Increased Sarcoplasmic Reticulum Calcium Leak but Unaltered Contractility by Acute CaMKII Overexpression in Isolated Rabbit Cardiac Myocytes

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Abstract—The predominant cardiac Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK) is CaMKII\(\alpha\). Here we acutely overexpress CaMKII\(\alpha\) using adenovirus-mediated gene transfer in adult rabbit ventricular myocytes. This circumvents confounding adaptive effects in CaMKII\(\alpha\) transgenic mice. CaMKII\(\alpha\) protein expression and activation state (autophosphorylation) were increased 5- to 6-fold. Basal twitch contraction amplitude and kinetics (1 Hz) were not changed in CaMKII\(\alpha\) versus LacZ expressing myocytes. However, the contraction–frequency relationship was more negative, frequency-dependent acceleration of relaxation was enhanced (\(\tau_{0.5L}/\tau_{3L}=2.14\pm 0.10\) versus \(1.87\pm 0.10\)), and peak Ca\(^{2+}\) current (\(I_{Ca}\)) was increased by 31\% (\(-7.1\pm 0.5\) versus \(-5.4\pm 0.5\) pA/pF, \(P<0.05\)). Ca\(^{2+}\) transient amplitude was not significantly reduced (\(-27\%\), \(P=0.22\)), despite dramatically reduced sarcoplasmic reticulum (SR) Ca\(^{2+}\) content (41\%; \(P<0.05\)). Thus fractional SR Ca\(^{2+}\) release was increased by 60\% (\(P<0.05\)). Diastolic SR Ca\(^{2+}\) leak assessed by Ca\(^{2+}\) spark frequency (normalized to SR Ca\(^{2+}\) load) was increased by 88\% in CaMKII\(\alpha\) versus LacZ myocytes (\(P<0.05\); in an multiplicity-of-infection–dependent manner), an effect blocked by CaMKII inhibitors KN-93 and autocamtide-2–related inhibitory peptide. This enhanced SR Ca\(^{2+}\) leak may explain reduced SR Ca\(^{2+}\) content, despite measured levels of SR Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchange expression and function being unaltered. Ryanodine receptor (RyR) phosphorylation in CaMKII\(\alpha\) myocytes was increased at both Ser2809 and Ser2815, but FKBP12.6 coimmunoprecipitation with RyR was unaltered. This shows for the first time that acute CaMKII\(\alpha\) overexpression alters RyR function, leading to enhanced SR Ca\(^{2+}\) leak and reduced SR Ca\(^{2+}\) content but without reducing twitch contraction and Ca\(^{2+}\) transients. We conclude that this is attributable to concomitant enhancement of fractional SR Ca\(^{2+}\) release in CaMKII\(\alpha\) myocytes (ie, CaMKII-dependent enhancement of RyR Ca\(^{2+}\) sensitivity during diastole and systole) and increased \(I_{Ca}\) (Circ Res. 2006;98:235-244.).

Key Words: calcium ■ CaMKII ■ excitation–contraction coupling ■ sarcoplasmic reticulum

Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase that phosphorylates numerous target proteins.\(^1,2\) The major cardiac isoform is CaMKII\(\alpha\), and the splice variant CaMKII\(\delta\) is primarily cytosolic, whereas CaMKII\(\beta\) is nuclear because of a nuclear localization sequence.\(^3\)

During excitation–contraction coupling (ECC), Ca\(^{2+}\) entry, mainly via voltage dependent L-type Ca\(^{2+}\) channels (\(I_{Ca}\)), triggers sarcoplasmic reticulum (SR) Ca\(^{2+}\) release via ryanodine receptors (RyRs), via Ca\(^{2+}\)-induced Ca\(^{2+}\) release.\(^4,5\) The resultant increase in intracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)), causes Ca\(^{2+}\) binding to troponin C, which activates myofilaments, leading to contraction. For relaxation to occur, Ca\(^{2+}\) must be removed from the cytoplasm. SR Ca-ATPase (SERCA) and Na\(^+\)/Ca\(^{2+}\)-exchanger (NCX) are the main mechanisms for Ca\(^{2+}\) removal.\(^4,5\) CaMKII can modulate ECC by phosphorylating several important Ca\(^{2+}\)-dependent regulatory proteins in heart, including Ca\(^{2+}\) transport proteins, such as RyR and phospholamban (PLB), and possibly L-type Ca\(^{2+}\) channels.\(^2,5\)

