S-Nitrosylation Induces Both Autonomous Activation and Inhibition of Calcium/Calmodulin-dependent Protein Kinase II δ

Received for publication, March 6, 2015, and in revised form, August 27, 2015. Published, JBC Papers in Press, August 27, 2015, DOI 10.1074/jbc.M115.650234

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Background: CaMKIIδ and NO can modulate cardiac signaling/pathology.

Results: NO treatment after calcium/calmodulin binding prolongs CaMKIIδ activation, whereas NO pretreatment inhibits CaMKIIδ activation, effects mediated by Cys-290 and Cys-273, respectively.

Conclusion: S-nitrosylation has a dual role in modulating CaMKIIδ in the heart.

Significance: Dual regulation by NO is a new pathway by which CaMKII can modulate cardiac function.

NO is known to modulate calcium handling and cellular signaling in the myocardium, but key targets for NO in the heart remain unidentified. Recent reports have implied that NO can activate calcium/calmodulin (Ca2+/CaM)-dependent protein kinase II (CaMKII) in neurons and the heart. Here we use our novel sensor of CaMKII activation, Camui, to monitor changes in the conformation and activation of cardiac CaMKII (CaMKIIδ) activity after treatment with the NO donor S-nitrosoglutathione (GSNO). We demonstrate that exposure to NO after Ca2+/CaM binding to CaMKIIδ results in autonomous kinase activation, which is abolished by mutation of the Cys-290 site. However, exposure of CaMKIIδ to GSNO prior to Ca2+/CaM exposure strongly suppresses kinase activation and conformational change by Ca2+/CaM. This NO-induced inhibition was ablated by mutation of the Cys-273 site. We found parallel effects of GSNO on CaM/CaMKIIδ binding and CaMKIIδ-dependent ryanodine receptor activation in adult cardiac myocytes. We conclude that NO can play a dual role in regulating cardiac CaMKIIδ activity.

The δ isoform of calcium/calmodulin (Ca2+/CaM)-dependent protein kinase II (CaMKIIδ) has emerged as a key mediator of a number of cellular functions in the heart, including ion channel regulation (1, 2), gene transcription (3, 4), and apoptotic cell death (5, 6). CaMKIIδ is expressed as a homodimeric protein, and each monomer consists of three domains: a C-terminal association domain that directs multimeric assembly of the holoenzyme, an N-terminal catalytic domain that interacts with and phosphorylates substrates, and a regulatory domain that binds to the kinase domain and is autoinhibitory of CaMKII activity at baseline. Activation of CaMKII requires the binding of Ca2+/CaM to the regulatory domain, which initiates a conformational shift that exposes the active face of the catalytic domain for substrate binding. Therefore, under normal physiological conditions, CaMKIIδ activation is typically driven by cardiac Ca2+ fluxes. However, CaMKIIδ is subject to a number of posttranslational modifications that prevent reassociation of the catalytic and regulatory domains of the kinase and, therefore, lead to autonomous activation of CaMKIIδ (7). These modifications, described previously, include autophosphorylation at the Thr-287 site (8), oxidation at the Met-281/282 sites (9), and O-GlcNAc modification at the Ser-280 site (10). Each of these modifications prolongs CaMKII activation in the heart and can induce cardiac pathology, including adverse structural remodeling, arrhythmia, and cell death.

New evidence suggests that NO may regulate CaMKIIδ activation via S-nitrosylation, the covalent attachment of an NO moiety to the thiol side chain of a cysteine residue. For example, β-adrenergic stimulation activates CaMKIIδ in part through an NO synthase-dependent pathway (11), which increases the sarcoplasmic reticulum Ca2+ leak via ryanodine receptors (11, 12, 13). Moreover, CaMKIIδ activity is enhanced by NO donors (14), there is evidence that myocyte CaMKII can be nitrosylated (11, 12), and several S-nitrosylation sites have been predicted by consensus modeling. Therefore, CaMKIIδ may be directly modified by NO (12).

Most recently, S-nitrosylation sites have been described for the neuronal (α and β) isoforms of CaMKII (15, 16). It has been reported that CaMKIIα required dual S-nitrosylation of the Cys-280 and Cys-289 sites to produce autonomous activity, whereas CaMKIIβ, which does not have a cysteine residue that is homologous to Cys-280 on CaMKIIα, could be activated by a single S-nitrosylation event at Cys-290. These findings suggest that different CaMKII isoforms are differentially regulated by...
NO, leaving a critical gap in our understanding of the mechanisms that underlie potential NO-induced activation of CaMKII\(\beta\), the primary cardiac isoform.

