Circulation Research

ORIGINAL RESEARCH

Nitric Oxide Modulates Ca^{2+} Leak and Arrhythmias via S-Nitrosylation of CaMKII

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BACKGROUND: Nitric oxide (NO) has been identified as a signaling molecule generated during β-adrenergic receptor stimulation in the heart. Furthermore, a role for NO in triggering spontaneous Ca^{2+} release via S-nitrosylation of CaMKIIδ (Ca^{2+}/calmodulin kinase II delta) is emerging. NO donors are routinely used clinically for their cardioprotective effects on the heart, but it is unknown how NO donors modulate the proarrhythmic CaMKII to alter cardiac arrhythmia incidence. We test the role of S-nitrosylation of CaMKIIδ at the Cysteine-273 inhibitory site and cysteine-290 activating site in cardiac Ca^{2+} handling and arrhythmogenesis before and during β-adrenergic receptor stimulation.

METHODS: We measured Ca^{2+}-handling in isolated cardiomyocytes from C57BL/6J wild-type (WT) mice and mice lacking CaMKIIδ expression (CaMKIIδ-KO) or with deletion of the S-nitrosylation site on CaMKIIδ at cysteine-273 or cysteine-290 (CaMKIIδ-C273S and -C290A knock-in mice). Cardiomyocytes were exposed to NO donors, S-nitrosoglutathione (GSNO; 150 μM), sodium nitroprusside (200 μM), and β-adrenergic agonist isoproterenol (100 nmol/L).

RESULTS: Both WT and CaMKIIδ-KO cardiomyocytes responded to isoproterenol with a full inotropic and lusitropic Ca^{2+} transient response as well as increased Ca^{2+} spark frequency. However, the increase in Ca^{2+} spark frequency was significantly attenuated in CaMKIIδ-KO cardiomyocytes. The protection from isoproterenol-induced Ca^{2+} sparks and waves was mimicked by GSNO pretreatment in WT cardiomyocytes but lost in CaMKIIδ-C273S cardiomyocytes. When GSNO was applied after isoproterenol, this protection was not observed in WT or CaMKIIδ-C273S but was apparent in CaMKIIδ-C290A. In Langendorff-perfused isolated hearts, GSNO pretreatment limited isoproterenol-induced arrhythmias in WT but not CaMKIIδ-C273S hearts, while GSNO exposure after isoproterenol sustained or exacerbated arrhythmic events.

CONCLUSIONS: We conclude that prior S-nitrosylation of CaMKIIδ at cysteine-273 can limit subsequent β-adrenergic receptor–induced arrhythmias, but that S-nitrosylation at cysteine-290 might worsen or sustain β-adrenergic receptor–induced arrhythmias. This has important implications for the administration of NO donors in the clinical setting.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: calcium ■ heart ■ nitric oxide

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The ability of the heart to rapidly enhance output is mediated in part by stimulation of β-adrenergic receptors (β-ARs), which trigger increased release of intracellular Ca^{2+} from the sarcoplasmic reticulum (SR) and accelerated reuptake within cardiomyocytes.[1,2] Excessive β-AR stimulation can lead to arrhythmias[3] and heart

failure[4]; therefore, an understanding of the downstream signaling pathways that lead to pathological Ca^{2+} handling is vital. Nitric oxide (NO) is a gaseous signaling molecule that can alter cardiac function[5] and is produced endogenously within cardiomyocytes following β-AR stimulation.[6] NO-releasing drugs (donors) have been used clinically for

Novelty and Significance

What Is Known?

- Calcium flux is altered in cardiac cells exposed to nitric oxide donors and other conditions associated with protein nitrosylation.
- The activity of CaMKII (Ca^{2+}/calmodulin kinase II), a cardiac signaling kinase associated with proarrhythmic pathways, is mediated by nitrosylation.

What New Information Does This Article Contribute?

- We show that cardiac CaMKII expression is necessary for increased calcium leak in myocytes treated with nitric oxide donors.
- Using novel mouse models that resist CaMKII nitrosylation, we demonstrate that proarrhythmic calcium leak can be prolonged OR inhibited depending on the site of CaMKII nitrosylation.
- Finally, we show that the C273 nitrosylation site on CaMKII protects against arrhythmias, both in vivo and ex vivo.

Previous groups have demonstrated that conditions favoring protein nitrosylation can alter myocyte and whole heart calcium handling. However, there is controversy on the direction of the effect, as nitric oxide donors have shown both positive and negative effects on the development of cardiac arrhythmias. Here, we show that nitrosylation of the cardiac kinase CaMKII at 2 distinct sites (C273 and C290) are intergral to nitrosylation-mediated calcium mishandling. Moreover, the 2 sites have opposing effects, as nitrosylation at C273 inhibits the kinase and prevents arrhythmias, while C290 nitrosylation prolongs CaMKII activity and worsens arrhythmic phenotypes in cells and isolated hearts. These findings link CaMKII to nitrosylative stress and suggest future clinical directions focussed on cardiac CaMKII inhibition to prevent arrhythmia.

Nonstandard Abbreviations and Acronyms

β-AR	β-adrenergic receptor
Akt	protein kinase B
CaM	Ca^{2+}/calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CaMKIIδ-KO	mice lacking the delta isoform of CaMKII
GSNO	S-nitrosoglutathione
LTCC	L-type Ca^{2+} channel
NO	nitric oxide
NOS	nitric oxide synthase
RyR2	ryanodine receptor type 2
SERCA	SR Ca^{2+} ATPase
sGC	soluble guanylyl cyclase
SNP	sodium nitroprusside
SR	sarcoplasmic reticulum
WT	wild type

over a century for their protective actions on heart function[7]; however, the direct effects of NO on cardiomyocytes are still under investigation. NO can exert positive inotropic effects due to protein modification by S-nitrosylation,[8] where NO covalently attaches to cysteine (cysteine) residues and alters protein activity.[9] NO exposure (either endogenously produced or exogenously applied) has been regarded as cardioprotective in the context of ischemia-reperfusion, due to the S-nitrosylation of cardiac proteins.[10]

Recently, evidence has demonstrated that endogenous NO production is linked to increased spontaneous release of Ca^{2+} from the SR during β-adrenergic stimulation.[11–13] These observations challenge the cardioprotective role of NO, as spontaneous Ca^{2+} leak is arrhythmogenic.[14] The function of several cardiac Ca^{2+} handling proteins is reported to be modulated by S-nitrosylation, including the RyR2 (ryanodine receptor type 2), LTCC (L-type Ca^{2+} channel), and SERCA (SR Ca^{2+} ATPase).[15] Interestingly, NO can alter the frequency of Ca^{2+} sparks in cardiomyocytes following β-AR stimulation in either a positive or negative manner,[16] although the detailed mechanism by which NO can both enhance and reduce Ca^{2+} spark frequency is not clearly understood.

An emerging target for cardiac NO is the CaMKIIδ (CaM [Ca^{2+}/calmodulin]−dependent kinase II delta),[11,13,17] a nodal regulator of cardiac Ca^{2+} handling.[18] The regulatory domain of CaMKIIδ contains 2 S-nitrosylation sites that alter its activity.[19] S-nitrosylation at cysteine-290, following initial activation by Ca^{2+}/CaM, causes autonomous activation of the kinase, consistent with the observation that NO exposure can enhance CaMKIIδ activity and increase Ca^{2+} sparks.[11,13,20] In contrast, S-nitrosylation at cysteine-273 inhibits CaMKIIδ by preventing activation by Ca^{2+}/CaM,[19] suggesting a dual role for NO in mediating CaMKIIδ activity that may be alternately protective or pathological depending on intracellular conditions.

β-AR stimulation increases spontaneous Ca^{2+} release from RyR2 in a CaMKIIδ-dependent manner.[4] β-AR stimulation also increases endogenous NO production in cardiomyocytes,[13] which is necessary for the enhancement of Ca^{2+} sparks.[11] We therefore hypothesized that

arrhythmogenic activity at the cellular and whole heart levels would be prolonged by NO after β-adrenergic stimulation due to *S*-nitrosylation of the cysteine-290 site on CaMKIIδ. Further, we hypothesized that exposure to NO before β-AR stimulation would lead to *S*-nitrosylation of the cysteine-273 site on CaMKIIδ, reducing β-AR induced Ca²⁺ mishandling. Here, we tested the role of NO and CaMKIIδ on altered Ca²⁺ handling in the context of β-AR stimulation in isolated mouse cardiomyocytes and Langendorff-perfused mouse hearts from transgenic mice lacking expression of CaMKIIδ or the *S*-nitrosylation sites at cysteine-273 (inhibitory) or cysteine-290 (activating).

METHODS

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Mouse Models

Experiments were performed using 12- to 16-week-old male and female mice with 4 genotypes: C57BL/6J wild-type (WT), knockout mice with deletion of CaMKIIδ (CaMKIIδ-KO), and novel knock-in mouse models with a single mutation of the cysteine-273 or cysteine-290 *S*-nitrosylation sites on CaMKIIδ (CaMKIIδ-C273S and CaMKIIδ-C290A). The CaMKIIδ-C290A knock-in mice were generated by the UC Davis Mouse Biology Core and have been described previously.[20] CaMKIIδ-C273S animals were generated using CRISPR/Cas9 genome editing at the Australian Phenomics Facility (Australian National University, Australia). For all mouse resources described here and materials listed below, please see the Major Resources Table in the Supplemental Material.

Protein Expression

The ventricles were dissected and snap-frozen in liquid nitrogen for protein analysis as previously described.[21] CaMKIIδ expression was assessed using a primary antibody against CaMKIIδ (1:5000, Thermo Fisher PA5-22168) and GAPDH (1:10000, GeneTex GTX627408) for a loading control. Blots were then incubated with secondary mouse or rabbit antibodies conjugated with horse-radish peroxidase (1:10000; Thermo Fisher 31430, 31460), visualized by chemiluminescence detection with Super-signal West Pico (Thermo Fisher), and imaged using a Syngene gel doc system.