CaMKII is directly associated with RyR and overexpression of CaMKII in transgenic mouse cardiomyocytes increases SR Ca\(^{2+}\) release as shown by increased frequency of spontaneous SR Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks).\(^6,7\) Blocking CaMKII (using KN-93) decreases Ca\(^{2+}\) spark frequency dramatically, providing evidence for a direct relationship between CaMKII activity and the increased spark frequency.\(^7\) These results in myocytes from CaMKII transgenic mouse hearts were confirmed by Currie et al,\(^8\) who showed that the
specific CaMKII peptide inhibitor autophosphorylation occurs without CaMKII phosphorylation. Wehrens et al. showed that CaMKII-dependent RyR phosphorylation increased RyR open probability (P_o), without alteration of FKBP12.6 association.9

CaMKII may also be involved in the pathogenesis of hypertrophy and heart failure.2 In human heart failure, CaMKII expression and phosphorylation is increased.10,11 In neonatal ventricular myocytes, overexpression of CaMKIIΔδ caused transcriptional activation of atrial natriuretic peptide gene expression (a hypertrophic signaling marker).12 Furthermore, overexpression of the cytoplasmic δδ isoform in mouse heart results in profound contractile dysfunction and heart failure.6,7 In our previous studies using these animals, we described major alterations in intracellular Ca^{2+} handling with marked reductions in Ca^{2+} transients, SR Ca^{2+} content, and SERCA, PLB, and RyR protein expression and enhanced NCX function and expression, all of which are typical for heart failure. Most remarkably, however, with respect to the RyR, the frequency of Ca^{2+} sparks (indicative of diastolic spontaneous SR Ca^{2+} release events or opening of RyR clusters) was greatly enhanced, demonstrating increased diastolic SR Ca^{2+} leak despite reduced SR Ca^{2+} load and diastolic [Ca^{2+}]_i (which by themselves would normally reduce SR Ca^{2+} leak).13 We showed that this was most likely attributable to increased CaMKII-dependent RyR phosphorylation increasing RyR openings, because Ca^{2+} spark frequency could be reduced back to normal levels by blocking CaMKII. Backphosphorylation and subsequent studies using phospho-CaMKII antibodies indeed showed increased RyR phosphorylation in transgenic wild type.6,7 Although these results show that CaMKIIΔδ overexpression can cause heart failure and altered cellular Ca^{2+} transport, it was unclear how direct effects of acute CaMKII-dependent protein phosphorylation alter Ca^{2+} handling functionally and with respect to protein expression, especially in the context of possible developmental changes or adaptive responses associated with heart failure induction as reported previously.6,7 Therefore, we have acutely overexpressed CaMKIIΔδ in ventricular rabbit myocytes and compared these with LacZ-expressing control cells to investigate intracellular Ca^{2+} handling. We demonstrate that acute CaMKIIΔδ overexpression enhances SR Ca^{2+} leak and reduces SR Ca^{2+} content. However, in acute CaMKIIΔδ overexpression, we do not see alterations in the protein expression levels or function of NCX and SERCA (in striking contrast to the failing transgenic mice), and twitch contractions and Ca^{2+} transients are unaltered. This is attributed to an increased fractional SR Ca^{2+} release (and I_{Ca}), which may result from the same CaMKIIΔδ-dependent enhancement of RyR Ca^{2+} sensitivity that enhances diastolic SR Ca^{2+} leak.

Materials and Methods

Generating Adenoviral Vectors and Cardiac Myocyte Isolation

Adenoviral vectors were generated as published previously.14,15 For adenoviral transfection, ventricular myocytes from rabbit hearts (female Chinchilla Bastards; 1.3- to 2.0-kg weight) were isolated using standard procedures6,13 with collagenase B (0.5 mg/mL, Boehringer-Mannheim, Mannheim, Germany) and protease (0.02 mg/mL, Sigma, St Louis, Mo). Cells were plated at a density of 4.2×10^{4} rod-shaped cells/cm^{2} on culture dishes (55 mm) and incubated for 24 hours in supplemented M199 tissue culture medium (Sigma-Aldrich Chemie, Taufkirchen, Germany). All procedures involving animals were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Initially, myocytes were transfected with multiplicities of infections (MOIs) of 1, 10, and 100 to test for CaMKIIΔδ protein overexpression and phosphorylation levels. For functional experiments, myocytes were then transfected with either CaMKIIΔδ, or LacZ using a MOI of 10 or 100 for 24 hours at 37°C in a humidified incubator (5% CO_{2}, 95% O_{2}). Myocyte volume was calculated from myocytes length×width×40% of width.7