Previous studies have reported a modulatory role for NO in cardiac calcium handling (17–19), and CaMKII\(\beta\) has been well established as a key mediator of altered calcium fluxes, ion channels, and pathological transcriptional regulation in the heart (2, 4). Moreover, CaMKII\(\beta\) is already known to be activated by oxidative stress (9), and perturbations of the balance between the nitroso and redox levels in the heart are thought to play a key role in cardiovascular pathology (20). Therefore, establishing the mechanism by which NO activates CaMKII\(\beta\) is critical for elucidating the underlying connection between NO signaling and cardiac function. There are three potential cysteine residues (Cys-290, Cys-273, and Cys-116) on CaMKII, with thiol side chains that are available for \(S\)-nitrosylation (12). We generated mutant variants of our FRET-based CaMKII activity sensor Camui lacking these potential \(S\)-nitrosylation sites and assessed Camui activation after exposure to the NO donor S-nitrosogluthathione (GSNO). Using Camui in conjunction with well established kinase assays, we measured CaMKII activity and changes in structural conformation under conditions favoring \(S\)-nitrosylation.

Here we show, for the first time, that \(S\)-nitrosylation of the Cys-290 site results in persistent autonomous CaMKII\(\beta\) activation and conformation. Moreover, we show that exposure to GSNO prior to treatment with Ca\(^{2+}\)/CaM inhibits CaMKII\(\beta\) activity, whereas mutation of the Cys-273 site ablates this effect. We also show that nitrosylation modulates CaM binding to CaMKII\(\beta\). Finally, we demonstrate that modulation of CaMKII\(\beta\) activation by nitrosylation plays a role in the generation of CaMKII\(\beta\)-dependent Ca\(^{2+}\) sparks in permeabilized mouse ventricular myocytes. Our results provide the first mechanistic details for CaMKII activation by NO in the heart and illuminate a novel mechanism by which NO can suppress cardiac CaMKII activity, suggesting a more complex and nuanced regulatory role for NO in CaMKII function than what has been described previously.

**Experimental Procedures**

**Generation of the Camui Constructs and Sequence Alignment**—The wild-type Camui\(\delta\)C construct was generated as described previously (21). In brief, mutagenesis of the CaMKII\(\delta\)C sequence was done to introduce a BglII site adjacent to the start codon and to replace the stop codon with a SalI site. The CaMKII\(\delta\)C sequence was inserted into the CFP-C1 vector with BglII and SalI, and CFP-CaMKII\(\delta\)C was inserted into the YFP-N1 vector creating CFP-CaMKII\(\delta\)C-YFP. QuikChange mutagenesis (Agilent Technologies) and InFusion HD Cloning Plus were used to generate the following mutations to the CaMKII\(\delta\)C sequence: C116A, C273A/C273S, C290A, C273S/290A, C116A/C290A, C116A/C273S, and C116A/C273S/C290A. CaMKII sequence alignment and analysis were used to generate Veiouse version 6.0.4 by Biomatters.

**HEK 293 Cell Transfection**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 5% fetal bovine serum and penicillin/streptomycin for 24 h, followed by transfection in Lipofectamine 3000 (Life Technologies) with expression plasmids encoding a Camui variant. Camui expression was checked after 48 h by fluorescence microscopy.

**Mouse Cardiac Ventricular Myocyte Isolation**—Single ventricular myocytes were enzymatically isolated from hearts of C57BL6 wild-type mice as described previously (22). All procedures were performed according to the Guiding Principles in the Care and Use of the Animals approved by the Council of the American Physiological Society. Briefly, after anesthesia (isoflurane, 5%), mice were injected with the anticoagulant heparin (8000 units/kg of body weight). Hearts were excised and retrogradely perfused on a constant flow Langendorff apparatus (4 min, 37 °C) with Ca\(^{2+}\)-free normal Tyrode solution and gassed with 100% O\(_2\). Ventricular myocytes were digested by Liberase (0.75 mg/ml, Roche) and trypsin (0.0138%, Gibco). Ventricular myocytes were filtered through a nylon mesh and allowed to sediment for 10 min. The sedimentation was repeated three times every 10 min, and [Ca\(^{2+}\)] was raised stepwise to 0.125, 0.25, and 0.5 mM. Myocytes were kept in 0.5 mM Ca\(^{2+}\) normal Tyrode solution at room temperature.

**Fluorescence of Camui in HEK Cell Lysates**—Transfected HEK cells were lysed by sonication in KCl-HEPES buffer (pH 7.2) with a protease inhibitor mixture (Calbiochem). After centrifugation (2000 rpm, 5 min), the lystate was removed and diluted into a basal incubation medium containing 140 mM KCl, 20 mM HEPES, and 1 mM MgCl\(_2\) with protease inhibitors (pH 7.2) with KOH at room temperature. Free [Ca] in solution was calculated using MaxChelator.

Camui variants in 0.3 ml of lystate were first incubated in a chamber (Warner Instruments) mounted on a coverslip on the stage of an inverted Leica microscope. Additions (1% volume) were prepared in the same parent buffer with 3- to 10-s mixing. The lystate was initially incubated with 50 \(\mu\)M EGTA to reduce [Ca\(^{2+}\)] to nominally Ca\(^{2+}\)-free conditions and establish a basal autoinhibited state of CaMKII. Free [Ca\(^{2+}\)] was then increased to 200 \(\mu\)M, and 10 \(\mu\)M CaM was added to directly activate CaMKII, as indicated under “Results.” To promote nitrosylation or oxidation, CaMKII lysters were exposed to either 150 \(\mu\)M GSNO or 1 \(\mu\)M H\(_2\)O\(_2\) for 7 min. To test for the autonomous CaMKII activation state, Ca\(^{2+}\) was chelated by addition of 10 mM EGTA.