Heart Fibrosis

Ventricles were dissected above the apex of the heart and fixed in 4% formalin (Sigma) for 24 hours. The fixed tissue was then transferred to PBS for 24 hours, followed by 30% sucrose in PBS for 48 hours. Masson trichrome staining of sections was performed by the Histology Unit at University of Otago. Slides were then scanned on an Aperio Slide Scanner (Leica) under 40× magnification. Blue target pixels (representing collagen) were then identified. Percent collagen is the sum of all regions from sample sections of target pixels divided by total pixels.

Measurement of NO Concentration

The amount of NO released following addition of NO donor *S*-nitrosoglutathione (GSNO) to our experimental buffer (Krebs-Ringer HEPES buffer; see below) was measured using an Apollo 1000 Free Radical analyser with an isoproterenol-NOPF100 NO microsensor (1 mm; World Precision Instruments). The sensor was calibrated with S-nitroso-N-Acetyl-D,L-Penicillamine (Toronto Research) in 0.1 M CuCl₂ solution.[22] Data were acquired using a Powerlab 2/25 and recorded in LabChart 8.1 (ADInstruments, New Zealand).

Calcium Imaging

Freshly isolated cardiomyocytes were loaded with 2 µM Fluo-4-AM (Thermo Fisher) for 20 minutes at room temperature, followed by wash and de-esterification for 30 minutes. Cardiomyocytes were field stimulated at 0.5 Hz for 30 s to establish a steady state before recording Ca²⁺ transients in line-scan mode (2 ms/line, 0.15×0.15 µm pixel size). Ca²⁺ sparks and the occurrence of waves was measured under quiescent conditions (30 seconds after termination of 0.5 Hz pacing). Total SR Ca²⁺ content was determined at the end of each experiment with a rapid 20-mM caffeine exposure in Ca²⁺-free Krebs-Ringer HEPES buffer following a 30-second train of 0.5 Hz stimulations. Representative images have been chosen as illustrative examples of the results.

Isolated Heart Function

Mouse hearts were excised and arrested in Ca²⁺-free buffer before being cannulated via the aorta and Langendorff-perfused. Baseline data were recorded for 10 minutes, followed by drug infusion for 10 minutes. Ventricular arrhythmic events from the LV pressure trace were evaluated and classified as outlined in Figure S1 and Table S1. The different types of arrhythmias observed were classified by an arrhythmia score, which indicated severity of the arrhythmias (Table S2).

ECG Recordings

ECGs were recorded from mice under 1.5% to 2% isoflurane. ECG measurements were acquired from lead II connected to a Powerlab and recorded in LabChart 8.1(ADInstruments). Recordings were made for 10 minutes with the last 5 minutes used for analysis performed in LabChart.

Data Analysis

All individual data points are shown for cardiomyocyte Ca²⁺ and isolated heart parameters along with the mean±SEM. For results reported in the text, the data are mean±SD. Isolated heart perfusion data were analyzed on laboratory Chart 8.1 (ADInstruments). Statistical analysis was performed using Prism 10 (GraphPad) and RStudio (Posit; McNemar test only). Numeric data (n>10) were analyzed for normality using Shapiro-Wilk test. Paired data (drug responses) with a normal distribution were analyzed using paired 2-tailed Student *t* test. Non-normally distributed data or n<10 were analyzed using a nonparametric alternative test (Wilcoxon matched-pairs signed rank test or a Friedman test). For group comparisons (and SR content), an unpaired *t* test or an ordinary 1-way ANOVA (Tukey multiply comparisons test) was used for normally distributed data, while a Mann-Whitney test or Kruskal-Wallis, was used for nonparametric data sets. Differences in the fraction of

cardiomyocytes displaying Ca²⁺ waves were determined by a λ^2 test (between groups) or a McNemar test (paired data). Values where $P \leq 0.05$ were considered statistically significant.

RESULTS

NO Donor Does Not Alter Baseline Ca²⁺ Transient Properties

To induce *S*-nitrosylation of target proteins, we used the NO donor GSNO, which spontaneously releases NO into the perfusate (Figure 1A). [NO] peaked in Krebs-Ringer

HEPES buffer at 0.54 ± 0.06 µM after 10 minutes and remained stable over the duration of our experimental timeframe (Figure 1B). Ventricular cardiomyocytes were isolated from WT and CaMKIIδ-KO mice, which had undetectable expression of CaMKIIδ (Figure 1C). The cardiomyocytes were exposed to 150 µM GSNO according to the protocol outlined in Figure 1D. There were no observed effects of GSNO on Ca²⁺ transient amplitude (Figure 1E; control versus GSNO WT: $P=3.1\times10^{-1}$, KO: $P=9.4\times10^{-2}$) or time constant Tau of Ca²⁺ decay (Figure 1F; control versus GSNO WT: $P=9.5\times10^{-1}$, KO: $P=1.6\times10^{-1}$) in either WT or CaMKIIδ-KO myocytes. We also measured Ca²⁺

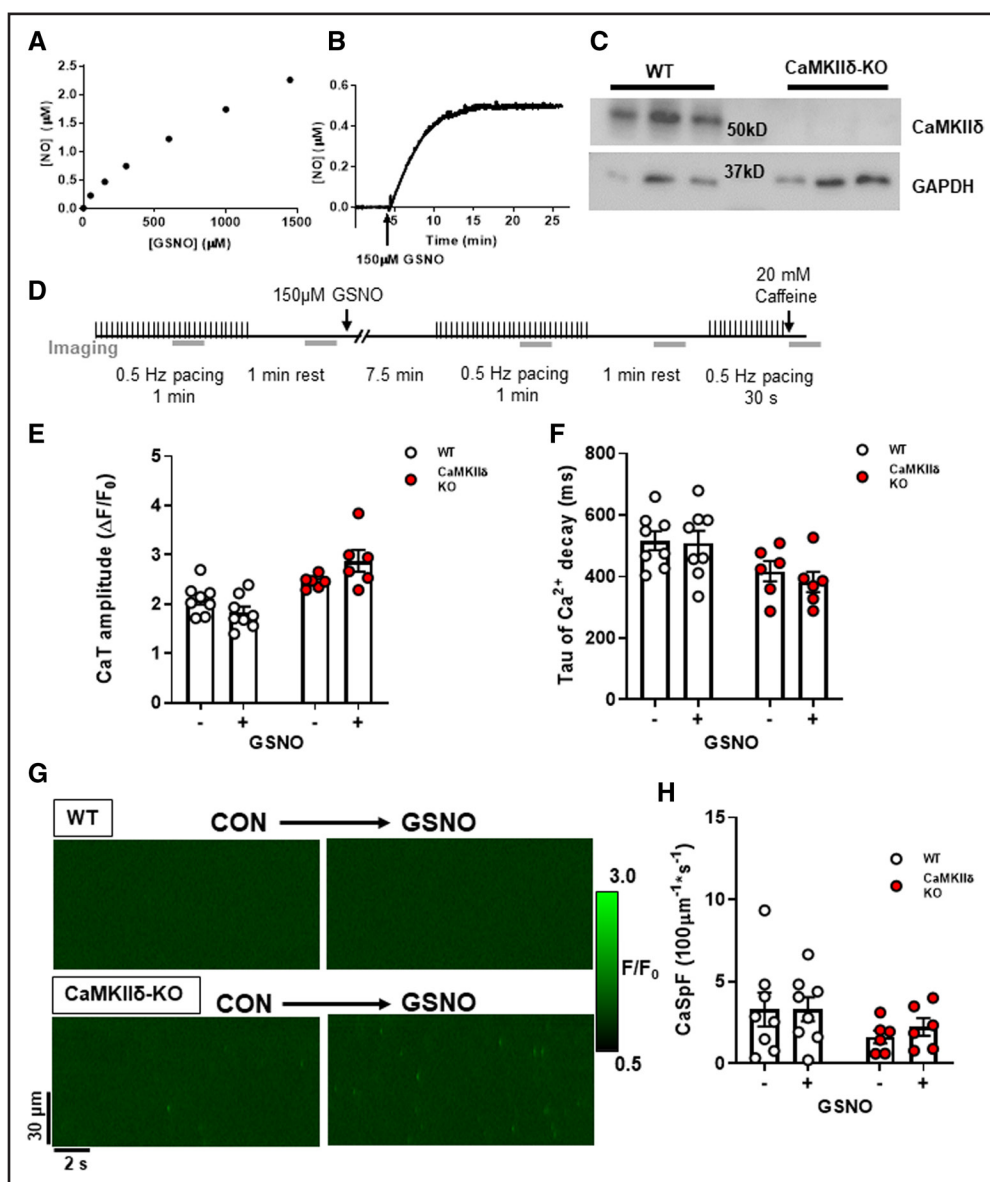**Figure 1. Cardiomyocyte Ca²⁺ transients and sparks with nitric oxide donor *S*-nitrosoglutathione (GSNO).**
A, Nitric oxide (NO) liberated from increasing GSNO concentrations in buffer was measured using a NO electrode. **B**, The kinetics of NO release following the addition of 150 µM GSNO to buffer. **C**, Western blot of ventricle tissue samples from wild-type (WT) and CaMKIIδ (Ca²⁺/calmodulin kinase II delta) KO mice (n=3 hearts) showing loss of CaMKIIδ protein expression in CaMKIIδ-KO vs WT hearts (with GAPDH loading controls). **D**, WT or CaMKIIδ-KO cardiomyocytes were treated with 150 µM GSNO and paced at 0.5 Hz. There was no change in Ca²⁺ transient amplitude (**E**) or decay kinetics (**F**). In quiescent cardiomyocytes, Ca²⁺ sparks were measured from line-scan images (**G**), and there was also no effect of GSNO on Ca²⁺ spark frequency in either WT or CaMKIIδ-KO cardiomyocytes (**H**; WT: n=8 cells, N=3 hearts; CaMKIIδ-KO: n=8 cells, N=3 hearts).

sparks in unpaced cardiomyocytes with confocal line-scan imaging (Figure 1G). We observed no effect of GSNO on Ca^{2+} spark frequency in cardiomyocytes of either genotype (Figure 1H; control versus GSNO WT: 5.5×10^{-1}, KO: $P=6.3\times10^{-2}$). There was also no effect of GSNO on SR Ca^{2+} content (Figure S2). Taken together, our data show that at the baseline low frequency stimulation used here, there is no major difference in electrically evoked or spontaneous Ca^{2+} transients in WT versus CaMKIIδ-KO, nor does exposure to GSNO alone alter these properties.

