Selective Modulation of Coupled Ryanodine Receptors During Microdomain Activation of Calcium/Calmodulin-Dependent Kinase II in the Dyadic Cleft

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Rationale: In ventricular myocytes of large mammals with low T-tubule density, a significant number of ryanodine receptors (RyRs) are not coupled to the sarcolemma; cardiac remodeling increases noncoupled RyRs.

Objective: Our aim was to test the hypothesis that coupled and noncoupled RyRs have distinct microdomain-dependent modulation.

Methods and Results: We studied single myocytes from pig left ventricle. The T-tubule network was analyzed in 3-dimension (3D) to measure distance to membrane of release sites. The rising phase of the Ca2+ transient was correlated with proximity to the membrane (confocal imaging, whole-cell voltage-clamp, K\textsubscript{fluo}-4 as Ca2+ indicator). Ca2+ sparks after stimulation were thus identified as resulting from coupled or noncoupled RyRs. We used high-frequency stimulation as a known activator of Ca2+/calmodulin-dependent kinase II. Spark frequency increased significantly more in coupled than in noncoupled RyRs. This specific modulation of coupled RyRs was abolished by the Ca2+/calmodulin-dependent kinase II blockers autocamtide-2–related inhibitory peptide and KN-93, but not by KN-92. Colocalization of Ca2+/calmodulin-dependent kinase II and RyR was not detectably different for coupled and noncoupled sites, but the F-actin disruptor cytochalasin D prevented the specific modulation of coupled RyRs. NADPH oxidase 2 inhibition by diphenyleneiodonium or apocynin, or global reactive oxygen species scavenging, also prevented coupled RyR modulation. During stimulated Ca2+ transients, frequency-dependent increase of the rate of Ca2+ rise was seen in coupled RyR regions only and abolished by autocamtide-2–related inhibitory peptide. After myocardial infarction, selective modulation of coupled RyR was lost.

Conclusions: Coupled RyRs have a distinct modulation by Ca2+/calmodulin-dependent kinase II and reactive oxygen species, dependent on an intact cytoskeleton and consistent with a local Ca2+/reactive oxygen species microdomain, and subject to modification with disease. (Circ Res. 2013;113:1242-1252.)

Key Words: calcium/calmodulin-dependent protein kinase type 2 • myocytes, cardiac • NADPH oxidase • reactive oxygen species • ryanodine receptor calcium release channel • sarcoplasmic reticulum

In ventricular myocytes of larger mammals, including humans, the density of T-tubules (TT) is much lower than in rodents, and many ryanodine receptors (RyRs) in the sarcoplasmic reticulum are not adjacent to sarcolemma.1–3 During an action potential, opening of L-type Ca2+ channels (LTCC) triggers activation of the coupled RyRs, whereas noncoupled RyRs are activated through propagated Ca2+ release with a delay. Reduced synchrony of RyR activation across sarcomeres slows the overall rate of rise of [Ca2+]\textsubscript{i} and amplitude of the [Ca2+]\textsubscript{i} transient.1 With disease and loss of disorganization of TT, the fraction of noncoupled, orphaned RyR increases, contributing to an apparent reduction of the gain of excitation–contraction coupling.4,6 Recent studies have started to document the timeline of such changes during the transition from hypertrophy to heart failure7 and the potential for reversal.8,9

We have recently started to investigate whether the intrinsic properties of RyR subpopulations that are coupled or at a distance of TT are different, by studying spontaneous RyR opening events at rest in release sites that were close to TT or at a distance.10 Ca2+ sparks were more frequent in sites close to TT, and this was not dependent on activity of LTCC, but a clear mechanism could not be identified.

Mechanisms that affect properties of subpopulations of RyR clusters can be structural or functional.11 Activation and gating

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of RyRs is dependent on local [Ca^{2+}], cytosolic and luminal, and is further modulated through modification such as oxidation, nitrosylation, and phosphorylation. Many proteins are involved; both kinases and phosphatases associate with the RyRs to form a macromolecule. Ca^{2+}/calmodulin-dependent kinase (CaMKII) is of particular interest as it will be activated by the Ca^{2+} released from RyRs, after first binding to calmodulin. Recent studies have documented the time course of CaMKII activation during increased frequency of stimulation and the simultaneous phosphorylation of RyRs. More recently, an in situ fluorescence resonance energy transfer reporter has been used to follow the activation of CaMKII during stimulation. The process takes tens of seconds to build up and also has a slow recovery. Modeling and experimental data have shown how the N-terminus of calmodulin is thought to serve as a low-affinity Ca^{2+} sensor, transducing Ca^{2+} in the dyad, allowing activation in response to the high Ca^{2+} local to LTCC and RyR in the dyad in a frequency-dependent manner. RyRs that are not in the dyad (noncoupled RyRs) may be less likely to experience such a local increase in Ca^{2+} because Ca^{2+} can freely diffuse away from the release site and the influx through LTCC is absent. This leads to a testable hypothesis regarding a functionally distinct role for CaMKII in these subpopulations of RyR clusters. It is reasonable to suggest that, in response to an increase in frequency of stimulation, activation of CaMKII in the dyadic microdomain would be higher in coupled RyRs.

In the present study, we have examined this hypothesis. We have first developed an improved approach for distinguishing coupled and noncoupled receptors based on 3-dimensional (3-D) distance mapping correlated to the properties of local Ca^{2+} release during membrane depolarization. We thus identify diastolic release events as resulting from coupled RyRs or from noncoupled RyRs during dynamic studies. Subsequently, we examine the response in coupled RyRs and noncoupled RyRs to increasing frequency of stimulation and whether differences in response are consequent to preferential activation of CaMKII in the microdomain of coupled RyRs. We further investigate underlying mechanisms.

### Methods

Cell isolation, confocal microscopy, and electrophysiological recording were performed as described previously. To construct a 3-D membrane distance map, cells were stained with wheat germ agglutinin linked to Alexa 594 (Invitrogen), and a 3-D version of the Euclidean distance transform was used as previously described using an ImageJ plugin (http://www.optinav.com/Local_Thickness.htm). Ca^{2+} was monitored using confocal linescan recording at 650 Hz, with K_{Fluo-4} loaded via the pipette. All experiments were performed at 37°C. During 0.5 and 2 Hz pacing, pixel-wise activation times were quantified as the time to reach half-maximal of the mean cellular peak fluorescence (T_{50}). Sparks were recorded in a 15-second rest period after 2 minutes of pacing. Alternatively, after pacing the sarcoplasmic reticulum (SR) Ca^{2+} content was measured as the integral of the current produced during rapid application of caffeine (10 mmol/L). Immunofluorescent imaging was performed in isolated cells, fixed and consecutively stained with primary antibodies for Na^{+}/Ca^{2+} exchanger (Swant, Switzerland), RyR (34C clone; Pierce), and CaMKII (a kind gift from Dr Donald Bers, UC Davis), and secondary antibodies labeled with Alexa 488, 568, and 647. In these fixed cells, Na^{+}/Ca^{2+} exchanger was used as a membrane marker and 3-D distance maps were constructed in a way similar to that described above. Colocalization of coupled and noncoupled RyRs with CaMKII was quantified by the Manders coefficient.

### Statistics

All data are presented as means±SEM. Data have been compared using a paired t test or 2-way ANOVA with Bonferroni post hoc testing where applicable. Data were considered significantly different when the probability value was <0.05.

Detailed methods are provided in the Online Supplement.

### Results

#### Differential Activation of Coupled and Noncoupled RyRs by Increasing Frequency of Stimulation

In order to reliably identify release sites that are coupled and non-coupled, we measured the distance of release sites to the membrane and correlated with the properties of the evoked Ca^{2+} release during voltage clamp. Figure 1A shows a typical myocyte labeled with wheat germ agglutinin. The 3-D geometry of sarcolemmal/T-tubular membrane was calculated from the vertical stack of 2-D confocal images. A 3-D form of the Euclidean distance transform was then applied, allowing the creation of a distance map. The graph at the bottom of Figure 1A and 1B shows the distance to the nearest membrane for the line of the central section of the cell to be sampled for Ca^{2+} release events. To have 2 clearly distinct populations, coupled and noncoupled sites were classified based on distances of <0.5 and >2 µm, respectively, from the membrane.

In this same cell, along this line, Ca^{2+} release was elicited by depolarizing steps and a line scan image was acquired. Ca^{2+} transients were averaged and normalized to the diastolic Ca^{2+} level (F_0/F). Latency of Ca^{2+} release was assessed as the time to reach half-maximal Ca^{2+} release (T_{50}) and calculated for each pixel along the line scan (Figure 1A and 1B). This allowed correlating the distance and T_{50} in the same cell. Averaged data showed that in coupled sites within 0.5 µm from the sarcolemmal membrane, T_{50} typically was <18 ms, when stimulating cells at 0.5 Hz (Figure 1B, left). When stimulating cells at 2 Hz, in coupled sites T_{50} typically was <14.5 ms. Coupled sites were most numerous as shown in the histogram (Figure 1B, middle). At both frequencies of stimulation, the fraction of release sites that qualified as coupled based on T_{50} was comparable (51.6% at 0.5 Hz; 49.7% at 2 Hz; n_cells=7; N_{pigs}=5; Figure 1B, right), validating the use of T_{50} as a parameter reflecting a structural property (ie, distance to the membrane) of the cell. For sites >2 µm from the sarcolemmal membrane, T_{50} typically was >27 ms for stimulation at 0.5 Hz (Figure 1B, left) and >21.5 ms at 2 Hz. Again, the fraction of these noncoupled sites based on T_{50} was comparable at both frequencies (26.8% at 0.5 Hz and 28.4% at 2 Hz; n_cells=7; N_{pigs}=5). These relationships were...
confirmed in paired experiments in a larger population of cells (n Cells=12; N Pigs=8; data not shown). An analysis in fixed cells of the distance of RyR to membrane in a 2-D image generated data that are in the same order of magnitude, with somewhat lower numbers for noncoupled RyRs (Figure 1C; see Discussion). Taken together, this data allowed the use of parameter TF50 as surrogate for a complete distance map during dynamic studies. The TF50 temporal thresholds were thus used to localize release sites as being coupled and noncoupled and identify Ca2+ sparks occurring after stimulation as resulting from coupled or noncoupled RyRs.

To test for the possible differential regulation by CaMKII, we determined the effects of increasing frequency of stimulation from 0.5 Hz to 2 Hz (Figure 2A). Myocytes were stimulated at 2 Hz for 2 minutes, as this has been shown to attain steady-state Ca2+ transient amplitude and activation of CaMKII.16 Sparks were then recorded during the 15-second period after stimulation. A pronounced (>3-fold) increase in spark frequency was observed in coupled sites (n cells=33; N pigs=16), whereas noncoupled sites showed a 2-fold increase. This response was reversed after another 2-minute stimulation period at 0.5 Hz (Figure 2B). An increase in spark frequency is in line with an increase in SR Ca2+ content at the higher frequency (Online Figure IIA) but cannot by itself explain the difference between sites. No significant differences could be detected in the frequency response for spark amplitude, width, or duration between coupled and noncoupled sites (data not shown).

**Localized CaMKII Activation Underlies the Different Response in Coupled and Noncoupled RyRs**

To examine our working hypothesis regarding the role of CaMKII, the experiment was repeated in the presence of a CaMKII-specific peptide, autocamtide–2–related inhibitory peptide (AIP). Myocytes were preincubated with myristoylated AIP for 1 hour, and AIP was also subsequently included in the pipette solution. The increase in SR Ca2+ content was not blocked by...
CaMKII inhibition (data not shown). This was somewhat unexpected because inhibiting the effect of CaMKII on phospholamban and LTCC would tend to reduce SR Ca\(^2+\) uptake and Ca\(^2+\) influx SR, respectively, to reduce SR content. However, this was not inconsistent with reports that show enhanced SR Ca\(^2+\) uptake with frequency to be independent of phospholamban. \(^{23}\) Another possibility is that a reduced LTCC Ca\(^2+\) influx may simply be balanced by a reduced RyR-mediated Ca\(^2+\) leak. Changes in spark behavior are, therefore, more likely related to changes in RyR properties than consequent on changes in SR Ca\(^2+\) content. As shown in Figure 2C, the increase in spark frequency in coupled RyRs with stimulation at 2 Hz was effectively suppressed by AIP, whereas noncoupled RyRs were unaffected (n\(_{\text{cells}}\)=12; N\(_{\text{pigs}}\)=7). Similar results were obtained with the CaMKII inhibitor KN-93 (n\(_{\text{cells}}\)=10; N\(_{\text{pigs}}\)=4), whereas the inactive analog KN-92 (n\(_{\text{cells}}\)=12; N\(_{\text{pigs}}\)=4) had no effect (Figure 2C).

One potential mechanism for the regional differences in RyR activation could be a differential distribution of CaMKII. To test this, we performed triple immunostaining for RyR, CaMKII, and Na\(^+\)/Ca\(^2+\) exchanger (to report on sarcolemmal geometry), as illustrated in Figure 3A. Images were segmented into coupled and noncoupled sites using the 3-D distance mapping method (Figure 3B). The Manders coefficient of colocalization revealed an equal association of CaMKII with coupled and noncoupled RyRs (n\(_{\text{cells}}\)=20; N\(_{\text{pigs}}\)=3). These data suggest that local activation of CaMKII, rather than a differential distribution and targeting of CaMKII, is responsible for the specific modulation of coupled RyRs.

**Activation of Local CaMKII Is Dependent on an Intact Structure at the Z-Disc**

If CaMKII is activated in the microdomain of the dyad, ultrastructural configuration of this microdomain is likely to be critical. We
examined whether disruption of the microfilament network (preincubation with 10 \(\mu\)mol/L of cytochalasin D [cyto-D] for 1 hour) affected the frequency response. Figure 4A-b illustrates how cyto-D removes the frequency response of coupled RyRs without affecting noncoupled RyRs (ncells=12; Npigs=5). To test whether cyto-D prevented the action of CaMKII on coupled RyRs, AIP was applied after pretreatment with cyto-D. No additional effect on spark behavior was observed (ncells=7; Npigs=3; Figure 4A-c).

To test whether cyto-D affected the close configuration of RyR and CaMKII, we examined colocalization after pretreatment with cyto-D and observed a significant reduction (Figure 4B).

Membrane-Bound Reactive Oxygen Species Production Contributes to Coupled RyR Modulation

Because both RyRs\(^{24}\) and CaMKII\(^{25}\) are known to be activated by reactive oxygen species (ROS), we investigated whether local ROS may be involved in modulation of coupled RyRs. Prosser et al\(^{26}\) recently described an increased spark frequency in response to stretch that was because of ROS produced by membrane-bound NADPH oxidase 2 (NOX2), and increasing the frequency of stimulation has also been shown to globally increase ROS production in isolated myocytes.\(^{27}\) We, therefore, investigated whether a membrane-bound source of ROS could be implicated in a differential response of coupled and noncoupled RyRs, possibly through oxidation of CaMKII. As illustrated in Figure 5, inhibition of NOX2 with the specific inhibitor diphenyleneiodonium also reduced the frequency response in coupled RyRs (ncells=11; Npigs=4; Figure 5B); apocynin had similar effects (ncells=7; Npigs=3; Figure 5C), as did the specific peptide gp91-ds-tat but not the scrambled peptide (Online Figure IA). Inhibition of mitochondrial ROS production by rotenone had no effect (Online Figure IB). Global ROS scavenging with N-acetyl cysteine also reduced the increased spark frequency in coupled RyRs and not in noncoupled RyRs (ncells=10; Npigs=3; Figure 5D). None of these interventions significantly affected the increase of SR Ca\(^{2+}\) content at 2 Hz stimulation. These observations imply that local ROS production, probably by NOX2, is involved in the modulation of coupled RyRs during increased frequency of stimulation.

Modulation of Coupled RyRs During Whole-Cell LTCC-Triggered Ca\(^{2+}\) Release

The amplitude of LTCC-triggered global Ca\(^{2+}\) transient at 2 Hz is actually not larger than the amplitude at 0.5 Hz (Online Figure IIIA). This reflects the sum of opposite changes during an increase in frequency. At 2 Hz, release occurs on a background of elevated diastolic Ca\(^{2+}\). On one hand, the SR Ca\(^{2+}\) content is increased (Online Figure II) but, on the other hand, the Ca\(^{2+}\) influx through LTCC is reduced.\(^{28-30}\) We confirmed that this was also the case in pig myocytes (Online Figure IIIB). As previously published, a 1-second pause allowed substantial recovery of the LTCC (Online Figure IIIC). Ca\(^{2+}\) transients measured after this 1-second recovery period were significantly larger and the overall rate of upstroke was larger than at 0.5 Hz (Online Figure IIID). Inhibition

Figure 3. Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) is equally associated to coupled and noncoupled ryanodine receptors (RyRs). A, Example of a confocal image of a pig ventricular myocyte stained for RyR (blue), Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX; red), and CaMKII (green) that shows the distribution of CaMKII with coupled and noncoupled RyRs. B, A specific colocalization method is used in which coupled (red) and noncoupled RyRs (blue) are marked to colocalize with CaMKII (left). Level of colocalization is shown using the Manders coefficient (ncells=20; Npigs=3; right).
of CaMKII had, however, no significantly different effect on the global Ca²⁺ transient amplitude or kinetics (Online Figure IID).

Nevertheless, regional analysis confirmed the modulation of coupled RyRs. For each cell, a coupled and a noncoupled region were selected and local Ca²⁺ transients analyzed (Figure 6A). The rate of upstroke increased significantly at 2 Hz in the coupled region only, and this increase was inhibited by AIP (Figure 6B). The higher rate of upstroke is consistent with a selective CaMKII-dependent increase in open probability of coupled RyRs, given that the trigger LTCC is actually reduced (Online Figure III).

Discussion

Through the use of a novel experimental method, we have shown that only coupled RyRs respond to activation of CaMKII during stimulation at elevated frequency with an increased open probability. This response requires an intact cytoskeleton. The CaMKII-dependent modulation of coupled RyRs is abolished by NOX2 inhibition, indicating the presence of a local, interdependent Ca²⁺/ROS microdomain in the dyadic cleft.

3-D Mapping of Coupled and Noncoupled RyRs in Living Cardiac Myocytes

In earlier studies, we and others have correlated areas of delayed release during a stimulated Ca²⁺ transient with areas of rarefaction of TT.¹⁴,³¹,³² That analysis also showed early release sites that were not matched to TT in the plane of analysis but were assumed to result from TT in another plane. In the current study, we have developed a novel method to correlate release events with the distance to TT and sarcolemma with the aim to distinguish more accurately between coupled and noncoupled RyRs. The method uses distance calculations, analogous to the method used to characterize distance to sarcolemma in atrial cells,²² but matches RyR release properties to the distance map.

This novel method allows more confidence in the use of Tₚ₅₀ of Ca²⁺ release as a surrogate parameter for the distance to the sarcolemmal and T-tubular membranes and allows investigating whether regulation of coupled RyRs is distinct from that of noncoupled RyRs.
The proportion of coupled release sites derived from functional analysis coupled to distance mapping is in the same order as found using RyR immunostaining. Small discrepancies likely reflect the differences in methodology. The immunostaining data are also in the same order for both coupled and noncoupled RyRs as recently reported for dog ventricular myocytes, indicating that there is a large degree of homology between these large animal models.

**Differential Modulation of Coupled and Noncoupled RyRs**

In atrial cells that lack TTs, a higher probability of Ca\(^{2+}\) sparks in sarcolemmal coupled versus central noncoupled RyRs has previously been described. In cat atrial cells, sarcolemmal Ca\(^{2+}\) sparks originating from junctional release sites are 4 times more frequent than sparks originating from nonjunctional release sites. Also in rat atrial cells, peripheral Ca\(^{2+}\) sparks had 5 times higher frequency than central-occurring Ca\(^{2+}\) sparks. In rat, Ca\(^{2+}\) channel block did not affect spark frequency in subsarcolemmal sites, whereas in the cat it reduced spark occurrence.

The current experiments explore the role of dyadic microdomain in modulating junctional spark properties in ventricular myocytes with a more complex network of coupled and noncoupled RyRs throughout the cells. The significant increase of sparks from coupled RyRs during high-frequency stimulation is fully inhibited by AIP and KN-93, whereas noncoupled RyRs are not affected. This indicates that the dyadic
Ca2+ monitoring should address this further. Either (Online Figure IIC), but future studies using intra-SR content. Earlier studies in rat and rabbit myocytes suggest that those evoked by sparks dissipate rapidly.37,38 We did not ob-
in nonstimulated cells there are no measurable gradients and
in coupled and noncoupled sites is local differences in SR Ca2+
In pig ventricular myocytes, we previously showed that Ca2+ channel block did not affect spark frequency,10 but we cannot exclude a role for the presence of LTCC in defining the microdomain for modulation.
Another potential mechanism for different spark frequencies in coupled and noncoupled sites is local differences in SR Ca2+
modeling.19 This will then phosphorylate RyRs to a greater ex-
with high-frequency stimulation in pig myocytes, selective modulation of coupled RyRs is dependent on CaMKII because AIP and KN-93 were able to inhibit the pacing-induced response fully. This response occurs and is reversible within minutes, consistent with earlier reported time course of CaMKII activation.16 A specific modulation of events near TT could result from the preferred location of membrane-associated signaling cascades. Nikolaev et al39 reported that β2-adrenergic receptors are only localized in TT in rat and mice myocytes, whereas β1-adrenergic receptors are located across the entire cell surface. However, we could not observe any such preferential localization of CaMKII using immunofluorescence techniques. More subtle differences may be beyond current detection. Further investigation may be possible with available fluorescence resonance energy transfer probes to detect localized CaMKII activation, a method that has already shown the time course at the whole cell and nuclear level, but not yet at higher spatial resolution.17

Mechanisms of Selective Activation of CaMKII in the Dyads
With high-frequency stimulation in pig myocytes, selective modulation of coupled RyRs is dependent on CaMKII because AIP and KN-93 were able to inhibit the pacing-induced response fully. This response occurs and is reversible within minutes, consistent with earlier reported time course of CaMKII activation.16 A specific modulation of events near TT could result from the preferred location of membrane-associated signaling cascades. Nikolaev et al39 reported that β2-adrenergic receptors are only localized in TT in rat and mice myocytes, whereas β1-adrenergic receptors are located across the entire cell surface. However, we could not observe any such preferential localization of CaMKII using immunofluorescence techniques. More subtle differences may be beyond current detection. Further investigation may be possible with available fluorescence resonance energy transfer probes to detect localized CaMKII activation, a method that has already shown the time course at the whole cell and nuclear level, but not yet at higher spatial resolution.17

The mechanisms that underlie CaMKII localization are still to be understood fully, yet the present evidence suggests that the microfilament/microtubule network is involved, because disruption of this system with cyto-D led to the total abrogation of the CaMKII-dependent activation of coupled RyRs. The role of this network has previously been demonstrated for the CaMKII regulation of LTCC.40 Also, more recent work suggested that an intact actin cytoskeleton was necessary for signaling microdo-
mains in other primary cells,41 and it has been shown that the actin-associated protein β-spectrin42 is a major requirement for proper targeting of a CaMKII/Na+ channel complex in cardiac cells. This evidence underscores the importance of the cytoskel-
one in the creation of subcellular signaling environments.

Our current working hypothesis is that CaMKII is activated during the repeated influx of Ca2+ during high frequency. The higher local Ca2+ is expected to bind to calmodulin and activate CaMKII, recreating the behavior predicted by computational modeling.18 This will then phosphorylate RyRs to a greater extent. The local Ca2+ transients with increased rate of upstream that are seen in coupled regions during depolarization and trig-
Figure 6. Ca2+/calmodulin-dependent kinase II inhibition reduces the rate of upstream of Ca2+ transient in coupled regions only. A. The protocol used to stimulate cells is shown. The rate of upstream of Ca2+ transient after 1 second of rest after stimulation is measured (left). Example of a confocal image with a coupled and noncoupled region is selected. The amplitude of Ca2+ transient is plotted for the selected coupled and noncoupled regions (right). B. Rate of upstream of the Ca2+ transient in coupled and noncoupled ryanodine receptors without (ncells=9; Npigs=3) and with autocamtide-2–related inhibitory peptide (AIP; ncells=7; Npigs=3).
physically organized into clusters, with adjacent channels interacting. However, many RyRs are in smaller clusters, which are more likely to have a higher open probability and more spontaneous Ca\(^{2+}\) release, and their activity may be more sensitive to phosphorylation. If RyRs near TTs would be in a less orderly array with fewer RyR interactions possible, this could contribute to the observed differences. This possibility will be further explored using high-resolution imaging. Lastly, it is possible that the modulation of RyRs is indirect through a CaMKII-dependent modulation of LTCC.

Although the study has highlighted a requirement for CaMKII in the modulation of coupled RyRs, it is possible that this is through indirect processes activating CaMKII, rather than Ca\(^{2+}\)-calmodulin. Membrane-bound ROS production by NOX2 was implicated in the modulation of RyRs during stretch in rat myocytes, where most RyRs reside near TT. In the present study, inhibition of NOX2, or scavenging of ROS, also inhibited the response to increased frequency stimulation, indicating a dual dependence for activation of CaMKII and NOX2. However, the present data do not allow putting one upstream of the other. Activation of CaMKII by ROS is well known, but activation of NOX2 by CaMKII is not to be excluded. To examine this interdependence will require more sophisticated tools that allow measuring local near-membrane ROS production and local CaMKII activation directly.

### Limitations

The match between immunostaining and functional data in terms of fractions of coupled RyRs suggests that we are sampling from 2 well-separated RyR populations. However, it is not possible to rule out that the sparks we assign to noncoupled RyRs contain some RyRs that are less distant than 2 \(\mu\)m but have a longer latency. The resolution of the measurement of Ca\(^{2+}\) is limited by the optical blurring of the microscope, but if anything the differences reported here would be more pronounced if higher-resolution methods could resolve them.

Pharmacological tools always carry a risk of nonspecific effects, though combining independent data of different agents, as we did for CaMKII inhibition and NOX2 inhibition, strengthens the data. CytoD has been reported to interfere with multiple signaling pathways, though not with baseline Ca\(^{2+}\) handling, and effects of CytoD are mostly assigned to its action on the cytoskeleton. We currently do not have complementary tools for studying the role of the cytoskeleton, because, for example, colchicine reportedly affects baseline Ca\(^{2+}\) transport. Therefore, the interpretation of the CytoD data must be made cautiously.

### Perspectives and Conclusions

This study represents the first measurement of localized CaMKII activation in ventricular myocytes. The findings underscore the subcellular nature of the regulation of CaMKII targets and may explain some of the current discrepancies in the effects of CaMKII. The limited impact on the whole-cell Ca\(^{2+}\) transient kinetics reflects the interaction of multiple Ca\(^{2+}\) transport mechanisms and is consistent with other studies reporting little effect of CaMKII inhibition on global Ca\(^{2+}\) kinetics. Yet, the present analysis indicates that locally Ca\(^{2+}\) is modulated and points to a role for CaMKII in local signaling rather than on global Ca\(^{2+}\) handling.

The upregulation of CaMKII is a common feature in human hearts with heart failure and atrial fibrillation. The present study presents evidence for inhomogenous RyR and CaMKII activation. The consequences for such activation gradients within the SR may lead to eager Ca\(^{2+}\) release sites where release is more active. We performed a first evaluation whether the local CaMKII modulation was affected in disease, examining myocytes from the chronic ischemic area adjacent to a myocardial infarction. The data illustrated in Figure 7 indicate that CaMKII-dependent modulation of coupled RyRs is lost during remodeling after myocardial infarction. Further studies are ongoing to explore the underlying mechanisms such as altered colocalization or altered ROS production.

![Figure 7. Loss of Ca\(^{2+}\)-calmodulin-dependent kinase II–dependent regulation of coupled ryanodine receptors (RyRs) in post-myocardial infarction (MI) remodeling. A, Frequency-dependent modulation of coupled RyRs after MI (n\(_{\text{cells}}=11; N_{\text{pigs}}=6). B, Effect of CaMKII inhibition with autocamtide-2–related inhibitory peptide (AIP; n\(_{\text{cells}}=8; N_{\text{pigs}}=4) on spark frequency in coupled and noncoupled RyRs.](http://circres.ahajournals.org/)

A Loss of frequency-dependent modulation of coupled RyR in post-MI remodeling

B CaMKII inhibition reduces the response in non-coupled RyR

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In conclusion, coupled RyRs have a distinct modulation by CaMKII and ROS, which is dependent on an intact cytoskeleton. The data are consistent with the presence of a Ca2+/ROS microdomain in the dyadic cleft that may be involved in local signaling. This microdomain-restricted modulation of RyRs is subject to modification with disease.

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Disclosures
None.

References

**Novelty and Significance**

**What Is Known?**

- Ventricular myocytes from large mammals such as pig, dog, sheep, and human have a sarcomemal T-tubular network that is more pronounced than in rat and mice.
- A significant fraction of the Ca2+ release channels of the sarcoplasmic reticulum, namely, the ryanodine receptors (RYRs) at the sarcomemal Z-lines, are not part of the dyad formed by T-tubules (TT) and sarcoplasmic reticulum (noncoupled RYRs).
- Dyadic cleft is thought to form a microdomain for local signaling, but direct evidence is scarce.

**What New Information Does This Article Contribute?**

- In heart muscle cells, the first step of excitation–contraction coupling is activation of channels that are responsible for release of Ca2+ from the intracellular Ca2+ store, the RYRs. Typically, RYRs are thought to be located at the dyad, where invaginations of the sarcolemma, the TT, are in close vicinity to L-type Ca2+ channels in the sarcolemma. However, in ventricular myocytes of larger mammals, including humans, many RYRs are not adjacent to TT, that is, they are noncoupled. The present study shows that only coupled RYRs are induced to have a higher probability of opening when the cells are paced at high frequency, as during an increase in heart rate. This is not observed in noncoupled RYRs, which are not coupled and involves several local signaling molecules, such as Ca2+/calmodulin kinase II and reactive oxygen radicals. This is the first direct observation of confined microdomains of RyR modulation in cardiac myocytes. In disease, such as the remodeling of the heart after myocardial infarction, this microdomain modulation is lost and may contribute to the increased sensitivity to arrhythmias.
Selective Modulation of Coupled Ryanodine Receptors During Microdomain Activation of Calcium/Calmodulin-Dependent Kinase II in the Dyadic Cleft
Eef Dries, Virginie Bito, Ilse Lenaerts, Gudrun Antoons, Karin R. Sipido and Niall Macquaide

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In the *Circulation Research* article by Dries et al (Selective modulation of coupled ryanodine receptors during microdomain activation of calcium/calmodulin-dependent kinase II in the dyadic cleft. *Circ Res*. 2013;113:1242–1252. DOI: 10.1161/CIRCRESAHA.113.301896), Figure 3 did not display properly. The correct display of Figure 3 is below.

The error has been corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/113/11/1242.full.
Supplemental Material

Dries et al. Selective modulation of coupled ryanodine receptors during microdomain activation of CaMKII in the dyadic cleft.

Methods

Animal care
Animals were housed and treated according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health, U.S.A.) and experimental protocols were approved by the in-house ethical committee (Ethische Commissie Dierproeven, KU Leuven), with permit numbers P02042, P06048 and P10139.

Cell isolation
Left ventricular cardiomyocytes were enzymatically isolated from control pigs (40-45 kg) as described before. Animals were killed under full anesthesia with an overdose of pentobarbital, after which the heart was quickly excised; a large ventricular wedge with the perfusing artery was prepared. The coronary artery was cannulated and perfused with a constant flow (4 ml/min) at 37 °C. The tissue was briefly perfused with normal Tyrode to washout remaining blood (in mmol/L: NaCl 137, KCl 5.4, MgCl2 0.5, CaCl2 1.8, Na-HEPES 11.8, and glucose 10; pH 7.4). Then the tissue was perfused with Ca²⁺-free Tyrode, followed by the enzymatic solution (collagenase A-Roche, Switzerland and protease XIV) and after digestion, perfusion with low Ca²⁺ Tyrode (in mmol/L: NaCl 130, KCl 5.4, KH2PO4 1.2, MgSO4 1.2, CaCl2 0.18, Na-Hepes 6, glucose 20, pH 7.2). The digested tissue was minced, the suspension was filtered and the isolated myocytes were resuspended in normal Tyrode. After isolation, the cells were allowed to recover for 1 h before experiments.

Electrophysiological recordings
Cells were stimulated in the whole-cell voltage-clamp configuration (Axon 200B amplifier, Axon Instruments). Cells were constantly perfused with normal Tyrode at 37 °C and patch pipettes (2-3 MΩ) (GB 200-8P, Science Products) were filled with (in mmol/L): K-aspartate 120, NaCl 10, KCl 20, K-HEPES 10, MgATP 5, and K5Fluo-4 0.05; pH 7.2. [Ca²⁺]i transients were elicited by depolarizing steps (200 ms) from -70 mV to +10 mV at two different frequencies, 0.5 Hz and 2 Hz for a period of 2 minutes. AIP (autocamtide-2 related inhibitory peptide, Tocris Bioscience, Bristol, UK), KN93 (Sigma-Aldrich, Bornem, Belgium) and KN-92 (Calbiochem, Millipore) were used at 10 µmol/L incubation for 1 h and in the patch pipette. Cytochalasin D (Tocris Bioscience) was used at 10 µmol/L
and cells were incubated for 1 h. Diphenyleneiodonium (DPI) was used at 3 µmol/L with incubation for 30 minutes, and DPI was also included in the patch pipette. N-acetylcysteine (NAC, 10 mM, Sigma-Aldrich) and apocynin (100 µM, Acros Organics) were used for 1h incubation and included in the patch pipette. Gp91 ds-tat and gp91 scrambled peptides (Eurogentec) were used at 10 µmol/L and cells were incubated for 1h. Rotenone (Sigma) was used at 10 µmol/L and cells were incubated for 30 minutes. Sarcoplasmic reticulum (SR) Ca²⁺ content was measured by integrating the inward Na⁺/Ca²⁺ exchanger (NCX) current during fast caffeine application (10 mmol/L).

Confocal microscopy
To study the spatial distribution of [Ca²⁺], and SR Ca²⁺ release events, confocal line scan images were recorded using a Zeiss LSM 510 confocal system. [Ca²⁺], transients and spontaneous release events were visualized in longitudinal line scan imaging. Line scans were recorded at 650 Hz with a pixel size of 0.2-0.3 µm. To study SL/TT membrane geometry, membranes were fluorescently labeled with wheat-germ agglutinin-Alexa594 (WGA; 10 µg/ml) for 30 min and vertical Z stacks were recorded in the presence of cytochalasin D (cyto-D) (10 µmol/L) to inhibit contraction and allow correlation with a line scan recording. Vertical images were recorded with a spacing corresponding to the pixel size of the line scans in the same cell.

Image analysis
To analyze the spatial distribution of [Ca²⁺], 5 consecutive [Ca²⁺], transient line scan images were averaged and fluorescence was normalized to the diastolic [Ca²⁺] level (F/F₀). From the averaged transient, the time to reach half-maximal [Ca²⁺] (T_F50) is calculated for each pixel along the line scan to establish the spatial distribution of the timing of the Ca²⁺ release. To assess 3-D geometry of sarcolemmal (SL) and TT membranes, vertical images stacks were further processed. To reduce noise detection, during analysis all images of the stack were convolved using a Gaussian blur 0.2 microns wide. TTs were localized using the Otsu thresholding method and skeletonized, using ImageJ. In an approach similar to that described previously a 3-D form of the Euclidean distance transformation (ImageJ plugin: http://www.optinav.com/Local_Thickness.htm) was used to calculate the distance to the nearest membrane in the cell. A profile of distance to membrane in a single line was extracted and subsequent Ca²⁺ measurements were taken from this line to correlate with Ca²⁺ release. Where this method was correlated with Ca²⁺ release, TT images were taken after cessation of stimulation. After the 2 minute train of stimulation, spontaneous Ca²⁺ sparks were routinely observed. Spark detection was carried out using an automated spark analysis program which utilizes the Cheng algorithm called MacSpark (written by N. Macquaide). The cri was set to 4.5, preventing detection of false spark events. A per-pixel spark frequency was calculated and normalised to give a measure in
sparks. $100\mu\text{m.s}^{-1}$. The mean of these in the coupled and non-coupled release sites could then be used to define spark frequencies in each type of RyR. When analyzing spark frequency in different sites, the frequency is normalized to the length of the line with such sites.

The distance to membrane of RyR in immunostaining (Fig. 1C) used the same algorithm.

The degree of colocalization of RyR and CaMKII in the immunostaining experiments was quantified using the Manders’ coefficient. Where colocalization was studied for coupled and for non-coupled RyRs, a mask of the skeletonized NCX signal was used $<0.5$ or $>2$ µm away respectively. This was calculated using the Euclidean method outlined for regional spark localisation.

**Immunofluorescent staining**

Freshly isolated myocytes were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Cells were washed 3 times and incubated with blocking buffer (10% goat serum and 1% BSA in PBS) for 1h on room temperature. Primary antibodies were incubated separately overnight at 4°C (mouse IgG anti-RyR, 1/200, Affinity BioReagents, Golden, CO, USA; rabbit IgG anti-CaMKII 1/500, provided by D. Bers lab; mouse IgG1 anti-NCX 1/500, Affinity BioReagents, Golden, CO, USA). Cells were washed 3 times and incubated with secondary antibodies (for RyR: goat anti-mouse Alexa 647, 1/200; for CaMKII: goat anti-rabbit IgG Alexa 488, 1/200; for NCX: goat anti-mouse IgM Alexa 568, 1/200, Invitrogen Molecular Probes) for 1.5h at room temperature. Cells were then washed 3 times before imaging.

**Pig model of chronic ischemia and myocardial infarction**

The procedure to induce chronic ischemia and myocardial infarction (MI) in a pig model is similar to the one described before. Briefly, a copper-coated stent was implanted in the left anterior descending (LAD) artery of young adult domestic pigs (20-25 kg) to induce intima proliferation and a high-grade stenosis. After 6 weeks, the global left ventricle function and the extent of myocardial infarction (MI) were measured with magnetic resonance imaging, showing a moderate MI (approximately 10% of the LV mass). Afterwards, pigs were sacrificed and myocytes were isolated from the ischemic area adjacent to the MI and subjected to the similar protocols as in the control pigs.
Supplemental Data

**NOX inhibition by gp91ds-tat**
As illustrated in Online Figure IA, the peptide prevented the frequency-dependent increase in sparks in coupled receptors while the scrambled peptide had no effect. This intervention did not affect the increase in SR Ca\(^{2+}\) content with frequency (data not shown).

**Inhibition of mitochondrial ROS production by rotenone**
As illustrated in Supplemental Figure S1B, rotenone had no effect on the frequency-dependent increase in sparks in coupled receptors. This intervention did not affect the increase in SR Ca\(^{2+}\) content with frequency (data not shown).

**Sarcoplasmic reticulum content at different frequencies**
SR Ca\(^{2+}\) content was measured during rapid application of caffeine (Online Figure IIA) and quantified as the integral of the Na/Ca exchange current. As shown in panel B, SR Ca\(^{2+}\) content was larger at 2 Hz, independent of the sequence in which the conditioning was applied.

When examining the amplitude of the local caffeine-induced Ca\(^{2+}\) transients, there was no difference for coupled or non-coupled sites, as shown in panel C.

**Global Ca\(^{2+}\) transients at different frequencies and effect of CaMKII**
We further analyzed the Ca\(^{2+}\) transient during voltage clamp and L-type Ca\(^{2+}\) triggered SR Ca\(^{2+}\) release. The amplitude of the global Ca\(^{2+}\) transient during 2 Hz stimulation is smaller than the amplitude at 0.5 Hz when measured as the difference between the peak and the inter-beat, elevated, diastolic Ca\(^{2+}\) (ΔF\(_S\)). When the full amplitude, peak value (F\(_{peak}\)) is considered there is no difference (Online Figure IIIA). The lack of a clear increase in the overall Ca\(^{2+}\) transient amplitude reflects the sum of many different changes during an increase in frequency. The triggered release at 2 Hz occurs on a background of elevated diastolic Ca\(^{2+}\) due to incomplete relaxation of the Ca\(^{2+}\) transient. Although the SR Ca\(^{2+}\) content is up (Online Figure II), the triggering Ca\(^{2+}\) influx through LTCC is reduced and this will contribute to reduced SR Ca\(^{2+}\) release. This was previously shown for human myocytes\(^6\), and for mouse\(^7\) and rat myocytes\(^8\). As this could also explain our observation in pig myocytes, we conducted further experiments that confirm that similar mechanisms operate in pig myocytes.

Online Figure IIIB illustrates the reduction of the LTCC at 2 Hz. Imposing a 1 s pause after the 2 Hz train allows substantial recovery of the LTCC as shown in Online Figure IIIC. Therefore, we further studied the Ca\(^{2+}\) transient after preconditioning at 0.5 and 2 Hz but with a 1 s pause to allow decline of
diastolic Ca\(^{2+}\) and partial recovery of the LTCC. Ca\(^{2+}\) transients after 2 Hz preconditioning measured after this 1 s recovery period are significantly larger and the overall rate of upstroke is larger at 2 Hz than at 0.5 Hz (Online Figure IIID). We examined the effect of CaMKII inhibition with AIP (n\(_{\text{cells}}\)=7) but could not detect significant changes in the global parameters of Ca\(^{2+}\) transient amplitude or upstroke.

**Figure Legends**

Online Figure I. NOX2 inhibition by gp91 ds stat, but not mitochondrial ROS inhibition, reduces spark frequency in coupled RyR

(A) The effect of NOX2 inhibition with gp91 ds-tat peptide (n\(_{\text{cells}}\)=11; N\(_{\text{pigs}}\)=4) and gp91 scrambled peptide (n\(_{\text{cells}}\)=11; N\(_{\text{pigs}}\)=4) on frequency-dependent modulation of Ca\(^{2+}\) sparks, with further analysis for coupled and non-coupled release sites at 2 Hz stimulation in control cells and treated cells (n\(_{\text{cells}}\)=13; N\(_{\text{pigs}}\)=4). (B) The effect of mitochondrial ROS inhibition with rotenone (n\(_{\text{cells}}\)=10; N\(_{\text{pigs}}\)=3) on Ca\(^{2+}\) spark frequency is shown in coupled and non-coupled release sites at 2 Hz stimulation (n\(_{\text{cells}}\)=11; N\(_{\text{pigs}}\)=3).

Online Figure II. SR Ca\(^{2+}\) content and local caffeine-induced SR Ca\(^{2+}\) release.

(A) An example of a confocal line scan image, integrated Ca\(^{2+}\) transients and the corresponding current trace is shown for a cell at 2 Hz stimulation. (B) Left, the mean values of the integrated NCX current show a significant increase in SR Ca\(^{2+}\) content with increasing frequency-stimulation (n\(_{\text{cells}}\)=11; N\(_{\text{pigs}}\)=5). Right, the higher SR Ca\(^{2+}\) content after 2 Hz is reduced again when the frequency-stimulation is reversed (n\(_{\text{cells}}\)=11; N\(_{\text{pigs}}\)=6). (C) Coupled and non-coupled sites were identified during the preceding Ca\(^{2+}\) transients as for the sparks analysis. The right panel shows the mean values of the local amplitudes of the caffeine-induced Ca\(^{2+}\) transients at coupled and non-coupled sites (n\(_{\text{cells}}\)=9; N\(_{\text{pigs}}\)=7).

Online Figure III. Ca\(^{2+}\) transients and L-type Ca\(^{2+}\) current analysis during stimulation at different frequencies.

(A) Examples of confocal line scan images and plots are shown for 0.5 and 2 Hz stimulation. Ca\(^{2+}\) transients during stimulation are analyzed by measuring the ∆F from the peak of the Ca\(^{2+}\) transient to the inter-beat diastolic value (∆F\(_{\text{S}}\)). Peak values of the Ca\(^{2+}\) transient are analyzed by measuring the ∆F from the peak of the Ca\(^{2+}\) transient to the diastolic value after full relaxation (F\(_{\text{peak}}\)). The right panel shows mean values (n\(_{\text{cells}}\)=22, N\(_{\text{pigs}}\)=5). (B). I\(_{\text{CaL}}\) at different frequencies. Left, the protocol used to
measure the I_{CaL} amplitude is shown. