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NO-dependent CaMKII activation during β-adrenergic stimulation of cardiac muscle

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Aims During β-adrenergic receptor (β-AR) stimulation, phosphorylation of cardiomyocyte ryanodine receptors by protein kinases may contribute to an increased diastolic Ca^{2+} spark frequency. Regardless of prompt activation of protein kinase A during β-AR stimulation, this appears to rely more on activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), by a not yet identified signalling pathway. The goal of the present study was to identify and characterize the mechanisms which lead to CaMKII activation and elevated Ca^{2+} spark frequencies during β-AR stimulation in single cardiomyocytes in diastolic conditions.

Methods and results Confocal imaging revealed that β-AR stimulation increases endogenous NO production in cardiomyocytes, resulting in NO-dependent activation of CaMKII and a subsequent increase in diastolic Ca^{2+} spark frequency. These changes of spark frequency could be mimicked by exposure to the NO donor GSNO and were sensitive to the CaMKII inhibitors KN-93 and AIP. *In vitro*, CaMKII became nitrosated and its activity remained increased independent of Ca^{2+} in the presence of GSNO, as assessed with biochemical assays.

Conclusions β-AR stimulation of cardiomyocytes may activate CaMKII by a novel direct pathway involving NO, without requiring Ca^{2+} transients. This crosstalk between two established signalling pathways may contribute to arrhythmogenic diastolic Ca^{2+} release and Ca^{2+} waves during adrenergic stress, particularly in combination with cardiac diseases. In addition, NO-dependent activation of CaMKII is likely to have repercussions in many cellular signalling systems and cell types.

Keywords CaMKII • Ca sparks • Ca waves • NO-synthase

1. Introduction

In cardiac excitation–contraction coupling Ca^{2+}-induced Ca^{2+} release (CICR) is the mechanism that amplifies the Ca^{2+} signal initiated by entry of Ca^{2+} via voltage-dependent Ca^{2+} channels.[1] During each systole, CICR generates a robust Ca^{2+} transient by releasing Ca^{2+} from the sarcoplasmic reticulum (SR) via Ca^{2+} release channels (a.k.a. ryanodine receptors or RyRs). These are macromolecular complexes located in diadic clefts, microdomains of junctional SR, in close apposition to L-type Ca^{2+} channels.[2] Each group of RyRs and L-type Ca^{2+} channels forms a unit called couplon which can create elementary Ca^{2+} release events, Ca^{2+} sparks.[3] Faithful RyR functioning is crucial in the context of cardiac muscle Ca^{2+} release, its synchronization, and strength of contraction. Disturbances of these elementary mechanisms have been observed during various cardiac pathologies.[4,5]

Upon β-adrenergic receptor (β-AR) stimulation during emotional stress or physical exercise, key proteins of Ca^{2+} signalling, such as the L-type Ca^{2+} channels, the RyRs, and phospholamban are phosphorylated by protein kinases,[6,7] enhancing Ca^{2+} cycling. There is recent evidence indicating that elevated phosphorylation levels of the RyR mediated by protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) may increase their activity[8–11] (for reviews see refs[7,12]). During chronic β-AR stimulation, this could constitute a Ca^{2+} leak and deplete the SR of Ca^{2+}, which would reduce the amplitude of Ca^{2+} transients, eventually contributing to weak heartbeats.[13]

Experimentally, it has proven difficult to clearly assign a role for the different kinases in modulating RyR behaviour. The generation of various transgenic mouse lines, specifically targeting phosphorylation by PKA and CaMKII, has not clarified the situation and provided apparently contradictory results and interpretations. However, analysis of Ca^{2+} spark frequencies in transgenic mice expressing constitutively phosphorylated RyRs (S2808D, S2814D) suggested that PKA- and CaMKII-dependent phosporylation may increase resting Ca^{2+} spark frequencies.[5,9]

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Here, we examined Ca^{2+} sparks as signals closely reflecting RyR open probability in their native environment. While there is controversial literature on the relative importance of PKA- and/or CaMKII-dependent RyR phosphorylation,[8,14] only few studies specifically investigated changes of Ca^{2+} spark frequencies resulting from β-AR stimulation during diastole or in resting cardiomyocytes.[5,9,15,16] Based on these findings, it has been suggested that PKA-dependent phosphorylation of RyRs may not be significantly involved in the observed increase in the resting Ca^{2+} spark frequency. It has been reported that this entirely hinged on the SERCA stimulation resulting from phosphorylation of phospholamban[17] or occurred via a pathway that could involve CaMKII.[14,16,18–20] Since the CaMKII-dependent increase in spontaneous spark frequencies was observed in resting cardiomyocytes without detectable Ca^{2+} signals, which are typically required for significant Ca^{2+}-dependent activation of CaMKII,[21,22] it remained unresolved by which pathway(s) CaMKII would become activated under these conditions.

The current study represents an effort to reveal the mechanism involved in the activation of CaMKII in resting cardiomyocytes during β-AR stimulation and to identify an alternative pathway that could underlie the observed increase in Ca^{2+} spark frequency.

In the literature, several possible mechanisms have been mentioned. The 'exchange protein activated by cAMP' (Epac) may participate in the modulation of RyR open probability, either directly or via CaMKII.[18] Another alternative is activation of CaMKII by reactive oxygen species (ROS), which is known to occur independently of detectable Ca^{2+} signals.[23] Therefore, we carried out experiments investigating Ca^{2+} sparks and CaMKII activity to characterize the putative involvement of these and other cellular signalling pathways.

Together our findings reveal that upon β-AR stimulation, CaMKII becomes activated in a manner that does not require Ca^{2+} transients, initiated by formation of endogenous nitric oxide (NO). This represents an unexpected and newly discovered mode of CaMKII activation occurring in parallel to stimulation of PKA by cAMP. Preliminary findings have previously been presented in the abstract form.[24]

2. Methods

For additional information on methods, see Supplementary material online.