CaMKIIδ-KO Cardiomyocytes Produce Fewer Ca²⁺ Sparks During β-Adrenergic Stimulation

Next we increased myocyte Ca^{2+} transients with exposure to 100 nmol/L isoproterenol, which is expected to strongly promote CaMKIIδ activation (Figure 2A). Representative confocal line-scans from Fluo-4-AM loaded cardiomyocytes isolated from WT and CaMKIIδ-KO hearts are shown in Figure 2B. Fluo-4 fluorescence was normalized to baseline (F_0) to determine the Ca^{2+} transient characteristics in WT and CaMKIIδ-KO cardiomyocytes during isoproterenol exposure (Figure 2C). Isoproterenol induced a 3-fold increase in Ca^{2+} transient amplitude (Figure 2D), while Ca^{2+} transient decay was twice as fast as in control conditions in both WT and CaMKIIδ-KO cardiomyocytes (Figure 2E). The SR Ca^{2+} content, as measured by peak Ca^{2+} release after caffeine application (Figure S2), was not significantly increased by isoproterenol for either mouse genotype (WT: $P=8.1\times10^{-2}$; KO: $P=1.5\times10^{-1}$) and did not differ between mouse genotypes (CON: $P=3.8\times10^{-1}$; isoproterenol: $P=5.5\times10^{-5}$); $P=$. isoproterenol increased Ca^{2+} spark frequency (WT: $P=1.7\times10^{-5}$; CaMKIIδ-KO: $P=1.7\times10^{-2}$) and amplitude (WT control 0.66 ± 0.49 ΔF/F_0 versus isoproterenol 1.21 ± 0.61 ΔF/F_0 $P=2.0\times10^{-3}$; CaMKIIδ-KO control 0.58 ± 0.15 ΔF/F_0 versus isoproterenol 0.83 ± 0.29 ΔF/F_0: $P=2.6\times10^{-2}$) in both WT and CaMKIIδ-KO cardiomyocytes; however, the increase in spark frequency was significantly attenuated in the CaMKIIδ-KO cardiomyocytes ($P=1.1\times10^{-5}$; Figure 2F and 2G), and there was a trend toward a smaller Ca^{2+} spark amplitude ($P=7.2\times10^{-2}$). Thus, while CaMKIIδ deletion did not prevent the effect of isoproterenol on overall Ca^{2+} transient properties, it limited spontaneous Ca^{2+} release during β-AR stimulation.

The cysteine-273 Site on CaMKIIδ Attenuates SR Ca²⁺ Release in Response to NO and Isoproterenol

We previously showed that S-nitrosylation of the cysteine-273 site of CaMKIIδ in vitro can prevent activation of the kinase in response to increased Ca^{2+}/CaM.[19] We hypothesized that this mechanism might protect

myocytes from the development of arrhythmogenic events by suppressing CaMKIIδ activity during transient periods of NO stress. To test this hypothesis, we generated knock-in mice that lack the inhibitory S-nitrosylation site (CaMKIIδ-C273S; Figure 3A through 3C). The CaMKIIδ-C273S mice had normal CaMKIIδ, RyR2 and SERCA2A expression in the ventricle (Figure 3D through 3H; WT versus C273S CaMKIIδ: $P=5.5\times10^{-1}$, RyR2: $P=5.5\times10^{-1}$, SERCA2A: 9.5×10^{-2}). There was no evidence of ventricular fibrosis (Figure 3G and 3I; $P=6.9\times10^{-1}$) or cardiomyocyte hypertrophy (Figure 3J through 3L; cell length: $P=5.9\times10^{-1}$, cell width: $P=6.9\times10^{-1}$) in the CaMKIIδ-C273S mouse hearts compared with WT. Isolated ventricular myocytes from WT and CaMKIIδ-C273S mice were treated with 150 µM GSNO for 7 minutes immediately before wash-in of 100 nmol/L isoproterenol without GSNO. Preincubation of WT cardiomyocytes with GSNO had no effect on baseline Ca^{2+} transient amplitude (Figure 4A; WT: $P=4.3\times10^{-1}$); however, there was a slight increase in Ca^{2+} transient amplitude in CaMKIIδ-C273S cardiomyocytes ($P=5.6\times10^{-3}$). Time constant of $[Ca^{2+}]_i$ decay was not affected by GNSO to cardiomyocytes in normal control Krebs-Ringer HEPES buffer (Figure 4B; WT: $P=9.2\times10^{-1}$; CaMKIIδ-C273S: $P=2.5\times10^{-3}$, not significant with a false discovery rate using a 2-stage step-up Benjamini, Krieger, and Yekutieli) relative. Moreover, WT cardiomyocytes pretreated with GSNO showed a typical Ca^{2+} transient response to isoproterenol, with Ca^{2+} transient amplitude ($P=5.6\times10^{-1}$) and decay ($P=9.2\times10^{-1}$) similar in magnitude to control WT cardiomyocytes not subject to GSNO pretreatment (Figure 4A and 4B). Notably, cardiomyocytes from CaMKIIδ-C273S mice pretreated with GSNO had larger isoproterenol-induced Ca^{2+} transients compared with CaMKIIδ-C273S cardiomyocytes not pretreated with GSNO (Figure 4A, bar 6 versus bar 8; $P=1.8\times10^{-4}$). These data indicate that genetic ablation of the inhibitory C273 S-nitrosylation site increases the effects of CaMKIIδ on SR Ca^{2+} release in the presence of NO and is consistent with our hypothesis that S-nitrosylation of the C273 site is protective in limiting CaMKIIδ activation and its effects on RyR2-related Ca^{2+} transient properties.

Representative line-scans to detect spontaneous Ca^{2+} leak with GSNO pretreatment followed by isoproterenol are shown for WT (Figure 4C) and CaMKIIδ-C273S (Figure 4D) cardiomyocytes. Pretreatment with GSNO did not alter baseline Ca^{2+} spark frequency for either WT ($P=5.4\times10^{-1}$) or CaMKIIδ-C273S ($P=3.1\times10^{-1}$) cardiomyocytes (Figure 4E). Isoproterenol increased the frequency of Ca^{2+} sparks in both WT and CaMKIIδ-C273S cardiomyocytes. However, this isoproterenol-induced increase was attenuated in WT cardiomyocytes pretreated with GSNO (Figure 4E, bar 3 to 4: 2.6 ± 2.0-fold versus 1 to 2: 4.6 ± 4.3-fold; $P=6.0\times10^{-2}$). In the C273S myocytes pretreatment did not alter the isoproterenol-induced
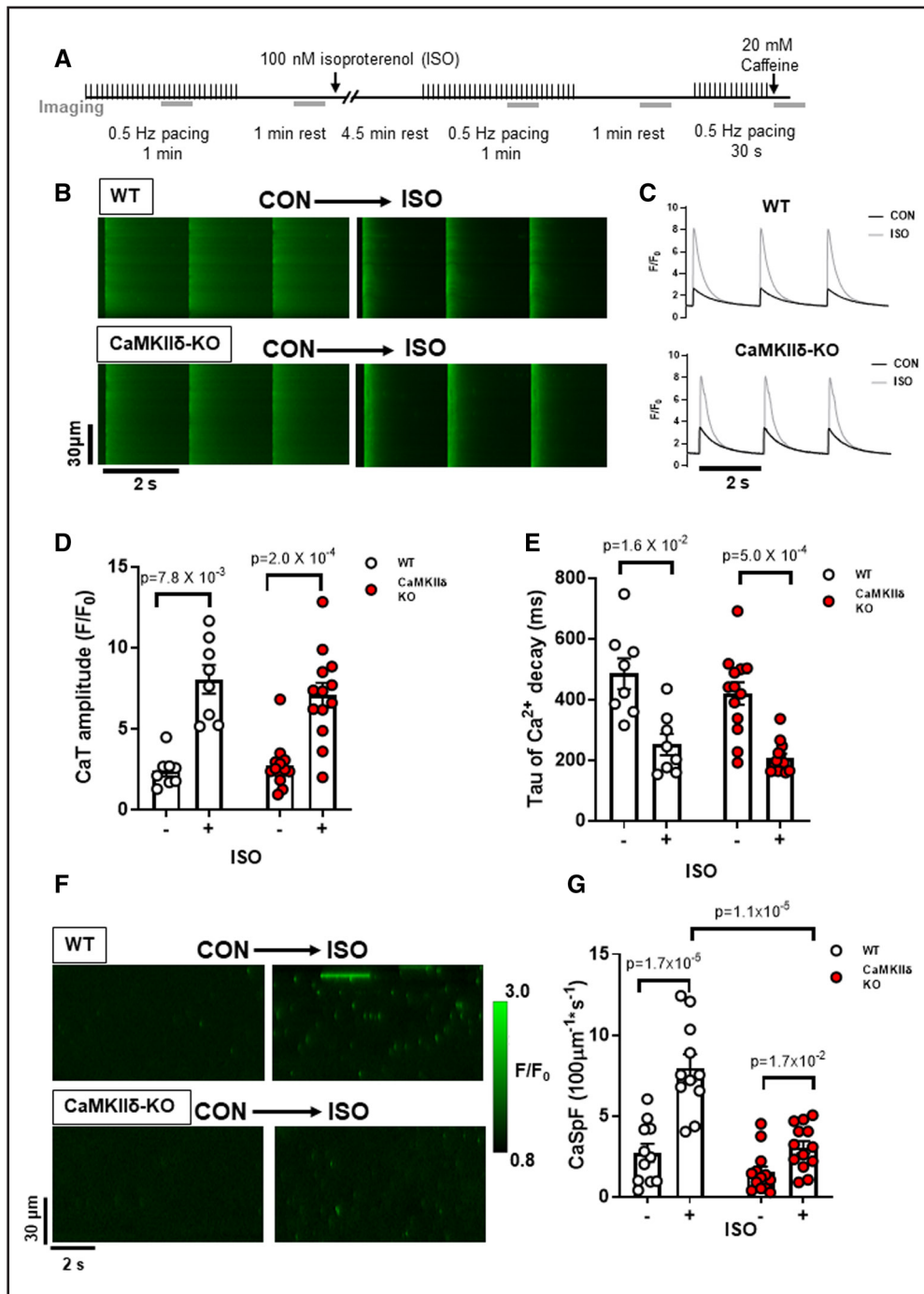
Figure 2. Cardiomyocyte Ca²⁺ transient and spark properties in response to β-adrenergic agonist isoproterenol.
A, Experimental protocol used for Ca²⁺ imaging, the gray bars represent when line-scan images were acquired. Representative line-scans during pacing (**B**) and resultant Ca²⁺ transients (**C**) from a WT and CaMKIIδ (Ca²⁺/calmodulin kinase II delta)-KO cardiomyocyte stimulated at 0.5 Hz under control conditions and with 100 nmol/L ISO. ISO increased Ca²⁺ transient amplitude (**D**) and accelerated decay (**E**) in both WT and CaMKIIδ-KO cardiomyocytes to a similar degree (WT n=8 cells, N=2 hearts; CaMKIIδ-KO n=13 cells, N=4 hearts). **F**, Representative line-scans from a WT and CaMKIIδ-KO cardiomyocyte show an increase in the number of Ca²⁺ sparks following exposure to ISO. Mean data show the ISO-induced increase in Ca²⁺ spark frequency (CaSpF) was attenuated in the CaMKIIδ-KO cardiomyocytes (**G**; WT: n=11 cells, N=2 hearts; CaMKIIδ-KO: n=13 cells, N=4 heats).