Shortening and Ca^{2+} Measurements Using Inverted Microscopes

Shortening and [Ca^{2+}], measurements were performed as reported previously.7,17 Briefly, myocytes were loaded with the Ca^{2+}-sensitive dye indo-1/acetoxymethyl ester to measure diastolic [Ca^{2+}]. Because no difference in diastolic Ca^{2+} was found, additional experiments used fluo-3/acetoxymethyl ester (10 μmol/L, respectively; Molecular Probes). Excitation wavelengths (360±5 nm for indo and 480±15 nm for fluo) using a 75-W xenon arc lamp on the stage of a Nikon Eclipse TE2000-U inverted microscope. Emitted fluorescence was measured using photomultipliers (at 405±15 nm and 485±12.5 nm for indo and 535±20 nm for fluo; IonOptix Corp, Milton, Mass). From the raw fluorescence, indo-1 ratio was calculated (405 nm/485 nm), and for fluo-3, F/F_{0} was calculated by dividing by the baseline fluorescence F_{0} after subtraction of the background fluorescence (IonWizard, IonOptix Corp). Myocytes were field-stimulated (voltage 25% above threshold) at 1 Hz and 37°C until steady state.

L-Type Ca^{2+} Current Measurements

I_{Ca} was recorded by voltage-clamp as reported previously.7 Briefly, low-resistance (~2 to 3 MΩ) electrodes were pulled and filled with K-free internal solution containing (in mmol/L) 105 CsCl, 20 HEPES, 5 BAPTA, 1 di-bromo-BAPTA, 1.49 CaCl_{2}, and 5 MgATP resulting in a free [Ca^{2+}] of 100 mmol/L (pH 7.2). Myocytes were superfused with K-free external solution containing (mmol/L) 140 NaCl, 4 CsCl, 5 HEPES, 10 glucose, 1 MgCl_{2}, and 2 CaCl_{2} (pH 7.4). Current–voltage relationships were established as follows: holding potential was ~ 90 mV; 5 prepulses were applied to 0 mV to ensure equal SR Ca^{2+} loading; brief Na^{+} current-inactivating pulses (50 ms, ~ 50 mV) preceded test potentials steps (between ~ 40 mV and ~ 40 mV, 200 ms, in 10 mV steps). I_{Ca} facilitation was assessed by repetitive depolarizations to 0 mV after a pause of 1 minute, and amplitudes and kinetics were measured and analyzed (EPC10, Heka Electronics Inc, Lambrecht, Germany).

Confocal Microscopy

Ca^{2+} signals were recorded in fluo-4 loaded myocytes on a laser scanning confocal microscope (Bio-Rad Radiance 2000MP). Fluo-4 was excited via an argon laser (488 nm) and emitted fluorescence (F) was collected through a 515 nm long-pass emission filter. [Ca^{2+}]_{i} was calibrated by the equation [Ca^{2+}]_{i}=K_{D}(F/F_{0} \cdot [Ca^{2+}]_{i}^{0})^{\frac{1}{n+1}}-1-F_{0} with K_{D}=1100 mmol/L and [Ca^{2+}]_{i}^{0}=100 mmol/L. Ca^{2+} sparks were analyzed by a program (IDL 5.3) that detects Ca^{2+} sparks as areas of increased fluorescence with respect to the SD of background fluorescence. We used a Ca^{2+} spark threshold of
3.8±SD, with human verification. Peaks of Ca²⁺ sparks were normalized as F/F₀ and duration was taken from the full-duration half-maximum (FDHM). Width or spatial size was taken as full-depth half-maximum (FWHM). Ca²⁺ spark frequency (CaSpF) was obtained by averaging the number of sparks in images recorded after 1 Hz stimulation and normalized to cell volume and scan rate as sparks (pL⁻¹s⁻¹), assuming voxel length and width of 0.2 µm and depth of 1 µm.