2.1 Isolation of Guinea-pig ventricular myocytes

For all electrophysiological and confocal Ca^{2+} and NO imaging experiments, we used freshly isolated Guinea-pig ventricular cardiomyocytes[16] following the animal handling procedures conforming with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and with the permission of the State Veterinary Administration and according to Swiss Federal Animal protection law (permit BE97/09). Animals were euthanized by stunning and cervical dislocation, followed by rapid removal and enzymatic dissociation of the cardiac tissue.

2.2 Experimental solutions

All drugs, inhibitors, and donors used during our experiments were freshly prepared daily from aliquots. Extracellular and intracellular (patch pipette) solutions were used from a ready-made stock.[16]

2.3 Electrophysiological recordings

I_{Ca} recordings were carried out in the whole-cell configuration of the patch clamp technique, resting membrane potential was set at −80 mV during Ca^{2+} imaging.[16]

2.4 SR Ca^{2+} content pre-conditioning

To ensure a constant SR Ca^{2+} content, we performed a SR Ca^{2+} loading protocol with a train of 20 membrane depolarizations from −80 to 0 mV (*Figure 1A*). Pharmacological interventions were applied, as indicated. Changes in SR Ca^{2+} content were compared with control conditions without the drug, after an identical loading protocol. In all cases, content was estimated by recording a Ca^{2+} transient triggered with caffeine.

2.5 Confocal Ca^{2+} and NO imaging

The Ca^{2+} spark frequency and SR Ca^{2+} content are shown after normalization and expressed as mean values ± SEM. For these recordings, we used fluo-3 as Ca^{2+} indicator. NO measurements were carried out using DAF-2DA. Confocal imaging was performed with either a FluoView-1000 (Olympus) or a MRC-1000 confocal laser-scanning microscope (Bio-Rad). Indicators were excited at 488 nm with a solid-state laser (Sapphire 488–10) and fluorescence was detected >515 nm.

2.6 *In vitro* $CaMKII_\delta$ activity and nitrosation assays

Ca^{2+}-independent, H_2O_2 and NO-dependent CaMKII activities were assessed by using an ELISA kit. Values are shown normalized to the maximal CaMKII activation levels reached in low Ca^{2+} (<10 nM Ca^{2+}; CaM/EGTA). Nitrosation of CaMKII was quantified using an antibody specifically detecting *S*-nitrosated cysteines.

2.7 Statistics

Paired or unpaired Student's *t*-tests were applied as appropriate to determine significance. In figures *P*-values of <0.05 or <0.01 are indicated by * or **, respectively. *N* refers to number of animals, and *n* to number of cells.

3. Results

3.1 Ca^{2+} spark frequency during β-AR stimulation is modulated by CaMKII but not PKA

We investigated cAMP-dependent pathways (e.g. PKA, Epac) to determine the involvement of PKA and/or CaMKII in the modulation of resting Ca^{2+} spark frequencies. Constant SR loading was achieved with Ca^{2+} pre-loading involving a train of L-type Ca^{2+} currents. SR Ca^{2+} content was estimated with caffeine before and after the experiment in each cardiomyocyte (*Figure 1A*).[16] As shown in *Figure 1B* and *C*, superfusion of resting cells with 1 μM isoproterenol (Iso) increased the frequency of Ca^{2+} sparks around 4-fold within 3 min, without significantly altering the $[Ca^{2+}]_{SR}$ in this time window (*Figure 1E*).[16] Next, cAMP was raised independently of the β-AR receptors by direct activation of adenylate cyclase with forskolin.[11] Surprisingly, and unlike Iso, 1 μM forskolin did not change the Ca^{2+} spark frequency significantly, even though the SR content increased to 125%, presumably resulting from SERCA stimulation without activation of sparks in parallel. Importantly, both drugs resulted in almost identical amplification of the L-type Ca^{2+} current (I_{Ca}), confirming that forskolin activated PKA to an extent corresponding to Iso application (*Figure 1D*). Inclusion of the specific PKA inhibitory peptide PKI in the patch solution did not prevent the increase in the Ca^{2+} spark frequency, but resulted in a drop of SR Ca^{2+} content, presumably resulting from the suppression of SERCA stimulation during Iso.

These results indicate that acute β-AR stimulation increases Ca^{2+} spark frequency, independently of cAMP and therefore makes an involvement of PKA and Epac very unlikely. Hence, we did not follow