increase in Ca²⁺ sparks (Figure 4E, bar 7 to 8: 3.4±4.8-fold versus 5 to 6: 2.9-fold; P=6.6×10⁻¹), consistent with our hypothesis that the inhibitory effect of *S*-nitrosylation at cysteine-273 was expected to be lost.

To further interrogate potential arrhythmogenic signaling, we quantified larger propagating proarrhythmic Ca²⁺ release events known as Ca²⁺ waves. Isoproterenol induced an increase in Ca²⁺ waves in WT cardiomyocytes,
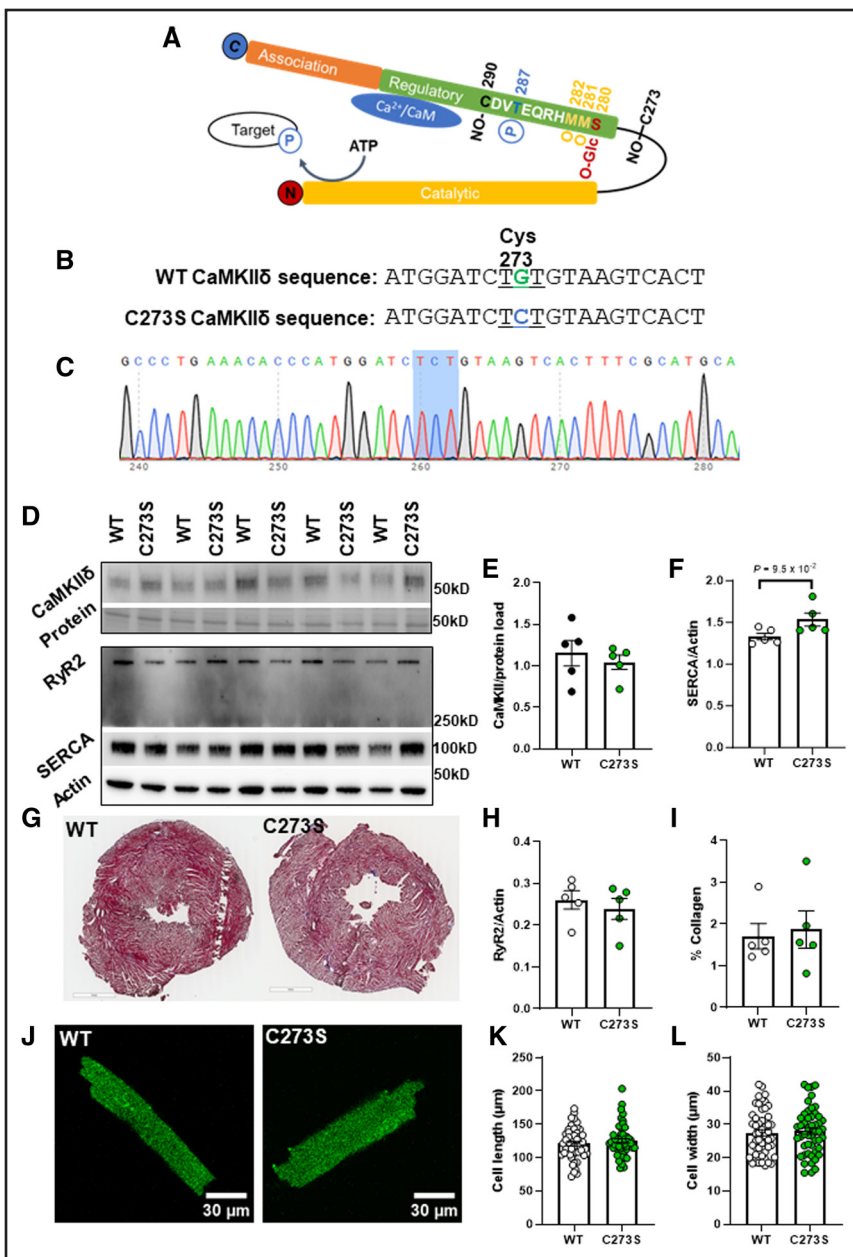
Figure 3. Characterization of the CaMKIIδ (Ca²⁺/calmodulin kinase II delta)-C273S knock-in mouse model.
Schematic of the CaMKIIδ monomer with the residue positions of various posttranslational modifications within the regulatory domain including S-nitrosylation (NO-), phosphorylation (P), oxidation (O), and O-GlcNAc modification, which are known to alter CaMKII activity (**A**). A mouse model was generated using CRISPR/cas9 causing a single point mutation in the cystine codon at position 273, resulting in replacement with a serine residue which cannot be S-nitrosylated (**B**). Example of a CaMKIIδ-C273S offspring confirmed with genotyping of ear notch (**C**). Protein expression in ventricular tissue (N=5 hearts) was measured using Western blots (**D**) for CaMKIIδ (**E**), SERCA (SR Ca²⁺ ATPase; **F**), RyR (ryanodine receptor type; **H**), and normalized to actin or protein load. Ventricular fibrosis was measured in fixed and stained tissue (**G**) by quantifying collagen content (**I**). Representative cardiomyocytes are shown in **J** for measurement of cell length (**K**) and width (**L**), wild type (WT): n=52, N=7 hearts; CaMKIIδ-C273S: n=50, N=8 hearts.

but in WT, this effect was suppressed by GSNO pretreatment (Figure 4F, left; $P=2.5\times10^{-1}$). In contrast, CaMKIIδ-C273S cardiomyocytes pretreated with GSNO robustly increased isoproterenol-induced Ca²⁺ waves (Figure 4F, right), suggesting that the inhibitory cysteine-273 S-nitrosylation site is important for preventing Ca²⁺ waves that can trigger action potentials and arrhythmias. We repeated the experiments with sodium nitroprusside (SNP), a NO donor used in clinical settings, which demonstrated a similar attenuation of Ca²⁺ sparks in the WT cardiomyocytes (as for GSNO pretreatment) in the presence of sGC (soluble guanylyl cyclase) inhibitor ODQ. This SNP-mediated reduction in Ca²⁺ sparks was lost in the CaMKIIδ-C273S cardiomyocytes, without altering the Ca²⁺ transient response to isoproterenol (Figure 5A through 5D). We infer that the SNP

(and GSNO)–mediated protection against isoproterenol-induced arrhythmogenic SR Ca release events occurs primarily via S-nitrosylation of CaMKIIδ-C273 rather than through an sGC-dependent pathway.