**Solutions and Experimental Protocol**

Normal Tyrode’s solution contained (mmol/L) 140 NaCl, 6 KCl, 10 HEPES, 10 glucose, 1 MgCl₂, and 2 CaCl₂ (37°C). SR Ca²⁺ load was evaluated by Ca²⁺ transient amplitudes induced by rapid caffeine (10 mmol/L) application. NCX function was assessed measuring Ca²⁺ elimination pathways (eg, mitochondrial uniporter, sarcolemmal Ca²⁺ ATPase, contributing <1% each) can be neglected. In a subset of experiments, KN-93 (1 µmol/L) or the membrane permeant AIP (20 µmol/L) was added to the external bath solution or the patch-pipette to inhibit CaMKII. Enough time was allowed for KN-93 and AIP to inhibit CaMKII. In another subset of experiments, tetracaine was used to inhibit SR Ca²⁺ leak (1 µmol/L).

For shortening-frequency measurements, stimulation frequency was varied stepwise (from 0.5 to 3 Hz), waiting at intermediate frequencies until steady state was reached. For postrest measurements, a rest interval of 30 s was applied measuring steady-state and posttwist contraction amplitude. Of note, rabbit myocytes are known to show rest decay of twitches.

**Protein Expression, Phosphorylation Levels, and Immunocytochemistry**

Western blot analysis was performed as described previously using an anti-CaMKIIα antibody (Santa Cruz), as well as antibodies for SERCA, NCX (Affinity BioReagents), and PLB (Upstate). For phosphorylation levels of CaMKII (Affinity BioReagents), PLB-Thr17, and PLB-Ser16 (Cyclacel) in transfected myocytes, phosphospecific antibodies were used. RyR expression and phosphorylation levels were investigated using antibodies kindly provided by Dr A. Marks (Columbia University, New York). For immunohistochemical experiments (epifluorescence), diaminobenzidine staining was performed (picture plus, Zymed) using a hemagglutinin (HA) antibody (Roche) against, which was coexpressed with CaMKII. In parallel, a fluorescent Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc) against anti-HA was used for confocal images.

**Coimmunoprecipitation and Immunoblotting**

Coimmunoprecipitation studies were performed to test for FKBP12.6/RyR2 interaction. CaMKIIα and LacZ transfected myocytes (5×10⁵) were lysed in 250 µL of lysis buffer containing (in mmol/L) 50 HEPES, pH 7.4, 500 KCl, 1% Triton X-100, and 5 EDTA and supplemented with protease inhibitors (0.2 mmol/L pefabloc SC, 100 nmol/L aprotinin, 1 µmol/L leupeptin, 1 µmol/L pepstatin A, 1 mmol/L benzamidine, 1 µmol/L of calpain inhibitor I, and 1 µmol/L of calpain inhibitor II). After centrifugation for 5 minutes (10 000g; 4°C), cell lysates (1 mg) were suspended in PBS (1 mL). Eight micrograms of anti-RyR antibody (34°C clone, Affinity BioReagents) and rabbit polyclonal anti-phospho-RyR (1:5000, RyR2-P2809 and RyR2-P28215; generous gifts of Dr A. Marks, Columbia University, New York). Secondary antibody used were donkey anti-rabbit whole Ig (1:10000, Amersham) and donkey anti-mouse affinity-purified IgG (1:500, Affinity BioReagents). Immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Statistics**

Results are expressed as mean±SEM. Significance (P<0.05) was determined using unpaired Student’s t test or 2-way repeated measurements ANOVA followed by Student–Newman–Keuls test as appropriate. Time constants of [Ca²⁺] decline, τₗₛ, were monoeXponential least-square fits. Time constants τₛ and τₛ of Iₛ inactivation were fitted biexponentially.

**Results**

**CaMKII Overexpression in Ventricular Rabbit Myocytes**

Figure 1A shows a typical CaMKII Western blot with rabbit myocytes infected at MOI100 (24 hours). Average overexpression of CaMKIIα was ~6-fold that of LacZ controls (P<0.05; n=5). In addition, myocytes showed MOI-dependent (1, 10, 100) increases in CaMKII protein expression, and also in CaMKII phosphorylation status, with maximum phosphorylation increases of ~5-fold at MOI100.
there was no change in diastolic $\text{Ca}^{2+}$ also not significantly decreased ($P<0.05$). On increasing stimulation frequency from 0.5 to 3 Hz, there tended to be a greater decrease in contraction amplitude in CaMKII$_C$-transfected myocytes (18%; $n=46$) versus LacZ myocytes (7%; $n=29$; $P=NS$). Frequency-dependent acceleration of relaxation (FDAR) (Figure 2D) was apparent in both CaMKII$_C$ and LacZ cells but was significantly enhanced in CaMKII$_C$ versus LacZ myocytes, consistent with a role for CaMKII in FDAR. The FDAR index $\tau_{\text{rest}}/\tau_{\text{SR}}=2.14 \pm 0.10$ in CaMKII$_C$ versus 1.87 ± 0.10 in LacZ ($P<0.05$). KN-93 pretreatment partially inhibited FDAR in both myocyte types (not shown).