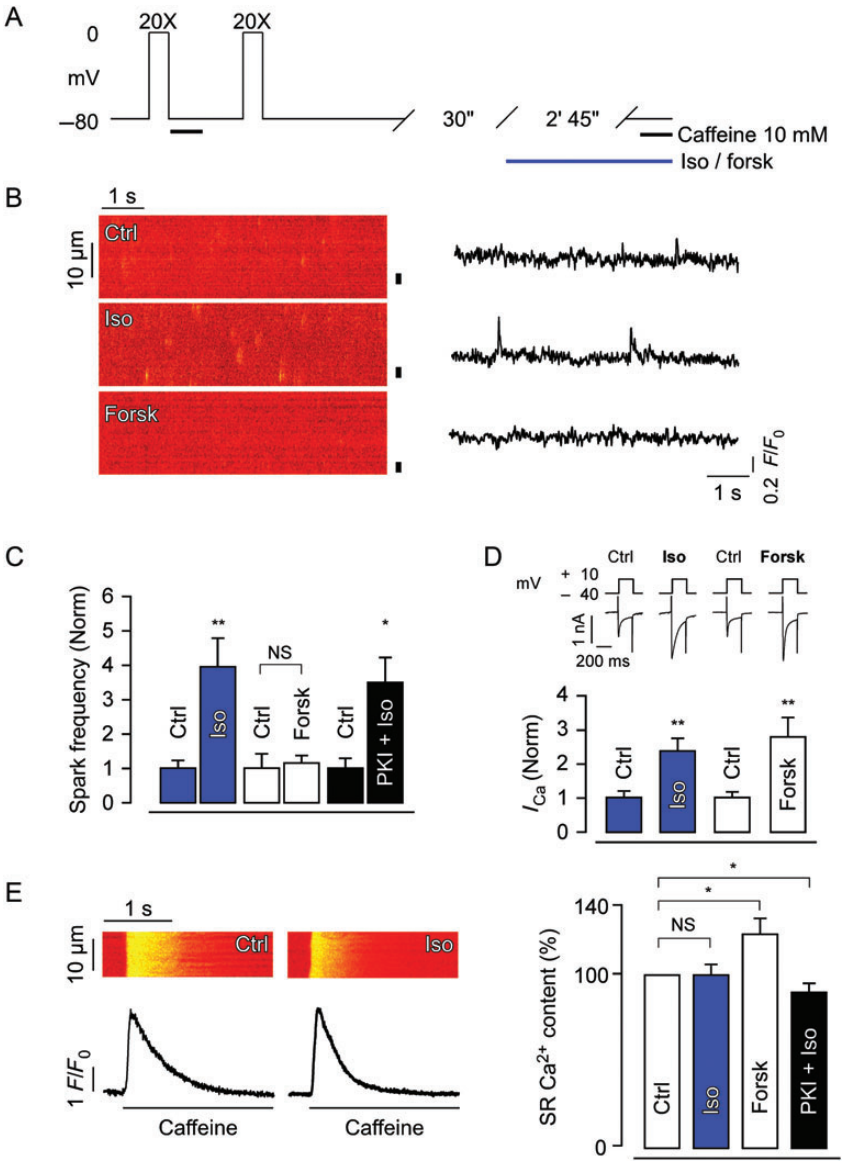

Figure 1 The increase in Ca^{2+} spark frequency by Iso is mediated by β-adrenergic receptors but not by cAMP. (*A*) The experimental protocol used trains of depolarizations to load the SR with Ca^{2+} and caffeine to estimate SR Ca^{2+} content (for details see Supplementary material online, information). (*B*) Confocal line-scan images showing Ca^{2+} sparks in control solution (Ctrl) and after ~3 min of 1 μM Iso or 1 μM forskolin. (*C*) Normalized Ca^{2+} spark frequency in Ctrl, after ~3 min of Iso ($n = 8$, $N = 8$) or forskolin ($n = 6$, $N = 2$) and after Iso in the presence of the PKA inhibitor PKI ($n = 7$, $N = 3$), respectively. In control, Ca^{2+} sparks are relatively indistinct and sparse in resting guinea pig cardiomyocytes[16] (around 1 s^{-1} 100 μm^{-1}). While Iso led to a ~4-fold increase in Ca^{2+} spark frequency, this was not observed with forskolin and was not prevented by the PKA inhibitor PKI. (*D*) Ca^{2+} current in Ctrl and after ~3 min Iso or forskolin. For this experiment, the cells were held at −40 mV to inactivate Na^+ currents. Normalized Ca^{2+} current in Iso ($n = 5$, $N = 4$) or forskolin ($n = 6$, $N = 5$). Current stimulation by forskolin was similar to that by Iso, documenting comparable PKA activation. (*E*) Typical SR Ca^{2+} content assessment by 10 mM caffeine. In forskolin, SR content is increased to 125 ± 8.8% ($n = 15$, $N = 5$) because of SERCA stimulation without activation of sparks. In contrast, in PKI the SR Ca^{2+} content decreased, partly because PKA inhibition prevents SERCA stimulation.

up on either of these pathways. A potential alternative pathway could be CaMKII, despite the fact that in quiescent cells, there were no Ca^{2+} signals that could activate this kinase. To confirm involvement of CaMKII, as also suggested by our previous study,[16] we carried out experiments in the presence of either KN-93 or KN-92 or included 10 μM of the specific CaMKII inhibitor autocamtide-2-related inhibitory peptide (AIP) in the patch solution. KN-93 and AIP prevented the increase in Ca^{2+} spark frequency in Iso without changing the SR Ca^{2+} content (*Figure* 2*A*–*C*). KN-92, the inactive negative control for KN-93, did not suppress the increase in spark frequency, as expected. Thus, under these experimental conditions, we can use the Ca^{2+} spark frequency as biological indicator for CaMKII activity.