FRET measurements used a Leica inverted microscope equipped with a Dualview2 and an Evolve Electron Multiplying Charge Coupled Device camera (Photometrics). Direct excitation of CFP was at 432 ± 10 nm, with emission measured simultaneously at 470 ± 15 nm (CFP) and 535 ± 15 nm (YFP FRET). Custom scripts using Metamorph software (Molecular Devices) enable synchronized control of the electronics for illumination, perfusion switching, and image acquisition. The protocol consisted of 250-ms acquisitions of CFP and FRET images (F\(_{\text{CFP}}\) and F\(_{\text{YFP}}\) every 20 s for 7 min/treatment. Average fluorescence intensity was quantified in ImageJ and corrected for background fluorescence (lysates from cells without Camui expression). The FRET ratio (F\(_{\text{CFP}}\)/F\(_{\text{YFP}}\)) for each time point within a trace was normalized to the average of the final 10 time points in 50 \(\mu\)M EGTA solution. Each replicate was from a separate HEK cell lysate, and data at each time point were averaged over replicates (n = 6) to provide error bars on these traces. Decreases of Camui FRET (increases of F\(_{\text{CFP}}\)/F\(_{\text{YFP}}\)) were con-
S-Nitrosylation and CaMKIIδ Activation

sidered to be indicative of the CaMKII conformational or activation state (21).

**CaMKII Activity Assays**—Parallel studies of CaMKII target phosphorylation also used lysates from HEK cells with different CaMKII mutants. CaMKII kinase activity was directly measured as the incorporation of 32P from [32P]ATP into an artificial substrate, syntide-2, as described previously (9, 10). HEK cells expressing Camui variants were lysed by sonication (as above) and treated for 7 min with 150 μM GSNO or 1 μM H2O2, followed by 10 mM EGTA, similar to the fluorescence experiments. Activation by Ca2+ (200 μM CaCl2) and CaM (10 μM) for 7 min was done either before or after GSNO exposure. Lysates were incubated with syntide-2 (a CaMKII substrate) and [32P]ATP for 10 min. Samples were blotted on Whatman filter paper (Sigma) to quench the reaction and washed three times to remove the remaining [32P]ATP, followed by scintillation counting to determine 32P incorporation into syntide-2. Control experiments were performed without syntide-2 to allow background subtraction of non-syntide-2 32P (typically <5%).

**Detection of CaMKII Nitrosylation**—Camui-transfected HEK293 cells were lysed in HENS buffer (containing 100 mM HEPES (pH 7.8), 1 mM EDTA, 0.1 mM neocuproine, 1% SDS, protease inhibitor mixture (Calbiochem), and 10 units/ml benzamidine). The SDS concentration in the lysate was then adjusted to 0.2% SDS before 30-min incubation at room temperature with intermittent vortexing. After centrifugation (2500 relative centrifugal force for 5 min), the fluorescence in the lysate was measured using a Spectramax M5 multimode plate reader (Molecular Devices; excitation, 500 nm; emission, 535 nm; cut-off at 515 nm; number of reads, 20). From this point on, the samples were protected from light. To induce nitrosylation, samples of equal fluorescence levels (~300 μg of protein) were exposed to 200 μM GSNO or reduced glutathione for 30 min in the presence of 200 μM free Ca2+ and 10 μM CaM. Nitrosylated proteins were then pulled down using 8 μl of anti-S-nitrosocysteine antibody (Sigma, catalog no. N5411). Samples were incubated end-over-end overnight at 4 °C before addition of 50 μl of pure proteome protein A/G mix magnetic beads (washed and resuspended in HEN buffer (100 mM HEPES (pH 7.8), 1 mM EDTA, and 0.1 mM neocuproine)). Samples were then incubated end-over-end for an additional 2 h at room temperature. The immunoprecipitates were subsequently washed five times with HEN buffer before elution from the beads with HEN buffer containing 10 mM dithiothreitol (which did not quench fluorescence). The fluorescence in the eluted samples was quantified using M5. The obtained fluorescence values were corrected for background fluorescence and the preimmunoprecipitation fluorescence of the sample. Data are presented as percent of fluorescence measured in the Camui-δC wild-type sample.

**Assessment of CaM/CaMKII Binding**—GFP-CaMKIIδC-transfected HEK293 cells were lysed in KCl-HEPES buffer with 1% Nonidet P-40. After centrifugation (2000 rpm, 5 min), the supernatant was removed and adjusted to 0.5 mg/ml protein and 0.1% Nonidet P-40. CaMKII was then immunoprecipitated by incubation overnight with anti-GFP antibody (Santa Cruz Biotechnology, catalog no. sc-9996, 1 μg/100 μg of lysate protein) at 4 °C, followed by incubation with protein A/G-coupled magnetic beads (Dynabeads, Thermo Fisher) for 1 h. The bead-protein complexes were washed several times with the Camui-FRET experiment parent buffer before examining the effect of CaMKII nitrosylation on the association with CaM. Drug additions to promote CaMKII activation and nitrosylation mimicked the Camui FRET experimental conditions (7-min exposure for each treatment). After treatment, bead complexes were washed three times by magnetic bead pulldown using CaM-free final buffer conditions before resuspension in sample buffer. Membranes were probed for CaM (Fitzgerald, monoclonal antibody 10R-C108C, clone 05-173, 1/5,000) and GFP and visualized with goat anti-mouse IgG (H+L) IRDye800CW (Licor) or light chain-specific Alexa Fluor 790 (Jackson Immuno-Research Laboratories). Signal intensities were quantified using ImageJ. All experiments were performed in duplicate.