### Ca$^{2+}$ Transients

Figure 3A shows $I_{Ca}$-voltage relationships, where peak $I_{Ca}$ was increased in CaMKII$_C$ versus LacZ by 31% at 0 mV ($7.1 \pm 0.5$ versus $5.4 \pm 0.5$ pA/pF; $P<0.05$). These effects can be significantly reversed by CaMKII inhibition with AIP ($5.8 \pm 0.5$ pA/pF; $P<0.05$ versus CaMKII$_C$). The $I_{Ca}$ inactivation time constants $\tau_1$ and $\tau_2$ were significantly prolonged in CaMKII$_C$ versus LacZ myocytes and could be completely reversed by AIP ($P<0.05$ versus CaMKII$_C$; Figure 3B). Repeated depolarization to 0 mV after a 1 minute rest causes a CaMKII-dependent $I_{Ca}$ facilitation (Figure 3C) that was enhanced in CaMKII$_C$-dependent vs LacZ myocytes ($P<0.05$). The CaMKII inhibitor KN-93 abolished facilitation in CaMKII$_C$ myocytes ($P<0.05$) and LacZ myocytes (not shown).

### SR Ca$^{2+}$ Content and NCX Function

Because no significant changes in twitch shortening or Ca$^{2+}$ transients were found, SR Ca$^{2+}$ load might be expected to be unchanged. However, SR Ca$^{2+}$ content measured by caffeine-induced Ca$^{2+}$ transients was dramatically reduced (by 41%) in CaMKII$_C$ versus LacZ (310 ± 79 versus 521 ± 120 mmol/L; $P<0.05$; Figure 4A). To assess NCX function, we measured the half-time of $[\text{Ca}^{2+}]_i$ decline during caffeine-induced Ca$^{2+}$ transients. No change was detectable for NCX function in CaMKII$_C$ myocytes ($P<0.05$) and LacZ myocytes (not shown).

**Figure 2. Twitches and intracellular Ca$^{2+}$ transients.**

A. Original traces showing twitch shortening at 1 Hz, as well as mean data for fractional shortening (resting cell length [RCL]) and $RT_{50\%}$ for LacZ ($n=29$) and CaMKII$_C$ ($n=46$). B. Original Ca$^{2+}$ transients at 1 Hz and average data for Ca$^{2+}$ transient amplitude ($\Delta[\text{Ca}^{2+}]$) and $RT_{50\%}$ for LacZ ($n=8$) and CaMKII$_C$ ($n=11$). C. Influence of increasing stimulation frequency (0.5 to 3 Hz) on fractional shortening in CaMKII$_C$-transfected myocytes ($n=39$) vs LacZ ($n=28$). D. FDAR index ($\tau_{\text{rest}}/\tau_{\text{SR}}$) was used to show differences in relaxation behavior between LacZ and CaMKII$_C$. *$P<0.05$ vs LacZ.

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**Twitch Shortening and Ca$^{2+}$ Transients**

Twitch fractional shortening at 1 Hz (Figure 2A) was not altered in CaMKII$_C$ versus LacZ (3.6 ± 0.2% versus 3.7 ± 0.3% resting cell length; $P=0.79$). Ca$^{2+}$ transients were also not significantly decreased ($P=0.22$; Figure 2B), and there was no change in diastolic $[\text{Ca}^{2+}]_i$, as measured by indo-1 (diastolic $F_{405}/F_{485}$ was 0.46 ± 0.02 versus 0.48 ± 0.01; $P=0.26$). Twitch relaxation and $[\text{Ca}^{2+}]_i$ decline were not changed, indicating unaltered basal SR Ca$^{2+}$ ATPase function (particularly because NCX function was also unaltered; see below). However, KN-93 significantly prolonged relaxation ($RT_{80\%}$) at 1 Hz to 225 ± 25 ms versus 187 ± 16 ms ($P<0.05$). On increasing stimulation frequency from 0.5 to 3 Hz, there tended to be a greater decrease in contraction amplitude in CaMKII$_C$-transfected myocytes (18%; $n=46$) versus LacZ myocytes (7%; $n=29$; $P=NS$). Frequency-dependent acceleration of relaxation (FDAR) (Figure 2D) was apparent in both CaMKII$_C$ and LacZ cells but was significantly enhanced in CaMKII$_C$ versus LacZ myocytes, consistent with a role for CaMKII in FDAR. The FDAR index $\tau_{\text{rest}}/\tau_{\text{SR}}=2.14 \pm 0.10$ in CaMKII$_C$ versus 1.87 ± 0.10 in LacZ ($P<0.05$). KN-93 pretreatment partially inhibited FDAR in both myocyte types (not shown).
SR Ca\(^{2+}\) Leak and Ryanodine Receptor Phosphorylation