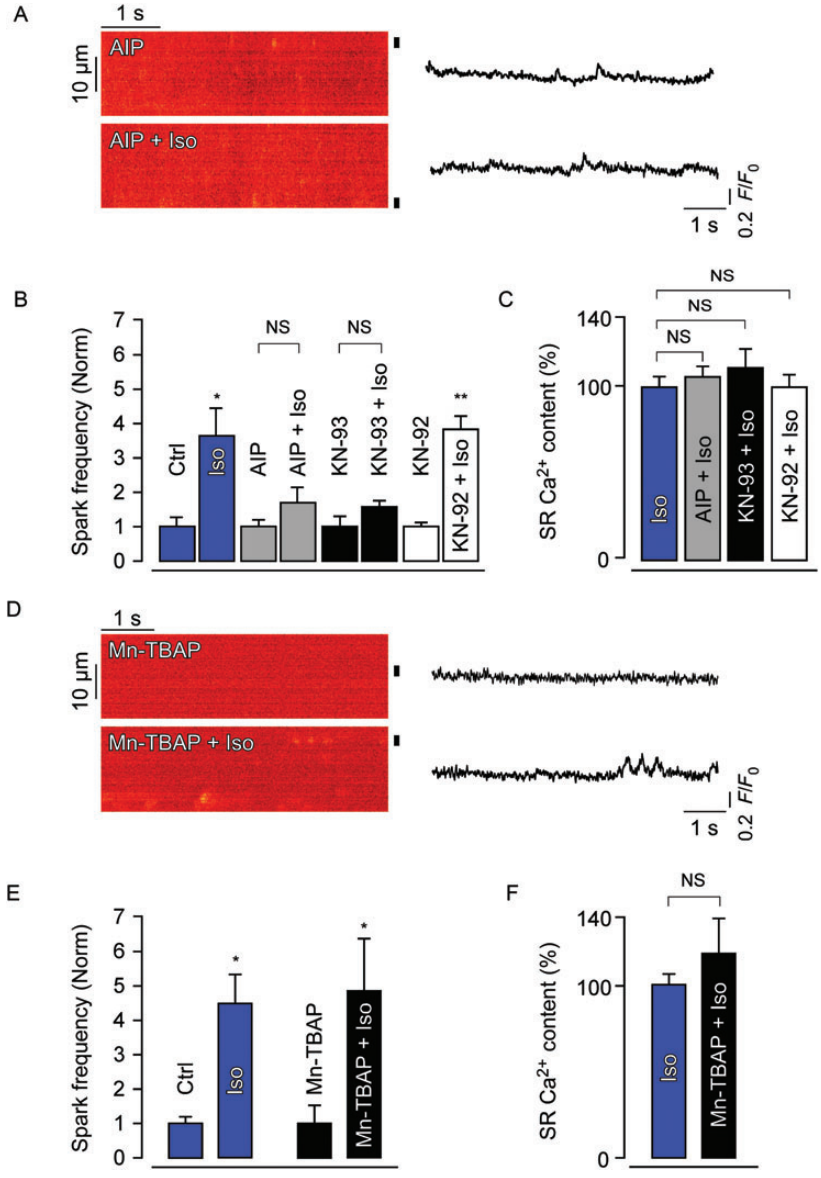

Figure 2 CaMKII is involved in spark frequency modulation, but not via ROS-dependent CaMKII activation. (*A*) Line-scan images in Ctrl and after 1 μM Iso, both in the presence of 10 μM of AIP in the patch pipette. (*B*) Ca^{2+} spark frequency in Ctrl and in Iso, in the absence ($n = 8, N = 8$) or presence of the CaMKII inhibitors AIP ($n = 9, N = 5$), KN-93 ($n = 5, N = 2$), and its inactive analogue KN-92 ($n = 5, N = 3$). (*C*) Unchanged SR Ca^{2+} content in the presence of AIP, KN-93, and KN-92. (*D*) Line-scan images recorded after 1 h pre-incubation with 100 μM ROS scavenger Mn-TBAP in Ctrl and after ~3 min of 1 μM Iso. (*E*) Ca^{2+} spark frequency during Iso without ($n = 8, N = 8$) and with Mn-TBAP ($n = 5, N = 5$). (*F*) Unchanged SR Ca^{2+} content in the presence of the ROS scavenger Mn-TBAP.

3.2 ROS scavengers fail to prevent the increase in Ca^{2+} spark frequency

We then examined whether Ca^{2+}-independent CaMKII activation by ROS[23] could underlie the higher spark frequency. For this, we used Mn-TBAP, a superoxide dismutase (SOD) mimetic, which has previously been shown in our experiments to reliably suppress Ca^{2+} sparks initiated by oxidative stress.[25] Interestingly, 100 μM Mn-TBAP failed to prevent the increase in Ca^{2+} spark frequency and did not significantly alter SR Ca^{2+} load *(Figure 2D–F)*, indicating that CaMKII activation by β-AR stimulation is not ROS dependent.

3.3 NO modulates Ca^{2+} spark frequency upon β-AR stimulation

It has been suggested that after β-AR stimulation the increase in SR Ca^{2+} leak, determined with a dedicated leak protocol, is dependent on NO production and independent of PKA, because NO-synthase (NOS) inhibition prevented the leak observed in the presence of Iso.[11,26] To examine whether a similar mechanism could be involved in the higher frequency of Ca^{2+} spark observed here, we inhibited the synthesis of NO while stimulating the cardiomyocytes with Iso, analogous to experiments described in *Figure 1*. Pre-treatment with 500 μM of the NOS