NO Treatment After β-AR Activation Does Not Alter Ca²⁺ Transients or Sparks

Previous literature has demonstrated a role for autonomous activation of CaMKIIδ by S-nitrosylation at cysteine-290[19] and enhanced Ca²⁺ release events mediated by CaMKII.[13] Due to the position of the cysteine-290 in the CaMKIIδ regulatory domain adjacent to the Thr-287 autophosphorylation site, the S-nitrosylation site is unlikely to be available under basal conditions when the kinase is autoinhibited by regulatory domain binding to
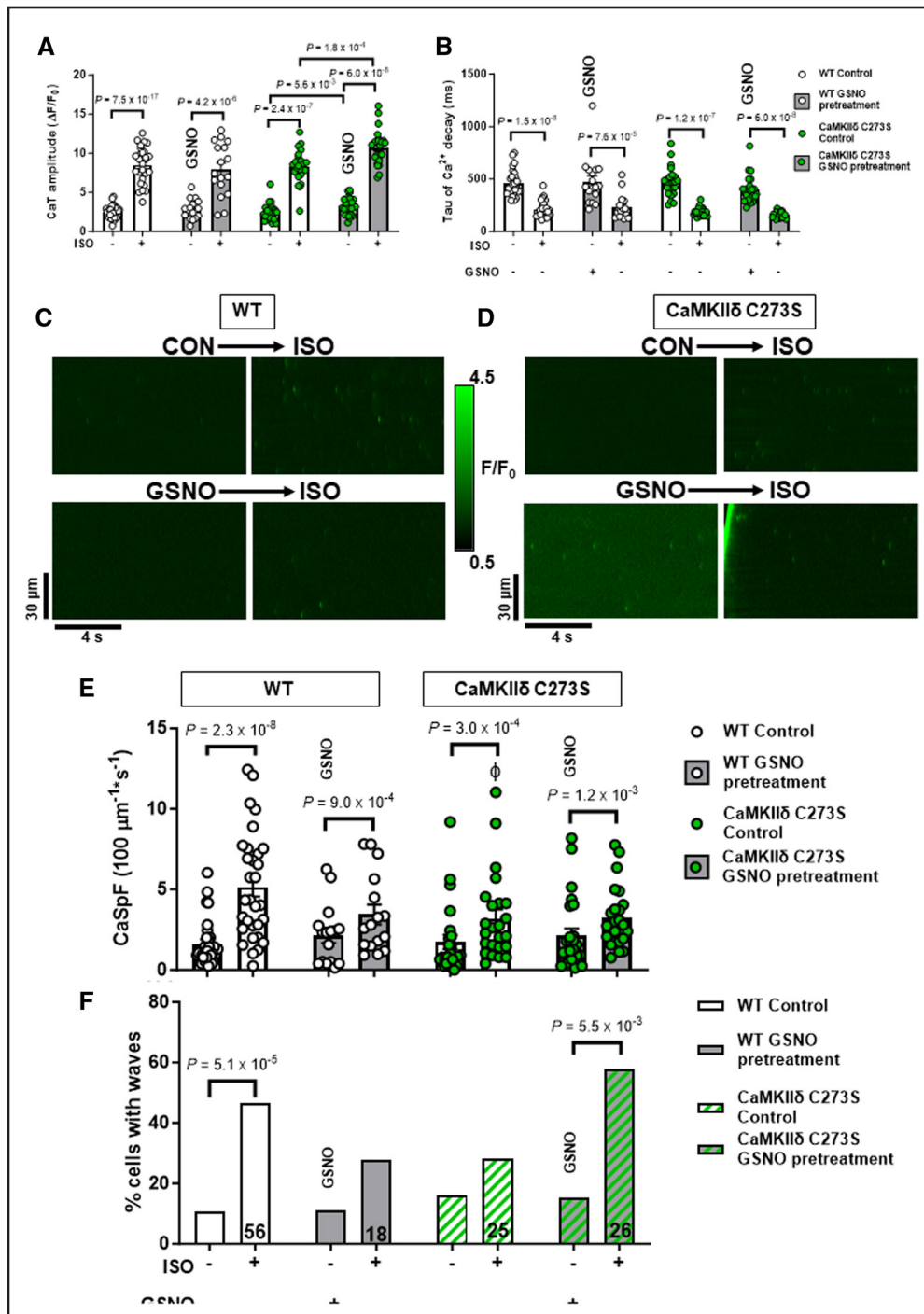
Figure 4. Pretreatment with *S*-nitrosoglutathione (GSNO) enhances Ca²⁺ transient response to isoproterenol in CaMKIIδ (Ca²⁺/calmodulin kinase II delta)-C273S cardiomyocytes.

Ca²⁺ transients from wild-type (WT) and CaMKIIδ C273S cardiomyocytes from were recorded at 0.5 Hz under control conditions and with 100 nmol/L ISO or with pretreatment of 150 µM GSNO before ISO exposure. Mean Ca²⁺ transient amplitude (**A**) and decay (**B**) data in response to ISO with control KRH buffer (white bars; WT: n=28 cells, N=14 hearts; CaMKIIδ-C273S: n=24 cells, N=14 hearts) or when pretreated with GSNO (gray bars; WT: n=18 cells, N=7 hearts; CaMKIIδ-C273S: n=24, N=9 hearts). Representative line-scans from quiescent WT (**C**) and CaMKIIδ-C273S (**D**) cardiomyocytes under control conditions and with ISO or with pretreatment of GSNO before ISO exposure. Mean Ca²⁺ spark frequency (**E**) data are shown for cardiomyocytes in response to ISO with control buffer (white bars; WT: n=31 cells, N=14 hearts; CaMKIIδ-C273S: n=24 cells, N=9 hearts) or when pretreated with GSNO (gray bars; WT: n=16 cells, N=7 hearts; CaMKIIδ-C273S: n=25 cells, N=9 hearts). Percentage of cardiomyocytes exhibiting Ca²⁺ waves in quiescent WT and CaMKIIδ-C273S cardiomyocytes (**F**). φ*P*<0.05 compared with WT ISO, ᵃ*P*=0.0002 vs CaMKIIδ-C273S ISO, ᵇ*P*<0.0001 vs WT ISO with GSNO pretreatment.
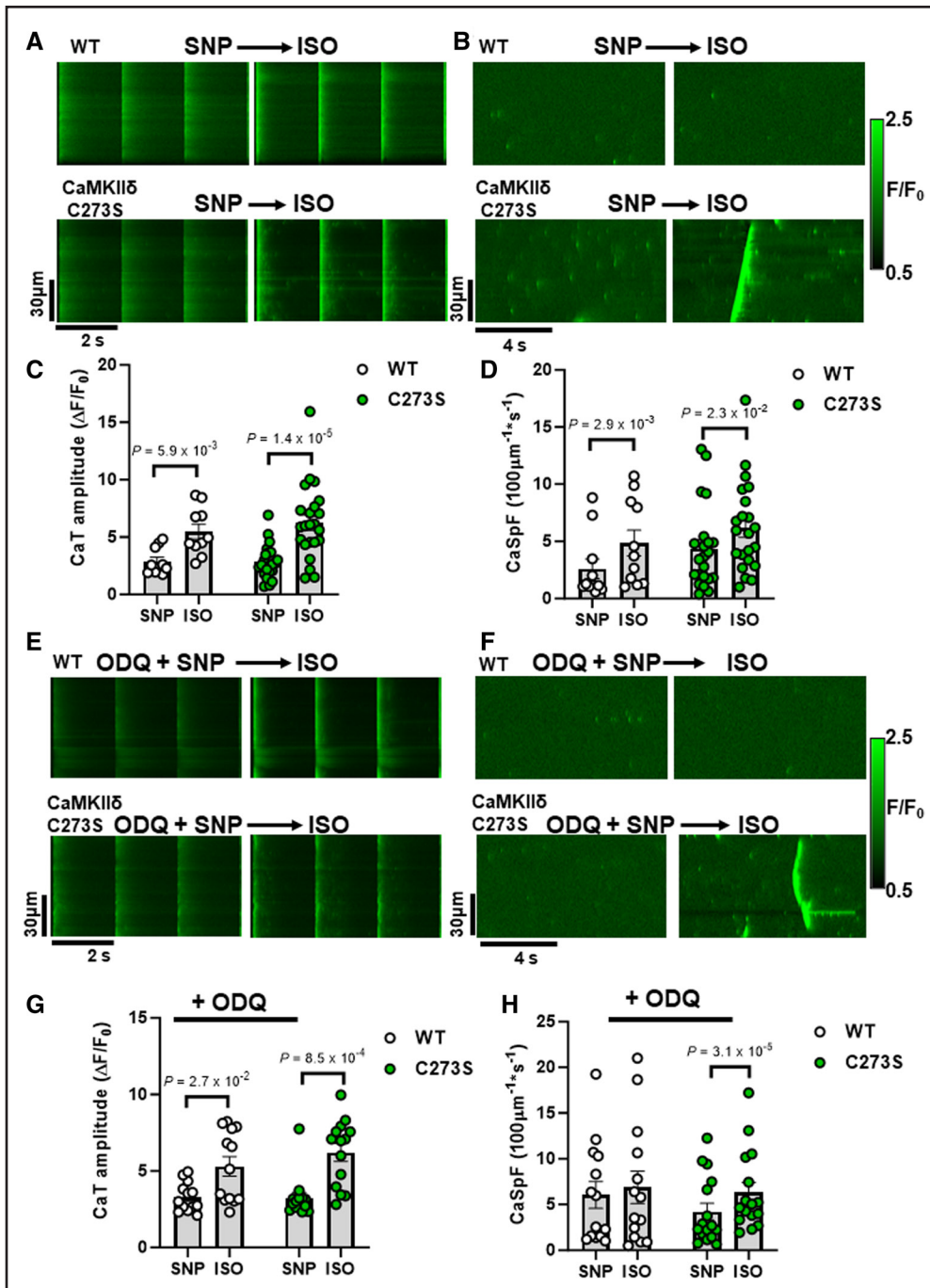
Figure 5. Pretreatment with clinical NO donor sodium nitroprusside (SNP) and sGC (soluble guanylyl cyclase) inhibitor elicits similar protection against isoproterenol-induced Ca²⁺ sparks.
Ca²⁺ transients (**A**) and sparks (**B**) from wild-type (WT) and CaMKIIδ (Ca²⁺/calmodulin kinase II delta)-C273S cardiomyocyte were recorded at the end of a 7-minute incubation with SNP (200 μM) followed by wash-in of 100 nmol/L ISO. Mean Ca²⁺ transient amplitude and spark frequency data are shown in **C** and **D**, respectively (WT: n=10 cells, N=3 hearts; CaMKIIδ-C273S: n=23 cells, N=6 hearts). The same experiments were performed following a pretreatment with sGC inhibitor ODQ (10 μM; 20 minutes incubation) with representative line-scans (**E** and **F**) and mean data (**G** and **H**) plotted for WT (n=13 cells, N=3 hearts) and CaMKIIδ-C273S (n=14 cells, N=4 hearts) cardiomyocytes. ISO indicates isoproterenol.

the catalytic domain.[19] Therefore, we tested the effect of GSNO following isoproterenol treatment when the CaMKIIδ activation state is increased in cardiomyocytes.[23] We also examined the effects of isoproterenol and GSNO on ventricular myocytes isolated from

CaMKIIδ-C290A knock-in mice, in addition to WT and CaMKIIδ-C273S animals.