To more directly assess SR Ca\(^{2+}\) leak, we measured Ca\(^{2+}\) spark frequency. Because SR Ca\(^{2+}\) content is a major determinant of Ca\(^{2+}\) spark frequency and SR Ca\(^{2+}\) content is very low in CaMKII\(_{\Delta C}\) myocytes, spark frequency was normalized to SR Ca\(^{2+}\) content (measured at the same time). Normalized Ca\(^{2+}\) spark frequency was increased by 88% in CaMKII\(_{\Delta C}\) versus LacZ (1.9±0.1 versus 1.0±0.1; *P<0.05; Figure 5A), indicating enhanced SR Ca\(^{2+}\) leak at a given SR Ca\(^{2+}\) content. Similarly, fractional SR Ca\(^{2+}\) release during a normal twitch was significantly increased in CaMKII\(_{\Delta C}\) cells (0.41±0.06 versus 0.26±0.03; *P<0.05; Figure 5B). Although part of this 58% increase in fractional release may be attributable to the 23% increase in peak \(I_{\text{Ca}}\) it may also reflect enhanced RyR sensitivity to Ca\(^{2+}\) (especially because the lower SR Ca\(^{2+}\) content by itself would tend to greatly reduce fractional SR Ca\(^{2+}\) release).

The enhanced SR Ca\(^{2+}\) leak and fractional release were associated with significantly increased RyR phosphorylation at Ser2809 and Ser2815 (71% and 70% respectively, *P<0.05; Figure 5C). To investigate whether FKBP12.6 association with RyR2 was altered, coimmunoprecipitation of equal amounts of myocyte lysates with anti-RyR antibody. At equal amount of RyR2 precipitated in each sample, no decrease in FKBP12.6 was observed in cells overexpressing CaMKII\(_{\Delta C}\) (Figure 5D), whereas RyR2 phosphorylation was increased. Thus, the increased phosphorylation of RyR elicited by CaMKII affects RyR function but does not dissociate FKBP12.6 from RyR2, in agreement with others.

Reversal of SR Ca\(^{2+}\) Leak and CaMKII-Dependent PLB Phosphorylation

The effects of CaMKII\(_{\Delta C}\) overexpression are MOI dependent. Figure 6A and 6B show a dose-dependent increase in SR Ca\(^{2+}\) spark frequency and amplitude. Figure 6C shows that Ca\(^{2+}\) spark frequency can be significantly decreased by the CaMKII\(_{\Delta C}\) inhibitors AIP or KN-93 or by inhibiting RyR gating by tetracaine. Figure 6D shows an alternative SR Ca\(^{2+}\) leak measurement, where abrupt RyR block by tetracaine (in Ca\(^{2+}\)-free, Na\(^{+}\)-free solution) causes [Ca\(^{2+}\)] to decline and SR Ca\(^{2+}\) content to rise (F/F\(_0\) decreased from 1.05±0.08 to 0.75±0.06; *P<0.05).

In addition, acute CaMKII\(_{\Delta C}\) overexpression at MOI100 resulted in increased PLB-Thr17 phosphorylation (+125±42%, *P<0.05) but a slight decrease in PLB-Ser16 phosphorylation (−44±26%; P=NS; Figure 6E). In contrast, PLB protein expression is unchanged (as SERCA and NCX in Figure 4D).