**Ca2+ Sparks in Saponin-permeabilized Ventricular Myocytes**—Fresh isolated myocytes were permeabilized by saponin (50 μg/ml) for 3 min as described previously (23). Briefly, ventricular myocytes were superfused with an internal solution containing 1 mM EGTA, 10 nM free [Ca2+]i (calculated using MaxChelator), 10 mM HEPES, 120 mM potassium aspartate, 1 mM free MgCl2, 5 mM ATP, 5 mM phosphocreatine disodium, 5 units/ml creatine phosphokinase, 8% dextran (Mw, 40,000) (pH 7.2). 10 mM reduced glutathione was excluded in all internal solutions. Cell protocols prior to Ca2+ spark measurements were devised to parallel (as far as practical in myocytes) the protocols used with Camui in HEK cell lysates.

After saponin permeabilization, myocytes were washed for 1 min with the same internal solution to establish a basal autoinhibited state of CaMKII (Control). Some cells were then exposed to 150 μM GSNO for 7 min at 10 nM [Ca2+]i (GSNO) to test for direct GSNO effects on Ca2+ sparks at baseline. For both Control and GSNO conditions, some cells were subjected to activation of endogenous CaMKII (24), and these groups are referred to as CaMKII and GSNO → CaMKII. To activate CaMKII, [Ca2+]i, was elevated for 1 min to 500 nM with 1.2 μM exogenous CaM, 2 μM phosphatase inhibitor okadaic acid, and 15 μM PKA inhibitor. Okadaic acid was included to prevent dephosphorylation of CaMKII and its targets, and PKA inhibitor was included to prevent PKA activation as described previously (24). After CaMKII direct activation, ventricular myocytes were washed for 1 min with 10 nM [Ca2+]i internal solution, including both okadaic acid and PKA inhibitor. Some myocytes exposed only to CaMKII activation were subsequently exposed to 150 μM GSNO for 7 min (at 10 nM [Ca2+]i), constituting the CaMKII → GSNO condition. Therefore, GSNO → CaMKII and CaMKII → GSNO had similar treatments, except for the order of GSNO and CaMKII.

For each of these five groups, cells were washed for 1 min in 10 nM [Ca2+]i, before switching to 30 nM free [Ca2+]i plus 1 μM EGTA and 25 μM Fluo-4 for 5 min to measure Ca2+ sparks. Note that [Ca2+]i, was never more than 30 nM, except during CaMKII activation (1 min at 500 nM [Ca2+]i).

Ca2+ sparks were recorded at room temperature by a laser-scanning confocal microscope (Radiance 2100 MP, ×40 objective, Bio-Rad) using line scan mode with an argon laser (excitation, 488 nm; emission, >500 nm). Data were analyzed using
SparkMaster (25), a spark analysis program that allows rapid and reliable spark analysis.

Statistics—Pooled data are represented as means ± S.D. (fluorescence) or S.E. (32P incorporation and immunoprecipitation experiments). Statistical comparisons were made using repeated two-way analysis of variance and paired Student’s t test where applicable (with p < 0.05 considered significant).

Results

Fig. 1 shows the amino acid sequence of several isoforms of CaMKII in the regulatory autoinhibitor domain, illustrating the CaM binding domain and known sites of posttranslational modification that are known to promote the autonomous conformation of CaMKII in which the kinase stays active even in the absence of Ca2+ (7). These CaMKIIδ sites include the autophosphorylation site (Thr-287), oxidation sites (Met-281/282), the O-GlcNAcylation site (Ser-280), and also the phosphorylation site that inhibits CaM binding (Thr-306/307). It also shows two of the three cysteines (Cys-116, Cys-273, and Cys-290) that have been proposed as potential sites for nitrosylation (12). Note that CaMKIIα lacks the analogous Cys-273 that is present in CaMKIIδ. We made mutations at each of these potential nitrosylation sites in the full-length CaMKIIα and CaMKIIδ that were also incorporated into the CaMKIIα and δ Camui reporters.