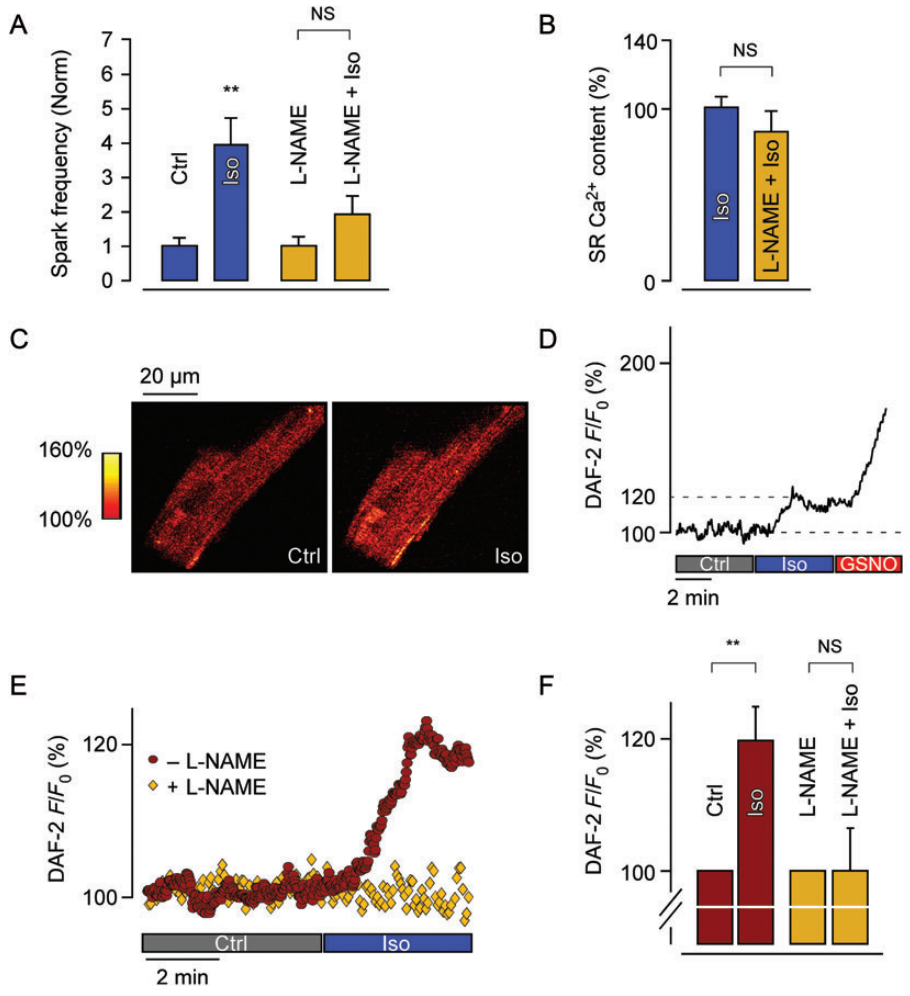

Figure 3 The increase in Ca^{2+} spark frequency is dependent on NO. (*A*) Comparison of the Ca^{2+} spark frequency stimulation by 1 μM Iso without ($n = 8$, $N = 8$) and with ($n = 7$, $N = 5$) 1 h pre-incubation with 500 μM L-NAME. (*B*) SR Ca^{2+} content remained unchanged in the presence of L-NAME. (*C*) Representative signal of DAF-2 fluorescence before (Ctrl) and after ~3 min of 1 μM Iso. (*D*) Representative time-course of NO production upon 1 μM Iso application. At the end, GSNO is applied as a positive control for NO detection. (*E*) Averaged time-course of DAF-2 fluorescence in Ctrl and during Iso recorded in the absence (red trace $n = 7$, $N = 6$) and presence of L-NAME (orange trace, $n = 6$, $N = 4$). (*F*) Normalized NO-induced DAF-2 fluorescence upon Iso (red bar) and after pre-incubation in 500 μM L-NAME (orange bar). L-NAME prevented the NO signal observed in control, confirming that the DAF-2 fluorescence resulted from NO production.

inhibitor L-NAME prevented a significant increase in Ca^{2+} spark frequency (*Figure 3A*), indicating that upon β-AR stimulation, the spark frequency is modulated by endogenous NO produced by NOS of the cardiomyocyte.

To confirm NO involvement, cells were loaded with diaminofluorescein (DAF-2) by exposure to the ester (DAF-2DA; 0.1 μM). After application of Iso, we recorded a substantial increase in DAF-2 fluorescence (19 ± 5%; *Figure 3C* and *E*). In some cells, the NO donor *S*-nitroso-L-glutathione (GSNO; 500 μM) was added at the end of the protocol, to confirm that DAF-2 resolves NO signals (*Figure 3D*). Please note that these recordings were corrected for dye bleaching and should not be taken quantitatively for NO concentrations. Because DAF-2 has been reported to detect other types of reactive species,[27] we repeated the same experiment in 500 μM L-NAME to suppress NO formation. This prevented the increase in DAF-2 fluorescence (*Figure 3E* and *F*, orange symbols and columns), confirming that the signal reflects NO production. Cellular ROS production induced by GSNO was excluded with the ROS sensitive fluorescent indicator CM-H_2DCF (see Supplementary material online, *Figure S1*). Together, these findings firmly establish a link between Iso-induced production of intracellular NO by the cardiomyocytes and the observed increase in Ca^{2+} spark frequencies mediated by CaMKII. This interpretation is in line with the inability of Mn-TBAP to prevent the increase in Ca^{2+} spark frequency (*Figure 2D* and *E*) and is consistent with the observation that Mn-TBAP does not significantly scavenge NO.[28]

3.4 The NO donor GSNO reproduces the increase in Ca^{2+} spark frequency induced by β-adrenergic stimulation

NO can affect the function of proteins, including the RyRs, via several known pathways.[29–31] This can occur directly as post-translational protein modifications, such as *S*-nitrosation (also referred to as *S*-nitrosylation or transnitrosylation).[12,32] Alternatively, NO can lead