Discussion

The present study shows for the first time that acute overexpression of CaMKII\(_{\Delta C}\) (24 hours) results in increased Ca\(^{2+}\) leak from the SR and decreased SR Ca\(^{2+}\) load most likely because of RyR phosphorylation. We conclude that the effects of CaMKII\(_{\Delta C}\) overexpression on RyR function and SR
Ca\(^{2+}\) leak as observed in transgenic mice previously are mimicked by adenoviral overexpression in myocytes. Most importantly, however, in contrast to chronic CaMKII\(^{\delta_c}\) overexpression in mice, these acute alterations in ECC together with increased \(I_{Ca}\) do not lead to decreased twitch contractions or Ca\(^{2+}\) transients. Therefore, CaMKII\(^{\delta_c}\) mediated phosphorylation directly increases diastolic RyR opening and enhances ECC efficacy.

**CaMKII\(^{\delta_c}\) Overexpression**

Using adenovirus-mediated gene transfer, we elevated CaMKII\(^{\delta_c}\) expression in rabbit cardiac myocytes, and overexpression was specifically in the cytosolic (versus nuclear) compartment, consistent with CaMKII\(^{\delta_c}\) lacking the 11 amino acid nuclear localization sequence in the \(\delta_h\) splice variant.\(^3\) Whereas our previous results in transgenic CaMKII\(^{\delta_c}\) mice show clear hypertrophy on the whole heart and myocyte level,\(^7\) there was no significant increase in myocyte size after 24 hours of overexpression of CaMKII\(^{\delta_c}\). In CaMKII\(^{\delta_c}\)-transgenic mice, the more prolonged overexpression of CaMKII\(^{\delta_c}\) or its possible multimerization with the nuclear CaMKII\(^{\delta_h}\) in vivo could contribute to the otherwise unknown hypertrophic mechanism. Notably, in both transgenic CaMKII\(^{\delta_c}\) mice and the present acute CaMKII\(^{\delta_c}\) overexpressing rabbit myocytes, a major functional finding was increased SR Ca\(^{2+}\) leak associated with enhanced RyR phosphorylation and reduced SR Ca\(^{2+}\) content. It is possible that the increased diastolic Ca\(^{2+}\) leak from the SR may activate Ca\(^{2+}\)-dependent hypertrophic signaling pathways.\(^3,21,22\)

**L-Type Ca\(^{2+}\) Current**

In the present study, CaMKII\(^{\delta_c}\) overexpression resulted in significantly enhanced peak \(I_{Ca}\), and also prolonged \(I_{Ca}\), inac-
tivation parameters $\tau_1$ and $\tau_2$. Because CaMKII can activate $I_{Ca}^{\text{p}}$ and we see enhanced $I_{Ca}$ facilitation, the 31% increase in peak $I_{Ca}$ may reflect a relatively direct CaMKII-dependent regulatory effect on $I_{Ca}$. This interpretation is supported by the observation that acute CaMKII inhibition by KN-93 or AIP blocks both amplitude and inactivation effects. Indeed, both higher $I_{Ca}$ amplitude and slowed inactivation are hallmarks of CaMKII-dependent $I_{Ca}$ facilitation, consistent with a common fundamental mechanism. The increased $I_{Ca}$, together with the increases in fractional Ca$^{2+}$ release from the SR, results in unchanged twitch contraction (at least at low stimulation rates), even in the face of decreased SR Ca$^{2+}$ content.

**SR Ca$^{2+}$ Content and Contractions**

The SR is central in cardiac ECC and CaMKII can accelerate SERCA function via PLB phosphorylation. Surprisingly, we did not detect altered SERCA function at baseline contraction frequency in CaMKII$\delta_c$ versus LacZ myocytes. However, we do see modestly enhanced FDAR in the CaMKII$\delta_c$ versus LacZ myocytes (as in transgenic CaMKII$\delta_c$ mice). FDAR is thought to reflect CaMKII-dependent enhancement of SR Ca$^{2+}$ uptake (even though it does not require PLB). Thus, acute CaMKII appears to have only modest effects on SERCA function here. These modest effects on the rate of [Ca$^{2+}$]; decline here and in our previous study may be because the absolute extent of CaMKII-dependent PLB phosphorylation may be small and the increased phosphorylation at PLB-Thr17 may be counterbalanced by less at Ser16, as seen in the present study.