NO Induces Autonomous CaMKIIδ Activation—Camui, our FRET-based CaMKII activation state sensor, assesses changes in CaMKII conformation associated with direct or autonomous activation of the kinase (21). Therefore, the activation state of CaMKII can be assessed dynamically in real time for intact cells expressing Camui. Using this technique, we were able to assess CaMKII activation online during sequential signaling events, which is not possible with destructive methods to measure target phosphorylation (used below). Treatment of the WT CaMKIIδ form of Camui with Ca2+/CaM resulted in a 40% increase in the FCFP/FYFP ratio (reduced FRET) to ~1.4 times the basal level (Fig. 2A), consistent with CaMKII activity assays measuring incorporation of 32P into a CaMKII substrate (Fig. 2D, black column). WT Camui activation by Ca2+/CaM was fully reversed after treatment with 10 mM EGTA, demonstrating that no autonomous activation of CaMKII had occurred (Fig. 2, A and D, gray column). When WT Camui was incubated with Ca2+/CaM and then the NO donor GSNO (150 μM) was added, FCFP/FYFP remained similar to that with Ca2+/CaM alone (FCFP/FYFP ~1.37, Fig. 2B). After GSNO treatment, however, addition of 10 mM EGTA did not return WT Camui FCFP/FYFP to baseline (FCFP/FYFP ~1.25, Fig. 2B). This observation indicates that the NO donor induced the autonomous CaMKII activation state, as assessed directly by CaMKII target phosphorylation (Fig. 2D, blue column).

Importantly, the order of incubation for Ca2+/CaM and GSNO was a critical determinant of NO-induced CaMKII activation. When WT Camui was incubated with GSNO for 7 min prior to addition of Ca2+/CaM, the robust FCFP/FYFP change was nearly abolished (FCFP/FYFP ~1.09) and no autonomous activation of the kinase was detected (Fig. 2, C and D, red col-
Our data demonstrate that NO induces autonomous activation of CaMKII when present in conjunction with Ca\(^{2+}/\text{CaM}\) but that NO inhibits CaMKII activation when present before Ca\(^{2+}/\text{CaM}\) is available.

**NO-induced CaMKII Activation Is Dependent on the Cys-290 Site**—To further elucidate the mechanism by which NO produces autonomous CaMKII activation, we used Camui with mutations at each of the three potential cysteine residues with available thiol side chains for S-nitrosylation. Initially, each of the cysteine residues was mutated to an alanine residue (C290A, C273A, and C116A). However, kinase assays revealed that the C273A form of Camui had severely compromised activity and could only be activated weakly by Ca\(^{2+}/\text{CaM}\) compared with WT CaMKII (Fig. 3A). This indicates that the C273A mutation disrupts normal CaMKII activation. The Cys-273 site on CaMKII is homologous to the Ser-272 site on CaMKII (Fig. 1), so we generated an alternate C273S Camui mutant for this study. Each of the Camui mutants used in subsequent experiments (C290A, C273S, and C116A) showed comparable Ca\(^{2+}/\text{CaM}\)-induced activity, and this activity was reversed by EGTA in a manner similar to the WT form (Fig. 3B and C).

We next assessed nitrosylation of WT and mutant Camui by GSNO treatment using immunoprecipitation with an anti-nitrosylation antibody (Fig. 3D). Without GSNO pretreatment (or under reducing conditions) there was very little GFP fluorescence in the immunoprecipitate compared with GSNO-treated WT δC Camui. After GSNO exposure, the C116A mutant was similar to the WT, but the C273S/C290A double mutant and C116A/C273S/C290A triple mutant had very low fluorescence. The single-site C273S and C290A mutants were intermediate. These data are consistent with GSNO-dependent nitrosylation occurring mainly at Cys-273 and Cys-290, with less robust nitrosylation at Cys-116.

Next, each of the mutant Camui forms was incubated with Ca\(^{2+}/\text{CaM} \) and 150 \(\mu\text{M}\) GSNO, followed by treatment with EGTA. The NO donor induced a persistent conformation change consistent with autonomous activation state in the C273S and C116A mutants, comparable with WT Camui. However, this autonomous activation state was abolished in the C290A mutant, where EGTA returned FCFP/FYFP to baseline conformation (FCFP/FYFP, \(1.04\), Fig. 4, A and B). The conformational effect observed in Camui was recapitulated at the functional level in the direct CaMKII activity assay (Fig. 4C).

Moreover, we generated double (C273S/C290A) and triple (C116A/C273S/C290A) Camui mutants to test the potential interdependency of nitrosylation events in inducing the autonomously active state. In both cases, the mutation of the Cys-290 site ablated GSNO-induced autonomous activation state (Fig. 4D and E). Therefore, our data demonstrate that the Cys-290 site is required for mediating NO-induced autonomy in CaMKIIδ and that neither Cys-116 nor Cys-273 contribute appreciably to this effect.