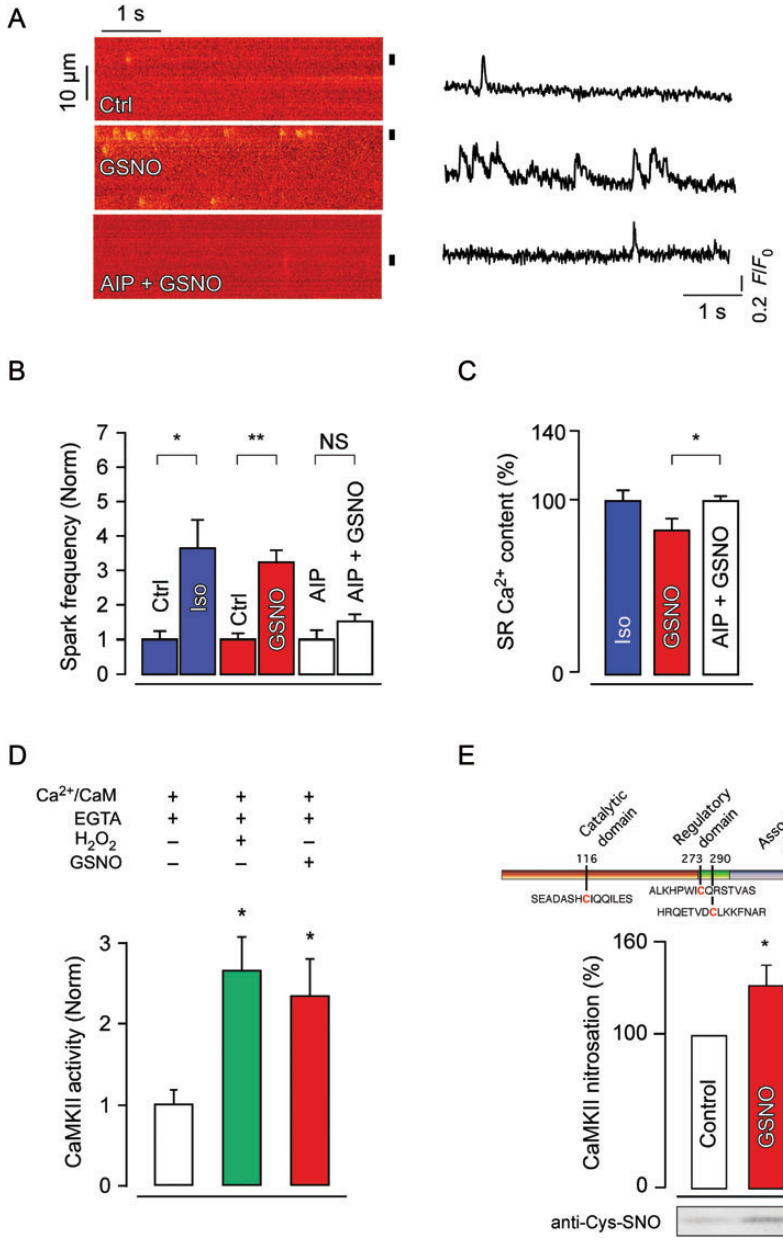

Figure 4 The NO donor GSNO increases Ca^{2+} spark frequency upon CaMKII activation via nitrosation. (*A*) Line-scan images of Ca^{2+} spark recordings in Ctrl (top), during 150 µM GSNO alone (middle), and GSNO in the presence of 10 µM AIP in the patch pipette (bottom). (*B*) Normalized spark frequencies after ~3 min of 1 µM Iso (blue bar $n = 6$, $N = 6$) and GSNO alone (red bar $n = 14$, $N = 11$) and with 10 µM AIP (white bar $n = 9$, $N = 4$). Inhibiting CaMKII with AIP prevented the higher frequency induced by GSNO. (*C*) After GSNO, the SR Ca^{2+} content was not maintained (83 ± 6% of control) due to a higher spark frequency without SERCA stimulation in parallel. (*D*) Quantitative *in vitro* CaMKII activation in response to 1 µM H_2O_2 (blue bar) or 500 µM GSNO (red bar) in comparison with control (white bar). CaMKII activities were normalized to full Ca^{2+}/CaM-dependent and control activities ($N = 5$). (*E*) Detection of nitrosated CaMKII *in vitro* by anti-Cys-SNO specific antibody reveals increased nitrosation in GSNO.

to the formation of cGMP and activation of protein kinase G (PKG), which has been shown to phosphorylate RyRs, but so far only *in vitro*.[33] The findings in *Figure 3* indicate that after Iso application, NO mediates the increase in Ca^{2+} spark frequency. To support this interpretation, we used a NO donor instead of Iso. One hundred fifty micromolar GSNO resulted in a 3.22 (±0.31)-fold increase in Ca^{2+} spark frequency, thereby quantitatively mimicking the changes observed in Iso (*Figure 4A* and *B*). Note that in these experiments, SR Ca^{2+} content did not maintain the control level, presumably because GSNO increased the Ca^{2+} spark frequency without concomitant SERCA stimulation (*Figure 4C*). To distinguish between a direct *S*-nitrosation of the RyRs and an indirect modification via CaMKII (suggested by the findings above), we tested whether AIP could prevent the GSNO-dependent occurrence of sparks, similar to what was observed in Iso. In the presence of AIP, GSNO did not significantly elevate spark frequency (*Figure 4B*). These findings confirm that the higher spark frequency in GSNO resulted largely from activation of CaMKII and not from a direct *S*-nitrosation of the RyRs or activation

of PKG. In summary, these data indicate that NO can activate CaMKII, in the absence of Ca^{2+} signals.

3.5 Quantification of NO-dependent CaMKII activation *in vitro*

To confirm that NO could activate CaMKII in the absence of elevated Ca^{2+}, we used an *in vitro* assay.[34] CaMKII activity was detected with an ELISA test and normalized to that observed in low Ca^{2+} (<10 nM; Ca^{2+}, CaM, and EGTA, *Figure 4D*). As already established,[23] addition of H_2O_2 activated CaMKII in low Ca^{2+}. The activity observed in 1 μM H_2O_2 was 2.65 (±0.47)-fold higher than in control. The NO donor GSNO resulted in comparable CaMKII stimulation of 2.31 (±0.39)-fold. CaMKII activity under these oxidative and nitrosative conditions represented ~16% of the maximal Ca^{2+}/CaM dependent activity (250 μM Ca^2 and 120 nM calmodulin). This confirms a direct activation of CaMKII by NO, as suggested by our findings in cardiomyocytes.

Each CaMKII monomer is predicted by GPS-SNO software[35] to have three potential sites for *S*-nitrosation (*Figure 4E*, upper panel). We used an antibody specifically recognizing *S*-nitrosated cysteines to quantify CaMKII nitrosation after pre-incubation of $CaMKII_\delta$ with GSNO (*Figure 4E*, lower panel). Indeed, a 32.1 ± 13% increase of CaMKII nitrosation was observed with this assay.

