as control. B. Voltage-dependence of $I_{Ca}$ density. The cell capacitance was 174.1 ± 9.2 pF for β-gal group and 175.9 ± 8.2 pF for CA-CaMKIIδC group. n=8 from 4~5 rats for each data points. C. The 63% decay time ($T_{63}$) as a function of membrane voltage. ANOVA test showed significant difference between two groups.
(P<0.001). D. Steady state inactivation curves of $I_{Ca}$, fitted by Boltzmann function. The voltages which cause 50% inactivation are $22.9 \pm 1.4 \text{mV}$ (n=8) for $\beta$-gal and $26.3 \pm 0.7 \text{mV}$ (n=8) for CA-CaMKII$\delta_C$ (p<0.05).

Supplement Fig 2. Histograms of the Ca$^{2+}$ spark rising time in cells expressing $\beta$-gal, wild type and mutant CaMKII$\delta_C$. n=250~800 sparks in 35~60 cells from 6 hearts for each group. Insert shows a typical result of Ca$^{2+}$ spark time course and its exponential fitting. Note the prominent mode at around 14-16 ms in the $\beta$-gal, WT- or CA-, but not the DN-CaMKII$\delta_C$ group, with the p value equals to 0.01 via KW_test.

Supplement Fig 3. Properties of Ca$^{2+}$ spark in resting cells expressing $\beta$-gal, WT-, CA- or DN-CaMKII$\delta_C$. A & B. the average data of spark width (full width at half maximum) and duration (full duration at half maximum), respectively (n=250~800 sparks for each group).

Supplement Fig 4. Properties of Ca$^{2+}$ wave in cells expressing $\beta$-gal, WT-, CA- or DN-CaMKII$\delta_C$ at 20mmol/L extracellular Ca$^{2+}$. A. Typical line-scan images of Ca$^{2+}$ wave in fluo-4 loaded cells. From top to bottom: raw image of a propagating Ca$^{2+}$ wave, after linear-alignment of the wave front, and the corresponding traces of spatially averaged time courses. B & C. the averaged wave speed and amplitude, respectively (n=30~37 cells from 3~5 hearts for each group).
Supplement figure 2
Supplement figure 3

A

Spark Width (μm)

<table>
<thead>
<tr>
<th>β-gal</th>
<th>WT</th>
<th>CA</th>
<th>DN</th>
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B

Spark Duration (ms)

<table>
<thead>
<tr>
<th>β-gal</th>
<th>WT</th>
<th>CA</th>
<th>DN</th>
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Supplement figure 4