**FIGURE 3. Generation of Camui mutants lacking potential nitrosylation sites.** Mutant forms of Camui lacking potential nitrosylation sites were generated and tested for normal CaMKII activity and reduced GSNO-induced nitrosylation. A, the C273A Camui-δ mutant had significantly reduced activity, as measured by kinase assay, compared with WT Camui-δ. Treatment with Ca\(^{2+}/\text{CaM}\) activated all three mutant forms of CaMKIIδ used for this study in a similar manner as WT CaMKII, as measured by Camui (B) and kinase (C) activity assay. D, Camui-δ nitrosylation induced by 200 \(\mu\text{M}\) GSNO, assessed by nitrosyl immunoprecipitation (IP) and detection of GFP fluorescence, for the CaMKII mutants was reduced but not significantly different from δC WT. Camui and activity data are from six experiments, whereas immunoprecipitation data are from three to four experiments. Data are shown as mean ± S.D. (A and S.E. (B and C). *, \(p < 0.05\).
FIGURE 4. The C290A mutation ablates NO-mediated CaMKIIδ activation. A–C, Camui fluorescence traces (A) and summary data (B) demonstrate that the C290A mutant form of Camui (but not C273S or C116A) prevents NO-mediated autonomous activation, a finding confirmed in kinase activity assays (C). D and E, double mutant (C273S/C290A, D) and triple mutant (C116A/C273S/C290A, E) variants of Camui showed no autonomous activation after treatment with Ca2+/CaM and GSNO, followed by EGTA. Data are from six experiments (A–C) and two experiments (D and E). Data are shown as mean ± S.D. (A, B, D, and E) and S.E. (C). *, p < 0.05.
Our data for the Cys-290 site of the cardiac CaMKIIδ are consistent with previous findings for neuronal CaMKIIα (15) and CaMKIIβ (16). Indeed, we found that GSNO treatment caused a conformational shift consistent with autonomous activation in a WT but not a C289A mutant of Camui on the basis of the CaMKIIα isoform (Fig. 5, A and B). A previous study indicates that the Cys-289 site in the CaMKIIα isoform has a role in oxidation-induced activation of the kinase (16). Because the balance of oxidation and nitrosylation is thought to play a key role in cardiovascular signaling (20), we sought to determine whether the Cys-290 site was similarly subject to oxidative effects in CaMKIIδ. Therefore, we also measured the effects of 1 μM H2O2 on the CaMKIIδ mutant forms of Camui. Surprisingly, we found that mutation of the Cys-290 site ablated H2O2-induced conformational change of CaMKIIδ (Fig. 5C), as described previously for the Met-281/282 sites (9). Our own activity data suggest that both the C290A and M281V/M282V mutations ablate oxidation-induced autonomous CaMKIIδ activity (Fig. 6). As observed in WT Camui (Fig. 2, C and D), GSNO pretreatment strongly suppressed the Ca2+/CaM-induced change in conformation and activity for the C290A and C116A mutants (Fig. 6A–C). Strikingly, GSNO pretreatment failed to inhibit the change in activation state of the C273S mutant by Ca2+/CaM (Fig. 6B), nor did it prevent GSNO-induced autonomous activity (Fig. 6C). Therefore, our data suggest a novel dual regulatory role for nitrosylation of CaMKIIδ, which inhibits the kinase through modification of Cys-273 when NO donors are present before Ca2+/CaM is available, but can promote autonomous CaMKIIδ through modification of Cys-290 when present after Ca2+/CaM has activated the kinase.

GSNO Alters CaM Binding to CaMKIIδ—It is known that autophosphorylation of CaMKIIα at the canonical Thr-286 stabilizes CaM binding to the kinase, which contributes to autonomous activation (26). To test whether nitrosylation enhances CaM binding to CaMKIIδ, we expressed and immunoprecipitated GFP-tagged CaMKIIδ in HEK cells and treated that MEGTA, white bar in Fig. 7A shows CaM that was pulled down with GFP-CaMKIIδ. No CaM was detected in the baseline pulldown (50 μM EGTA, white bar in Fig. 7B). However, when 10 μM CaM and 200 μM [Ca2+] were included, CaM was readily detected in the precipitate and was reversed upon Ca2+ quench by EGTA (Fig. 7B, black and gray columns). Therefore, CaM binds reversibly to CaMKIIδ as expected. GSNO addition after Ca2+/CaM resulted in an even
higher level of CaM bound to CaMKII\(\text{H}^{9254}\), which was only partially reversed by EGTA (Fig. 7B, light and dark blue columns, respectively). In marked contrast, pretreatment with GSNO before the addition of Ca\(^{2+}/\text{CaM}\) reduced the amount of CaM in the precipitate (Fig. 7B, dark red column). Moreover, that lower CaM level was readily removed by EGTA (Fig. 7B, dark red column). Taken together, these data suggest that nitrosylation of CaMKII\(\text{H}^{9254}\) at the Cys-290 site enhances and prolongs CaM binding, whereas nitrosylation of the Cys-273 site reduces CaM binding.