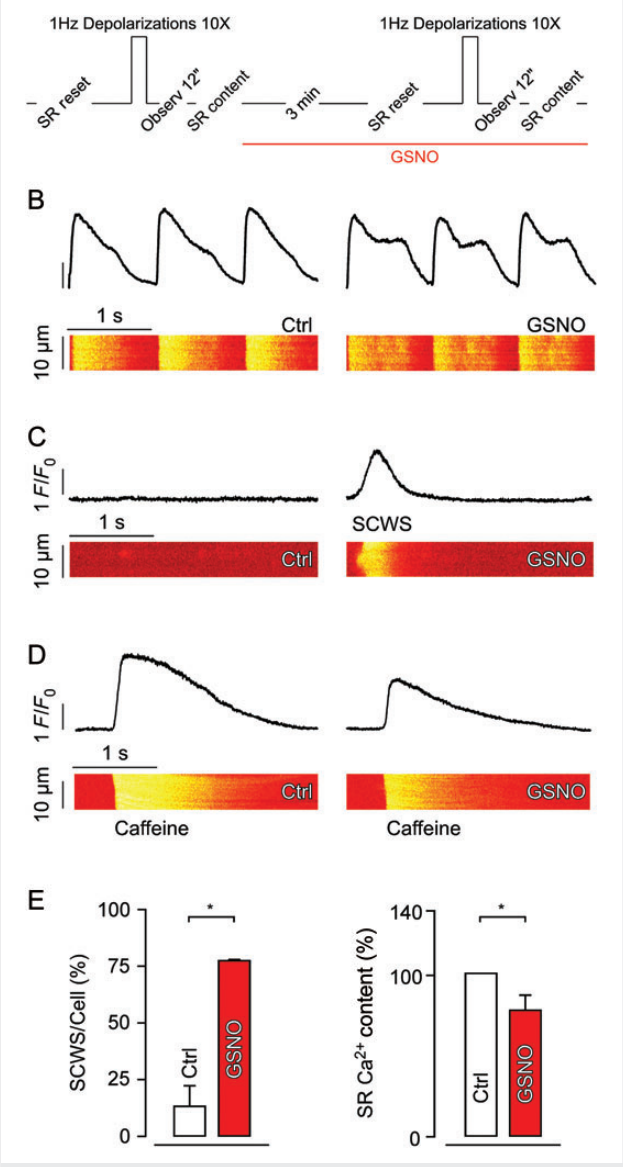

Figure 5 In beating cardiomyocytes, the NO donor GSNO increased diastolic Ca^{2+} spark frequency and induced arrhythmogenic diastolic Ca^{2+} waves. (*A*) Protocol used to record Ca^{2+} transients and Ca^{2+} waves from myocytes during and immediately after stimulation, with or without the presence of GSNO. (*B*) GSNO resulted in more diastolic Ca^{2+} sparks,(*C*) in a higher propensity for spontaneous Ca^{2+} waves (SCWS) and (*D*) in reduced SR Ca^{2+} content. (*E*) Statistical analysis of SCWS ($n = 9$, $N = 5$) and SR content ($n = 9$, $N = 5$).

3.6 GSNO leads to arrhythmogenic Ca^{2+} signals in beating cardiomyocytes

At the cellular level, spontaneous Ca^{2+} waves (SCWS) are considered to be indicators for arrhythmogenic conditions. Since the elevated Ca^{2+} spark frequencies shown above could result in SCWS, we tested the arrhythmogenic potential of the NO donor in field stimulated cardiomyocytes (*Figure 5*). In these experiments, NO presumably modified several relevant Ca^{2+} signalling proteins and membrane channels. However, the recordings revealed an increase in the diastolic Ca^{2+} spark frequency, similar to what was observed in resting cells. Furthermore, after a train of 10 depolarizations, 13.3% of the control myocytes exhibited SCWS, while in the presence of GSNO 77.8% showed waves. This elevated wave frequency was accompanied by a reduced SR content (to 77 ± 6.5% of control), confirming that the waves were resulting from altered function of the RyRs and not from SR Ca^{2+} overload.

Taken together, our results provide compelling evidence that the observed increase in Ca^{2+} spark frequency upon β-AR stimulation results from an activation of CaMKII, which is mediated by NO but is not dependent on Ca^{2+} transients (see *Figure 6* for a diagram of this pathway). This represents a new mechanism for CaMKII activation that may have far reaching implications.

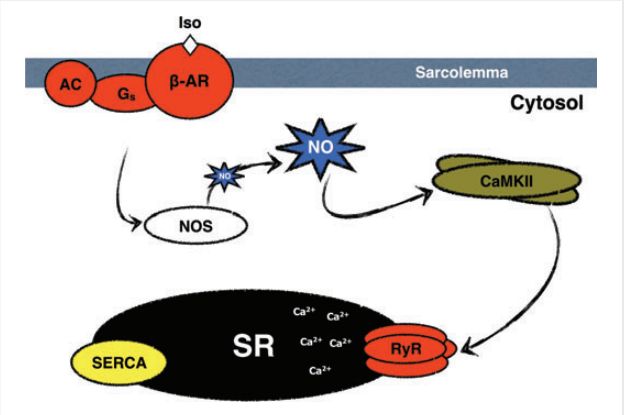

Figure 6 Diagram of the involved pathways during β-AR stimulation of resting cardiomyocytes. Our results show that during β-AR stimulation by Iso, endogenous production of NO derived from NOS activates CaMKII and subsequently modulates the RyRs open probability, as reflected by the higher Ca^{2+} spark frequency.

4. Discussion

RyRs have attracted considerable research interest, due to discoveries such as RyR mutations causing life-threatening arrhythmias.[36] Alterations of their behaviour are observed during several diseases and are often caused by post-translational modifications, most notable phosphorylation and oxidation/nitrosation (for review see ref.[12]). The participation of the RyRs in diseases such as catecholaminergic polymorphic ventricular tachycardias (CPVTs) and heart failure suggests that they may be potential drug targets.[37,38]

4.1 Modulation of RyR function

Changes of RyR function are expected to have a significant impact on cardiac Ca^{2+} signalling. Several laboratories have examined functional consequences of RyR phosphorylation on various levels of complexity, from single channels to isolated cells, partly using transgenic animal and disease models.[7,39,40] These studies have resulted in a considerable controversy and confusion regarding the functional role of the involved protein kinases PKA and CaMKII.[14,41] The reasons for this are not clear, but may arise from different disease models, protocols, and experimental designs. As suggested by the present study, they may partly arise from unexpected cross-talks between complex cellular signalling pathways.