Cardiomyocytes underwent wash-in of 100 nmol/L isoproterenol for 5 minutes followed by washout with control buffer, or buffer containing 150 μM GSNO. Ca²⁺

transients were measured at baseline, during isoproterenol wash-in, and following washout with GSNO buffer (Figure 6A). Isoproterenol increased Ca²⁺ transient

amplitude and accelerated decay in WT and CaMKIIδ-C273S cardiomyocytes as expected, and these effects were sustained during washout in the presence of GSNO
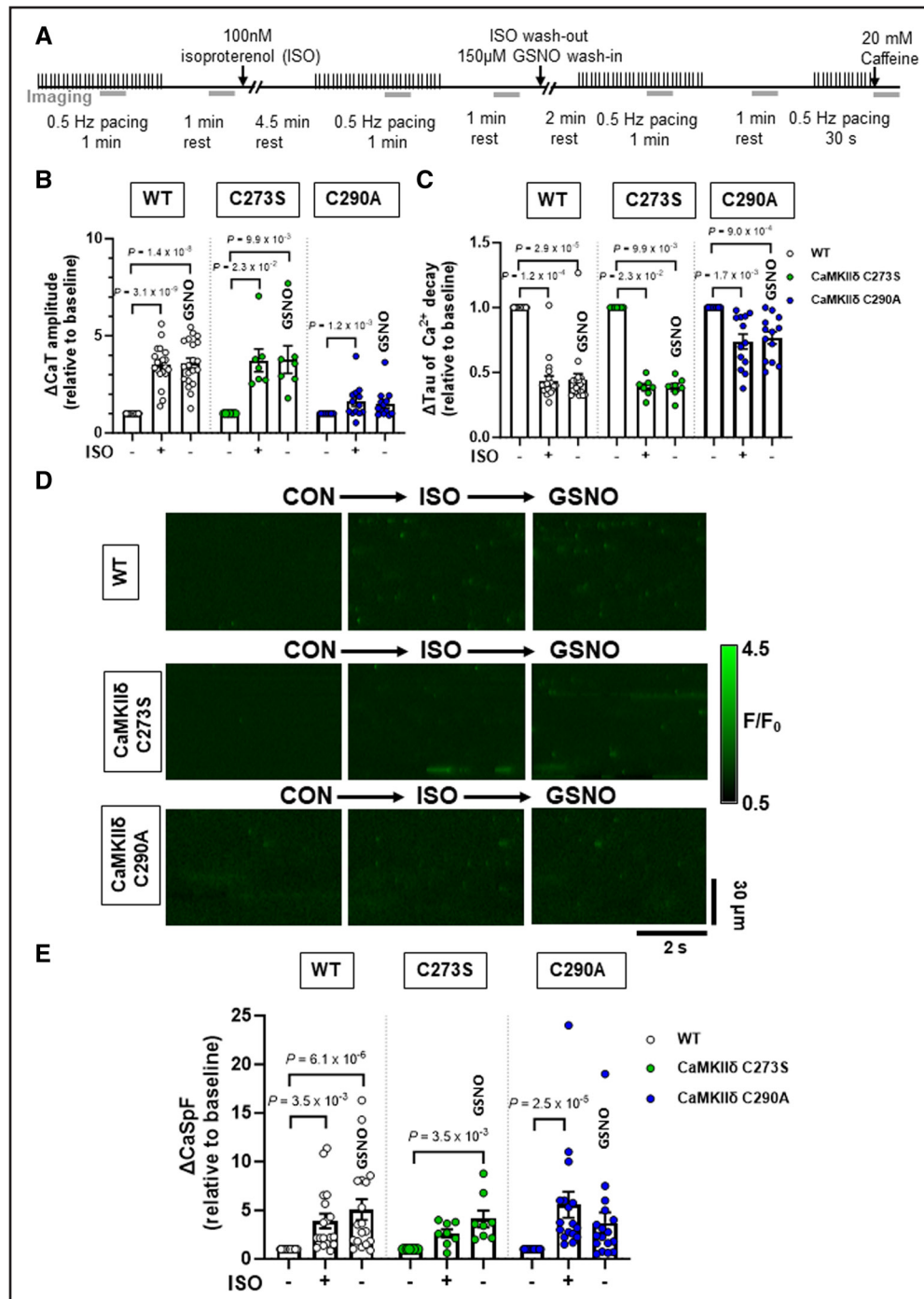

Figure 6. Effects of *S*-nitrosoglutathione (GSNO) after ISO on Ca²⁺ transients and sparks in wild-type (WT) or CaMKIIδ (Ca²⁺/calmodulin kinase II delta)-C273S and -C290A cardiomyocytes.
WT, CaMKIIδ-C273S, and -C290A myocytes were stimulated at 0.5 Hz and exposed to 100 nmol/L ISO, then following ISO exposure myocytes were exposed to 150-μM GSNO. Experimental protocol used for Ca²⁺ imaging, the gray bars represent when line-scan images were acquired (**A**). Mean Ca²⁺ transient amplitude (**B**) and decay (**C**) data are shown for cardiomyocytes in response to ISO and washout with GSNO (WT: n=20 cells, N=9 hearts; CaMKIIδ C273S: n=7 cells, N=5 hearts; CaMKIIδ-C290A: n=13 cells, N=5 hearts). Representative line-scans from quiescent WT, CaMKIIδ-C273S and -C290A cardiomyocytes under indicated conditions (**D**). Mean Ca²⁺ spark frequency data (**E**) are shown for cardiomyocytes in response to ISO with washout with GSNO (WT: n=18 cells, N=9 hearts; CaMKIIδ C273S: n=8 cells, N=5 hearts; CaMKIIδ-C290A: n=17 cells, N=5 hearts). All data are normalized to baseline control before addition of ISO. ISO indicates isoproterenol.

(Figure 6B and 6C, white and green symbols). Similar Ca^{2+} transient results were observed in CaMKIIδ-C290A cardiomyocytes; however, the Ca^{2+} transient amplitude increase was not sustained with GSNO (Figure 6C, blue symbols, $P=2.3\times10^{-1}$).

When spontaneous Ca^{2+} release was measured in quiescent cardiomyocytes (Figure 6D), isoproterenol resulted in an increase in the Ca^{2+} spark frequency (Figure 6E) for both WT and C290A genotypes. Surprisingly, this was not observed for C273S myocytes ($P=1.4\times10^{-3}$) until the addition of GSNO. Addition of GSNO during the isoproterenol washout prevented Ca^{2+} spark frequency from returning to baseline in both the WT and CaMKIIδ-C273S myocytes, while increased Ca^{2+} spark frequency was not maintained in myocytes from the CaMKIIδ-C290A mice during the isoproterenol washout, despite the presence of GSNO. These data are consistent with the hypothesis that isoproterenol-induced CaMKIIδ activation regulates Ca^{2+} handling in myocytes, and subsequent exposure to GSNO causes *S*-nitrosylation at the cysteine-290 site, thereby prolonging the enhancement of CaMKIIδ activity and Ca^{2+} sparks.

Knock-In CaMKIIδ-C273S Mice Have Impaired Cardiac Function and Altered ECG Characteristics at 12 Weeks of Age

The myocyte Ca^{2+} spark and wave data (Figure 4) suggest that the CaMKIIδ-C273 *S*-nitrosylation site might offer basal protection from stress associated with sympathetic activation of β-adrenergic receptors (ie, that is lost in CaMKIIδ-C273S mice). To test whether there is chronic cardiac adaptation in terms of cardiac function, we measured these parameters in anesthetized WT and CaMKIIδ-C273S mice at 12 weeks of age (Table S3). The echocardiography data indicated unaltered body weight, heart rate, and septal thickness between the groups. However, both end diastolic and systolic volumes of the LV were significantly increased in the CaMKIIδ-C273S mice, with reduced fractional shortening and ejection fraction. The ratio of early to late ventricular filling velocities was also significantly impaired in the CaMKIIδ-C273S hearts. Taken together, these data show that the lack of 1 *S*-nitrosylation site in the CaMKIIδ-C273S mice results in a modest reduction in both systolic and diastolic function, even in the absence of a significant cardiac challenge.

We also measured conduction characteristics through the hearts of anesthetized WT (Figure 7A) and CaMKIIδ-C273S (Figure 7B) using ECG. Even at 12 weeks of age, we observed significant differences in a number of ECG parameters (Figure 7C through 7K) for the CaMKIIδ-C273S mice, including prolongation of P wave duration and P-R interval, as well as increased Q, R, and S wave amplitudes. There was also a trend toward

increased T wave amplitude in the CaMKIIδ-C273S mice ($P=5.8\times10^{-2}$). In addition to these baseline alterations to conduction, we observed spontaneous arrhythmic events in the ECG traces of the CaMKIIδ-C273S mice. Figure 7L shows an example of periodic spontaneous variability in heart rate, which was observed in several of the CaMKIIδ-C273S mice but none of the WT mice (Figure 7M), leading to occasional periods of bradycardia (Figure 7N). These arrhythmic events were excluded from our analysis of ECG parameters (Figure 7C through 7K), as this would have greatly exaggerated the differences in these values. Nonetheless, our observations that conduction is altered and spontaneous arrhythmic events are occurring in the CaMKIIδ-C273S mice suggest that the cysteine-273 site on CaMKIIδ is playing a protective role in the heart.

NO Mediates Arrhythmogenic Response to β-AR Stress in Langendorff-Perfused Mouse Hearts

Our observation that GSNO pretreatment could suppress isoproterenol-induced Ca^{2+} sparks in isolated cardiomyocytes, motivated us to test the arrhythmogenic consequences at the whole heart level using Langendorff-perfused mouse hearts (Figure S3A). To ensure that the GSNO treatment was sufficient to induce *S*-nitrosylation during Langendorff-perfusion, we snap-froze hearts after perfusion and used a modified biotin switch assay to measure total *S*-nitrosylation (Figure S3B). Hearts that received GSNO after isoproterenol had a significant increase in total *S*-nitrosylation compared with control (Figure S3C, red versus white bar; $P=9.1\times10^{-3}$), and isoproterenol only (Figure S3C, red versus gray bar; $P=1.0\times10^{-2}$). This effect was partially reversed when GSNO was followed by 10-minute isoproterenol perfusion (Figure S3C, white versus blue bar; $P=1.2\times10^{-1}$).