In transgenic CaMKII$\delta_c$-overexpressing mouse hearts, SR Ca$^{2+}$ content was also reduced, but that could have been...
attributable to the enhanced SR Ca$^{2+}$ leak (and RyR phosphorylation), increased NCX function, or reduced SERCA function that are associated with the heart failure phenotype, as in other heart failure models. With acute CaMKII expression here, a more modest reduction in myocyte SR Ca$^{2+}$ content occurs with enhanced SR Ca$^{2+}$ leak but unaltered NCX and SERCA function and protein expression. This argues strongly in favor of enhanced SR Ca$^{2+}$ leak causing reduced SR Ca$^{2+}$ content here. However, the more severe reduction in SR Ca$^{2+}$ content in heart failure (whether induced by transgenic CaMKII overexpression or otherwise) is attributable not only to enhanced SR Ca$^{2+}$ leak but also to enhanced NCX function and reduced SERCA function.  

In isolated single-channel RyR recordings, CaMKII has been shown to increase cardiac RyR open probability. At an intermediate level of isolation, CaMKII greatly enhanced Ca$^{2+}$ spark frequency in permeabilized PLB-KO mouse myocytes (without enhanced SR Ca$^{2+}$ content). In addition, CaMKII is associated with the RyR in the cell, and can phosphorylate the RyR. The cardiac RyR has been reported to be phosphorylated by CaMKII at both Ser2809 and Ser2815 sites. Mark and colleagues have reported that these sites are segregated (CaMKII only at 2815 and PKA only at 2809) and that Ser2809 phosphorylation causes dissociation of FKBP12.6 from RyR and consequent RyR opening, whereas CaMKII-dependent phosphorylation activates RyR without causing FKBP12.6 dissociation. Likewise, here we do not see FKBP12.6 dissociation from the RyR in myocytes overexpressing CaMKIIoC, despite some increase in RyR phosphorylation at Ser2809. Thus RyR phosphorylation appears to cause enhanced SR Ca$^{2+}$ leak and reduced SR Ca$^{2+}$ content in CaMKIIoC versus LacZ myocytes.
Acute Cytoplasmic CaMKIIδ Overexpression

A remarkable finding here is that twitch contractions and Ca\(^{2+}\) transients are almost unaffected by the dramatically reduced SR Ca\(^{2+}\) content. This may be attributable in part to the enhanced \(I_{C}\), (as above), but a major factor is probably the sort of autoregulation described previously by Trafford et al\(^{15}\) in the presence of low caffeine concentration (which causes diastolic SR Ca\(^{2+}\) leak and sensitizes the RyR to Ca\(^{2+}\)). They showed that altered RyR gating only produces transient changes in Ca\(^{2+}\) transients (but sustained changes in SR Ca\(^{2+}\) content and fractional release). That is, abruptly CaMKII may enhance SR Ca\(^{2+}\) release, but this causes more Ca\(^{2+}\) extrusion (via NCX) and reduces SR Ca\(^{2+}\) content. With the lower SR Ca\(^{2+}\) content the enhanced fractional release only results (in the steady state) in the same amount of SR Ca\(^{2+}\) release. This may be what is happening here with acute CaMKII\(\delta\) over-expression. Indeed, Shannon et al\(^{16}\) recently simulated Ca\(^{2+}\) homeostasis mathematically showing that enhanced RyR Ca\(^{2+}\) sensitivity (as by caffeine or phosphorylation) increased SR Ca\(^{2+}\) leak and reduced SR Ca\(^{2+}\) content but enhanced SR fractional release without decreasing the size of the steady-state [Ca\(^{2+}\)], transient (as seen here).

Thus, enhanced RyR Ca\(^{2+}\) sensitivity by itself may contribute substantially to SR Ca\(^{2+}\) unloading on CaMKII over-expression (or in heart failure), without itself being appreciably negatively inotropic. Other factors must be largely responsible for the systolic dysfunction seen in CaMKII\(\delta\), transgenic mice or other heart failure models (eg, reduced SERCA function and enhanced NCX function).\(^{2,9,21}\) Indeed, in heart failure, CaMKII\(\delta\) is overexpressed,\(^{1,17}\) SR Ca\(^{2+}\) leak is enhanced,\(^{1,17}\) and block of CaMKII in heart failure can greatly enhance SR Ca\(^{2+}\) content without improving systolic function.\(^{22}\) We conclude that CaMKII-dependent enhancement of RyR Ca\(^{2+}\) sensitivity (and thus leak) does not contribute appreciably to systolic dysfunction, but the enhanced diastolic SR Ca\(^{2+}\) leak could possibly increase the propensity for triggered arrhythmias.\(^{28}\)

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