4.2 Modes of CaMKII activation

When examining the importance of protein kinases for changes of diastolic Ca^{2+} spark frequencies after β-AR stimulation, we made a surprising observation. Even though there were no visible Ca^{2+} signals in resting cells that could lead to significant CaMKII activation, the increase in Ca^{2+} spark frequencies could be prevented by pharmacologically blocking CaMKII (but not PKA). This immediately raised the question how under these circumstances CaMKII could become activated? In the literature, several possibilities have been reported, including a pathway involving 'exchange factor directly activated by cAMP' (Epac),[15,42] or requiring oxidative modification of the CaMKII.[23] The observation that the application of forskolin did not elevate the propensity of diastolic Ca^{2+} sparks, unlike β-AR stimulation, is in line with our conclusion, based on the experiments with protein kinase inhibitors for CaMKII and PKA, that any PKA involvement is highly unlikely. Furthermore, the negative result with forskolin regarding spark frequencies makes activation of CaMKII via the Epac pathway improbable and is consistent with the finding that forskolin does not increase SR Ca^{2+} leak.[11] Therefore, we carried out experiments to test for the second possibility of Ca^{2+} spark activation, oxidative stress. Of note, redox modifications of the RyRs are well known to increase their openings,[43,44] although direct RyR oxidation would not be sensitive to the CaMKII inhibitor AIP, as observed in this study. Thus, we were left with the alternative that an oxidative modification of CaMKII could be responsible for its activation. However, we were unable to prevent the Ca^{2+} sparks with Mn-TBAP, a SOD mimetic which we found to reliably suppress ROS induced sparks in a model of oxidative stress.[25] This finding suggests that ROS generation after β-AR stimulation is not involved in CaMKII activation and is consistent with a recent report showing that ROS production does not increase during β-AR stimulation of resting cells.[45]

4.3 NO activates CaMKII in cardiomyocytes

An interesting observation has been reported in a study examining SR Ca^{2+} leaks in cardiomyocytes during β-AR stimulation using a dedicated leak protocol.[11] In this study, the SR Ca^{2+} leak has been quantified from the drop of the cytosolic Ca^{2+} concentration after blocking the RyRs with tetracaine. These authors showed that the function of CaMKII, but also NO synthases, were important determinants for the SR Ca^{2+} leak.[26] Surprisingly, our experiments along these lines revealed that inhibition of NOS also prevented the increase in spark frequency, suggesting an involvement of NO signalling. To confirm a key role of NO, we used a multi-pronged approach. We were able to detect endogenous NO production by the cardiomyocytes upon Iso application, while the NO donor GSNO mimicked the effects of Iso on the spark frequency. In contrast, the GSNO effect was almost completely inhibited by the specific CaMKII inhibitor AIP, suggesting that direct RyR nitrosation was not involved. Rather this appeared to be mainly mediated by CaMKII. Could it be that NO maintains CaMKII active at resting Ca^{2+} concentrations, similar to what has been reported for ROS?[23] For this to occur, an initial Ca^{2+}-dependent activation of CaMKII is required, which could be mediated by invisible Ca^{2+} signals, such as the Ca^{2+} quarks suggested to underlie a fraction of the SR Ca^{2+} leak.[46,47] To address the intriguing question of NO-dependent CaMKII activation directly, we applied a biochemical *in vitro* assay of CaMKII activity. The results obtained confirmed that NO can maintain CaMKII active to an extent similar to H_2O_2, without requiring elevated Ca^{2+} concentrations. Since this occurred *in vitro* and was initiated by significant *S*-nitrosation of the CaMKII protein, it seems very likely that NO can directly activate CaMKII. Interestingly, a strikingly different regulation by NO and ROS has been reported for the $CaMKII_{\alpha}$ isoform of this kinase in pituitary tumour GH3 cells. This isoform was inhibited after nitrosation, but became activated in reducing conditions.[48]

4.4 Relevance of NO-dependent CaMKII activation

Taken together, these findings provide compelling evidence for a stimulation of endogenous NO generation by cardiomyocytes upon β-AR stimulation. In the beating heart, this presumably additive mechanism may be even more pronounced, since further CaMKII activation will occur by enhanced Ca^{2+} transients. Several studies have previously reported positive or negative inotropic effects of NO, which may be partly related to modifications of CaMKII and RyR function, involved NOS isoforms, or crosstalk between activated pathways.[49,50] Related to this, it has been suggested that NO may have a biphasic effect on RyR open probability, depending on the extent of pre-existing β-AR stimulation.[51] Although the precise mechanism of this phenomenon remains unresolved, it may involve CaMKII and could be related to changes of the nitroso/redox balance, as several post-translational modifications will modulate RyR function in an exceedingly complex fashion.[52]

The pathway characterized here represents a newly discovered mechanism for CaMKII activation. This results in a surge of diastolic Ca^{2+} sparks and increased wave propensity. Since Ca^{2+} sparks are signals faithfully reporting the function of the RyRs, this likely occurs via CaMKII-dependent modulation of the RyRs. In addition, the direct activation of CaMKII by NO observed here and the pathways participating downstream of CaMKII are expected to have additional repercussions for cardiomyocyte Ca^{2+} signalling. For example, NO-mediated CaMKII activation contributes to the modulation of Ca^{2+} cycling upon β-AR stimulation, such as during physical exercise or emotional stress. During sustained β-AR stimulation, such as during heart failure, this could lead to SR Ca^{2+} depletion, weaker heartbeat, and

arrhythmias.[40,53] The additional CaMKII activation prompted by NO may be particularly detrimental if it occurs in conditions with already hypersensitive RyRs, for example in the presence of CPVT mutations or oxidative RyR modifications.

Finally, the identification of this pathway adds to the experimental complexity of studies with cardiac muscle because it represents a possibility for crosstalk between PKA and CaMKII activation, downstream of β-AR stimulation. The existence of such a cross-talk may explain some of the controversial experimental results and interpretations regarding the regulation of the RyRs by PKA or CaMKII, respectively.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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