We observed different types of arrhythmias in the isolated hearts treated with isoproterenol and GSNO (Figure 8A). Treatment with 100 nmol/L isoproterenol induced a significant increase in total arrhythmic events (Figure 8B) and arrhythmia score (Figure 8C) in the WT hearts. After isoproterenol washout, arrhythmic events remained significantly higher in the WT but not in the CaMKIIδ-C273S hearts. The arrhythmia score also remained elevated above baseline in WT hearts even after isoproterenol washout, indicating that some isoproterenol-induced sensitization of the hearts persisted 10 minutes after washout. We then repeated the experiment with GSNO added to the perfusate during the isoproterenol washout (analogous to Figure 6) to test whether GSNO would stabilize the arrhythmic phenotype after the isoproterenol stress. GSNO treatment did not seem to prolong the increase in arrhythmias during the isoproterenol washout phase
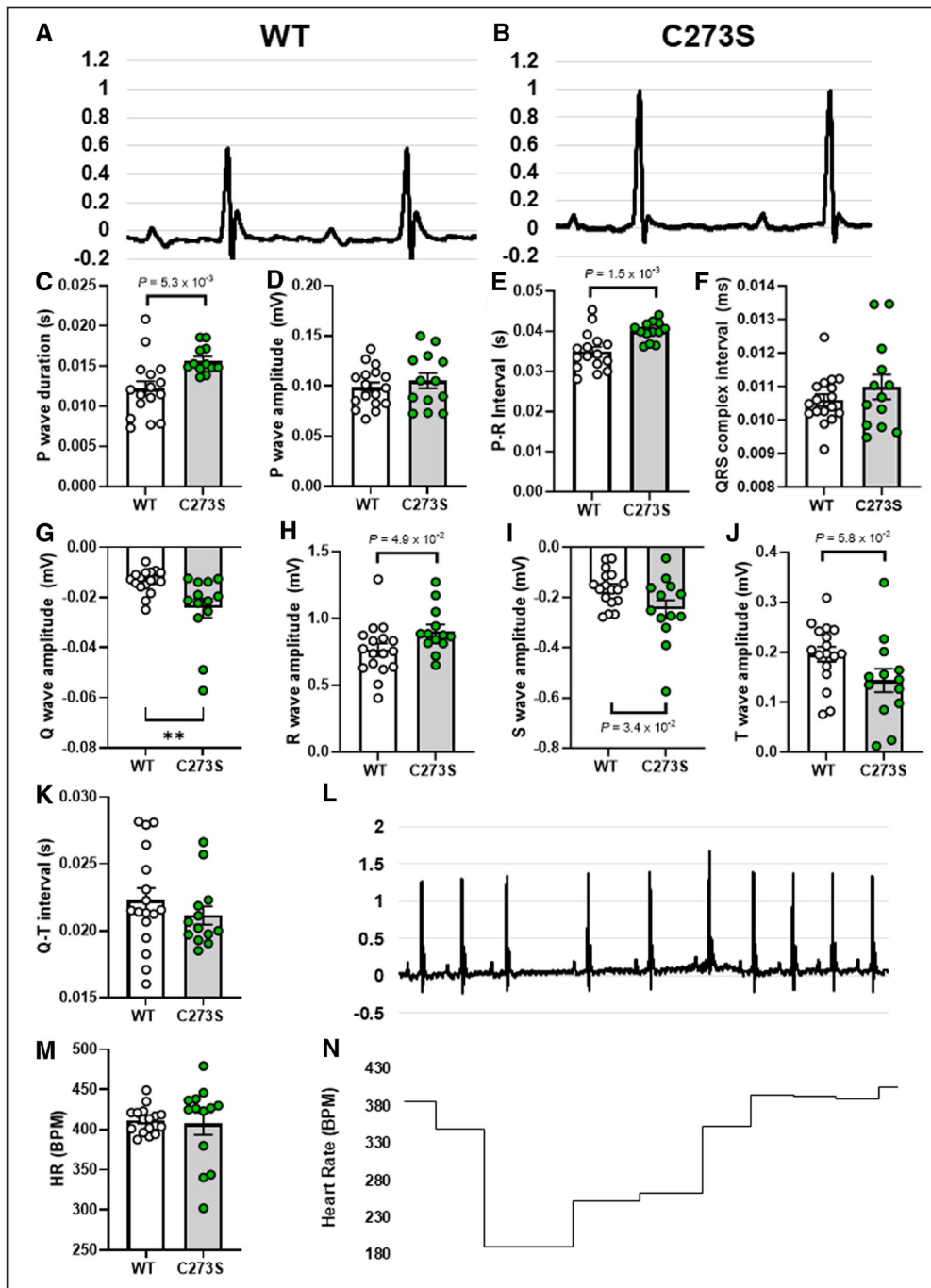
Figure 7. ECG characteristics for wild-type (WT) and CaMKIIδ (Ca²⁺/calmodulin kinase II delta)-C273S mice.
Representative electrocardiograms from a WT (**A**) and CaMKIIδ-C273S (**B**) mouse under anesthetic and mean data for waveform amplitudes and durations (**C** through **K**). N=13 to 17 per group. ECG traces for some of the CaMKIIδ-C273S mice showed spontaneous arrhythmic events (**L**). These events led to variability in total heart rate (HR) (**M**) and acute periods of bradycardia (**N**).

for both WT (P=5.8×10⁻²) and CaMKIIδ-C273S hearts (P=3.9×10⁻¹; Figure 8D). However, arrhythmia score remained significantly increased above baseline after isoproterenol treatment in the CaMKIIδ-C273S hearts (Figure 8E). These data are consistent with our hypothesis that isoproterenol-mediated stress activates CaMKIIδ to induce cardiac arrhythmia and that S-nitrosylation of activated CaMKIIδ (at cysteine-290)

can prolong this activation state, independent of cysteine-273 availability.

Our myocyte data showed that GSNO treatment before isoproterenol exposure could limit arrhythmogenic Ca²⁺ wave activity in myocytes from WT but not CaMKIIδ-C273S mice (Figure 4F). To test this at the whole heart level, we exposed the hearts to GSNO first (10 minutes) before administration of isoproterenol. Strikingly,
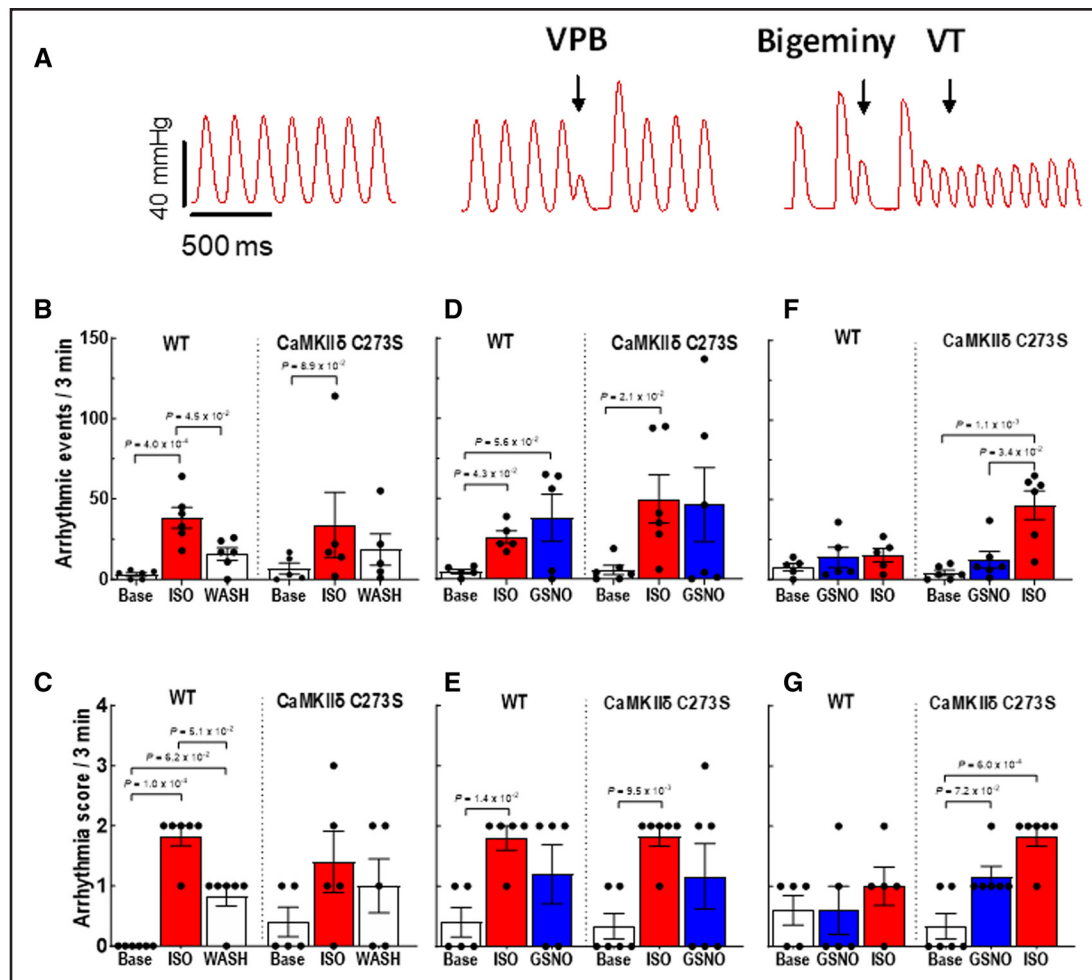
Figure 8. Pretreatment with *S*-nitrosoglutathione (GSNO) prevents ISO-induced arrhythmias in Langendorff-perfused hearts from wild-type (WT), but not CaMKIIδ (Ca²⁺/calmodulin kinase II delta)-C273S, mice.
Arrhythmias were measured in Langendorff-perfused hearts following protocols outlined in Figure 7A. **A**, Examples of arrhythmic events observed in the isolated hearts. We measured (**B**) quantity of arrhythmic events and (**C**) arrhythmia score during ISO perfusion only, (**D**) quantity of arrhythmic events and (**E**) arrhythmia score during ISO pretreatment before GSNO, and (**F**) quantity of arrhythmic events and (G) arrhythmia score during GSNO pretreatment before ISO. White bar=buffer, red bar=ISO, blue bar=GSNO. n=5 to 6 hearts per group. ISO indicates isoproterenol.