Nitrosylation-dependent CaMKII\(\text{H}^{9254}\) Activity Modulates Ca\(^{2+}\) Spark Frequency in Mouse Ventricular Myocytes—To test whether nitrosylation-dependent CaMKII\(\text{H}^{9254}\) activation affects physiological signaling in myocytes, we examined the effects of GSNO treatment on CaMKII\(\text{H}^{9254}\)-mediated activation of Ca\(^{2+}\) sparks. Activation of CaMKII\(\text{H}^{9254}\) is known to enhance Ca\(^{2+}\) leak from the sarcoplasmic reticulum in myocytes (24, 27), and CaMKII\(\text{H}^{9254}\)-mediated sparks can be enhanced by posttranslational modifications that increase CaMKII\(\text{H}^{9254}\) activity (10). We know from previous work that the protocols used here to activate endogenous CaMKII\(\text{H}^{9254}\) (which is associated with the ryanodine receptor) results in an increased Ca\(^{2+}\) spark frequency that is mediated by endogenous CaMKII\(\text{H}^{9254}\)-dependent phosphorylation of RyR2 at Ser-2814 (i.e. the effects were blocked by the CaMKII peptide inhibitor autocomptide-2-related inhibitory peptide (AIP) and in RyR2-S2814A/D knockin myocytes, 27, 28). Therefore, we treated permeabilized mouse ventricular myocytes with GSNO and/or Ca\(^{2+}/\text{CaM}\) to measure the effects of nitrosylation-dependent and CaMKII\(\text{H}^{9254}\) activation on the frequency and amplitude of spontaneous sarcoplasmic reticulum Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks).
Activation of CaMKIIδ with Ca²⁺/CaM resulted in a significant increase in Ca²⁺ sparks (CaMKII, Fig. 8A), consistent with prior observations. Conversely, GSNO alone did not affect Ca²⁺ sparks. However, treatment with Ca²⁺/CaM first and then GSNO (GSNO → CaMKII) followed by Ca²⁺ chelation with EGTA, resulted in a persistent and significant increase in Ca²⁺ spark frequency (Fig. 8B) and amplitude (Fig. 8C). These findings were consistent with our hypothesis that nitrosylation of CaMKIIδ results in autonomous activation of the kinase, which is larger and more frequent spontaneous Ca²⁺ release events. Importantly, when the permeabilized myocytes were pretreated with GSNO at 10 nM [Ca²⁺], before exposure to Ca²⁺/CaM, neither Ca²⁺ spark frequency or amplitude were enhanced by CaMKII activation (GSNO → CaMKII), indicating that nitrosylation of inactive CaMKIIδ inhibited subsequent activation of the kinase by Ca²⁺/CaM. Moreover, these functional effects in cardiac myocytes parallel the direct measurements of GSNO effects on CaMKII activation and conformational changes measured above in HEK cell lysates.

Discussion

Our data demonstrate that the primary cardiac isoform of CaMKII (CaMKIIδ) is activated by NO, a novel regulatory mechanism for CaMKII in the heart. Moreover, we show that the Cys-290 site on CaMKIIδ is critical for NO-induced activation. These results are consistent with observations in neuronal forms of CaMKII (α and β), which are also activated by NO (15, 16). The nitrosylation-sensitive Cys-290 site is situated in the hinge region between the regulatory and catalytic domains of CaMKII (Fig. 1). Other modifications in this area have been shown previously to autonomously activate CaMKIIδ, including phosphorylation at Thr-287, oxidation at Met-281/282, and O-GlcNAc modification at Ser-280 (8-10). These findings suggest a potential “hot spot” on CaMKII for activation of the kinase by posttranslational modification.

In contrast to previous studies on CaMKII nitrosylation, we discovered a novel regulatory mechanism by which NO inhibits CaMKIIδ activity through S-nitrosylation of the Cys-273 site on the basal inactive CaMKIIδ. This previously unknown mechanism suggests a more complex and nuanced role for mediation of CaMKII activity by NO in the heart. Indeed, the discovery of a distinct nitrosylation site that inhibits CaMKII activity mirrors similar observations for phosphorylation at Thr-307 (29) and oxidation at Met-308 (9). The α isoform of CaMKIIδ has a serine at position 272 (the homologous site to Cys-273 on CaMKII) and, therefore, is not subject to NO-mediated inhibition, whereas inhibition of CaMKIIβ or γ (which have this site) by S-nitrosylation has not been reported. Therefore, modification of the Cys-273 site and NO-mediated CaMKII inhibition may be unique to tissue types expressing CaMKIIδ, such as the myocardium. However, this Cys-273 site, the Met-281/282 sites, and the Cys-290 site are all conserved in CaMKIIδ, γ, and β (differing only in CaMKIIα, Fig. 1). Therefore, the sufficiency of Cys-290 for activation by S-nitrosylation and an inhibitory role of Cys-273 nitrosylation, which we demonstrate here for CaMKIIδ, may be similar in CaMKIIβ and γ (although that needs to be tested).

The discovery that GSNO can either inhibit or prolong CaMKII activation implies that the sensitivity and/or availabili-
ity of the Cys-273 and Cys-290 sites changes after Ca\textsuperscript{2+}/CaM binding. In particular, the Cys-290 site may be less available for nitrosylation in its basal, inactive form because of steric hindrance by the association between the regulatory and catalytic domains. On the other hand, the Cys-273 site may be available already in the basal state. Future studies will be needed to discern the sensitivity of each site to GSNO and other NO donors. Moreover, NO-induced activation and inhibition of CaMKII\textsubscript{a} may be affected by the presence of other posttranslational modifications, including autophosphorylation, oxidation, and O-GlcNAcylation. Indeed, O-GlcNAcylation seems to promote autophosphorylation (10). Therefore, the complex interplay between these modulatory mechanisms of CaMKII activity will require further examination.

In recent years, posttranslational modifications that result in autonomous CaMKII\textsubscript{a} activation have been implicated in several forms of cardiac pathology. Oxidation-dependent activation of CaMKII\textsubscript{a} contributes to myocyte apoptosis during myocardial infarction (9), atrial fibrillation (30), and vascular smooth muscle cell migration after vascular injury (31). CaMKII\textsubscript{a} is also activated by O-GlcNAc modification, which contributes to hyperglycemia-induced Ca\textsuperscript{2+} leaking and arrhythmia (10). NO-mediated signaling is known to play a role in cardiac physiology and pathology (17–19), but the underlying mechanisms are not completely understood. Here we show that nitrosylation of CaMKII\textsubscript{a} plays a key role in modulating Ca\textsuperscript{2+} release events from the sarcoplasmic reticulum in myocytes (Fig. 8), demonstrating just one mechanism by which nitrosylation-dependent CaMKII activation may influence cellular physiology.

Interestingly, like CaMKII, NO synthases 1 and 3 (but not 2) require Ca\textsuperscript{2+}/CaM binding for the production of NO (32). Therefore, both the timing and the localization of Ca\textsuperscript{2+} spikes may be critical in determining whether CaMKII\textsubscript{a} is activated or inhibited by nitrosylation. That is, if [NO\textsubscript{3}] rises in one cellular microdomain where Ca\textsuperscript{2+}/CaM is not sufficient to activate local CaMKII\textsubscript{a}, \( \beta \), or \( \gamma \), then it could suppress CaMKII activation. In another locus, where CaMKII and NO synthase are coactivated by local Ca\textsuperscript{2+}/CaM, it could promote stronger CaMKII activation (like a coincidence detector), and the combination could allow more locally discrete CaMKII regulation in cells. Therefore, our discovery that CaMKII\textsubscript{a} activity can be both prolonged and inhibited by S-nitrosylation suggests that CaMKII activation may be a critical node in how local NO and reactive oxygen species production translate into downstream physiological (or pathological) events in the heart.

An additional finding from our study was the observation that the Cys-290 site appears to play a role in modulating oxidation-dependent CaMKII\textsubscript{a} activity. This finding agrees with prior observations in the C289A mutant form of CaMKII\textalpha (16) but differs from a previous study focused on CaMKII\textsubscript{a} (9), which found no effect on H\textsubscript{2}O\textsubscript{2}-induced activity after mutation of Cys-290. The prior study that found no effect on redox-dependent activation after Cys-290 mutation was done in mouse CaMKII\textsubscript{a} purified from a baculovirus system, whereas the current measurements were made using Camui, on the basis of the human CaMKII\textsubscript{a} isoform, in cell lysates. Therefore, the observed differences may indicate NO-mediated effects that require an intact cellular milieu, or they may reflect the differing redox environments between mammalian and insect-based expression systems.

Autophosphorylation of CaMKII at Thr-287 greatly enhances CaM affinity for the kinase (26, 33). In this study, we observed that nitrosylation of CaMKII\textsubscript{a} at the Cys-290 site enhances CaM-binding (Fig. 7), suggesting similarities between the mechanisms by which Thr-287 phosphorylation and Cys-290 nitrosylation convert CaMKII\textsubscript{a} to an autonomously active form. There are now four posttranslational modifications in the regulatory domain of CaMKII\textsubscript{a} that are known to induce autonomous activity: phosphorylation (Thr-287), oxidation (Met-281/282), O-GlcNAcylation (Ser-280) and nitrosylation (Cys-290). Interestingly, nitrosylation of the Cys-273 site (or mutating it to Ala) appears to reduce CaM binding to CaMKII\textsubscript{a}, perhaps through steric occlusion of the CaM-binding site. Indeed, similar observations have been made for phosphorylation of the Thr-306 site of CaMKII\textsubscript{c}, which blocks CaM binding and inactivates the kinase (34). Future studies are needed to assess how posttranslational modifications throughout the regulatory domain of CaMKII\textsubscript{a} synergize functionally to modulate Ca\textsuperscript{2+}/CaM affinity.

Inhibition of CaMKII\textsubscript{a} has been suggested as a potential therapeutic strategy for the prevention or amelioration of heart disease (35, 36). Therefore, understanding the basic mechanisms that underlie CaMKII\textsubscript{a} activation and inhibition is critical for the development of future therapies. We have demonstrated that NO mediates CaMKII\textsubscript{a}, and the novel discovery that S-nitrosylation can either activate or inhibit CaMKII\textsubscript{a} activity depending on the timing of NO exposure relative to Ca\textsuperscript{2+}/CaM binding suggests a complex interaction between NO and CaMKII in cells with transient calcium changes, as in the myocardium. Further research will be necessary to elucidate the role of NO-mediated CaMKII activity in physiological and pathological cardiac signaling.

**Author Contributions**—J. R. E., C. B. N., and D. M. B. conceived and coordinated the study. J. R. E. and D. M. B. wrote the paper. H. U. performed and analyzed the experiments shown in Fig. 8. J. B. and M. L. S. designed, performed, analyzed, and drafted figures for the experiments shown in Figs. 3 and 7.

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