WT mice were protected from isoproterenol-induced arrhythmic events (or increases in arrhythmia score; Figure 8F and 8G), but this protection was not observed in the CaMKIIδ-C273S knock-in mice, which lack the *S*-nitrosylation site that limits CaMKIIδ activation (Figure 8F). In the CaMKIIδ-C273S mice, GSNO alone was sufficient to enhance the number of arrhythmic events (Figure 8G), a response that could be mediated through cysteine-290, which promotes autonomous CaMKIIδ activation and would be unopposed in the absence of the inhibitory cysteine-273 site. These data demonstrate that the cysteine-273 site on CaMKIIδ is critical to the protective effect of GSNO pretreatment with respect to isoproterenol-induced arrhythmic response in the heart.

DISCUSSION

One main finding of this study was that NO donors promote CaMKIIδ-dependent spontaneous SR Ca²⁺ release following β-AR stimulation in cardiomyocytes and ventricular arrhythmias in the intact heart. Conversely, pretreatment of cardiomyocytes or hearts with NO donors before isoproterenol exposure limited the β-AR-induced increase in spontaneous Ca²⁺ release events and ventricular arrhythmias. Furthermore, this protective effect of NO pretreatment was mediated by the cysteine-273 site on CaMKIIδ since it was attenuated when this site was mutated and not available for *S*-nitrosylation. Exposure to GSNO did not alter baseline Ca²⁺ handling or heart function. Notably, pretreatment with GSNO conferred protection against β-AR stimulation of pathological Ca²⁺ leak and arrhythmias without compromising β-AR induced gain of function (enhanced Ca²⁺ transient amplitude and decay rate). These data provide a new understanding of how NO treatment influences β-AR signaling and cardiac function with both protective and proarrhythmic consequences depending on the timing of NO exposure.

While the β-AR signaling system is a vital arm of the fight-or-flight response responsible for increasing cardiac output on demand, activation of the pathway can lead to aberrant Ca^{2+} release[4,11,12,17] and trigger arrhythmias.[24] Activation of NOS (nitric oxide synthase) has been identified as a downstream signaling mechanism of β-AR stimulation, as endogenous NO levels increase following cardiomyocyte isoproterenol exposure[13,17] and inhibition of NOS prevents an increase in Ca^{2+} leak with isoproterenol.[11,13,17] It has been proposed that NOS is activated following β-AR stimulation by a pathway independent of protein kinase A, apparently involving Epac and Akt (protein kinase B).[11] Here, we found that isoproterenol increased both Ca^{2+} spark frequency and amplitude in isolated cardiomyocytes, exposure of cardiomyocytes to GSNO alone under baseline conditions had no effect on triggered or untriggered Ca^{2+} events (Figures 2 and 4). This result was in contrast to the findings that GSNO (or SNP) increased Ca^{2+} sparks and arrhythmogenic waves in cardiomyocytes and was mediated by CaMKII nitrosylation and activation (rather than direct *S*-nitrosylation of RyR2).[11,13] As we have shown here, the directional effect of GSNO on CaMKII-dependent signaling is highly dependent on the basal state of CaMKII activation and whether Cys273 or Cys290 is the prime mediator, and that can depend on time, pacing rate, oxidative stress, Na$^+$, and Ca^{2+} levels. That, and species differences[25] could readily explain baseline differences in basal GSNO effects. Indeed, cardiac CaMKIIδ activity modulates the function of many myocyte targets,[18,26,27] including ion channels associated with arrhythmias and excitation-contraction coupling such as RyR2,[28] SERCA (via phospholamban[29]) and LTCC.[30]

Under healthy resting conditions, CaMKIIδ is largely but not completely autoinhibited and becomes progressively activated when intracellular Ca^{2+} levels rise and Ca^{2+}/CaM is bound to the regulatory domain.[18] Several posttranslational modifications[31–33] of CaMKIIδ, including *S*-nitrosylation,[19] enable an autonomously active form of the kinase that is associated with pathological Ca^{2+} mishandling and can be driven by high oxidative stress[32] or hyperglycemia.[33] The degree of CaMKIIδ *S*-nitrosylation is increased in cardiomyocytes treated with either GSNO[13] or isoproterenol[17] as determined by immunoprecipitation of CaMKIIδ and probing with anti-*S*-nitrosylation antibodies. However, this method is not able to discriminate between the 2 cysteine residues within the regulatory domain of CaMKIIδ that have opposite effects on CaMKIIδ activity.[19] *S*-nitrosylation at cysteine-290 causes autonomous activation of the kinase, consistent with the observation that NO exposure can enhance CaMKIIδ activity and increase Ca^{2+} sparks.[11,13] Conversely, *S*-nitrosylation at cysteine-273 inhibits CaMKIIδ by preventing Ca^{2+}/CaM binding,[19] suggesting a dual role for NO in mediating CaMKIIδ activity.

When cardiomyocytes are stimulated at 0.5 Hz, CaMKIIδ activation can be low[23]; therefore, the cysteine-290 residue within the regulatory region of CaMKIIδ may not be accessible for *S*-nitrosylation to induce autonomous activation.[19] Stimulation of cardiomyocytes at a physiological rate (6–8 Hz in mice) would likely increase the sensitivity of the cells to the NO and CaMKIIδ-mediated effects reported here in isolated cardiomyocytes; however, physiological rates are obtained in the isolated whole hearts and exposure to GSNO alone caused an increase in arrhythmia score in CaMKIIδ-C273S hearts (Figure 8G). Under the baseline conditions for isolated cardiomyocytes used in this study, we propose that GSNO pretreatment of cardiomyocytes results in *S*-nitrosylation of CaMKIIδ at cysteine-273, which can prevent Ca^{2+}/CaM dependent CaMKIIδ activation when subsequently stimulated with β-AR agonist isoproterenol. We found that pretreatment with GSNO prevented an isoproterenol-dependent increase in Ca^{2+} spark frequency and amplitude (Figure 4), which is known to be induced by CaMKIIδ-mediated phosphorylation of RyR2.[4] However, we cannot rule out attenuation of Ca^{2+} cycling of other Ca^{2+} handling proteins, such as SERCA[34] or LTCC[35] by direct *S*-nitrosylation.

Interestingly, our novel CaMKIIδ-C273S animals showed a trend toward increased expression of SERCA compared with WT littermates (Figure 3), despite their relatively young age and lack of a directed cardiac challenge. This observation could be consistent with the C273S mutant mice undergoing compensation to increase SR Ca^{2+} uptake, potentially in response to increased baseline CaMKIIδ activation and enhanced calcium leak during diastole. Future studies in older mice or after a significant cardiac challenge (ex. AngII pump implantation) might show a more pronounced shift in SERCA expression. Moreover, we acknowledge that CaMKII is subject to oxidation,[33] and new evidence suggests that cysteine residues on CaMKIIδ may be targets for oxidative stress.[36,37] Moreover, conditions of oxidative stress that would be conducive to CaMKIIδ activation would also alter both the mechanism and rate of thiol nitrosylation.[38] Another possibility for future consideration is that, during chronic cardiac stress, nitrosylation events on CaMKII are replaced with more stable oxidative modifications (ex. disulfide bonds). Therefore, our generation of knock-in mice that either lack the cysteine-273 or cysteine-290 sites, potentially used in conjunction with existing cellular or mouse models that lack the oxidation-sensitive methionine residues on CaMKII, will be valuable tools to further investigate the effect of NO and ROS-mediated CaMKIIδ activity on cardiac function in both normal and pathological settings.[39]

NO donors (eg, nitroglycerin, SNP) used for their vasodilatory actions have been administered clinically for almost a century, initially for angina pectoris and later for myocardial infarction.[7] NO donors are considered cardioprotective due in part to the ability of NO donors to mimic the benefits of ischemic preconditioning.[40] However, we

found GSNO or SNP pretreatment suppressed isoproterenol-induced cardiac arrhythmias. When the order of treatment was reversed in the isolated hearts, we found that NO donors administered after isoproterenol could enhance the number of arrhythmias, suggesting that NO donors should be used with caution when β-AR stress is elevated. Conversely, our data demonstrate that a well-timed acute dose or low-dose chronic treatment with an NO donor (potentially paired with a CaMKII inhibitor) could be a powerful antiarrhythmic strategy in the clinic. Importantly, our data demonstrate that the effects of NO donors on isoproterenol-induced Ca^{2+} sparks persist even in the presence of ODQ, a soluble guanylyl cyclase inhibitor. Guanylyl cyclase is known to play a role in mediating the inotropic effects of NO in myocytes,[41] and our findings suggest that CaMKII nitrosylation works independently of guanylyl cyclase, though the 2 pathways may be complimentary in modulating myocytes function. These findings present new evidence for dual and opposing effects of NO signaling in the whole heart with regard to triggered arrhythmias.

ARTICLE INFORMATION

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Acknowledgments

Author Contributions

Experiments were performed in the laboratory of J.R. Erickson, D.M. Bers, and M.J. Kohr within the Department of Physiology at the University of Otago, the Department of Pharmacology at the University of California, Davis, and the Department of Environmental Health and Engineering at Johns Hopkins Bloomberg School of Public Health, respectively. J.R. Erickson and D.M. Bers are responsible for the conception of the work and interpretation of the results. A.S. Power, E.U. Asamudo, L.P.I. Worthington, C.C. Alim, O.V. Ebenebe, R.E. Parackal, and R.S. Wallace contributed to study design, performed and analyzed experiments, prepared figures, and drafted the article. J. Heller Brown contributed to the interpretation of the work and revised the article. All authors contributed to critical revisions of the article.

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Disclosures

None.

Supplemental Material

Expanded Materials and Methods
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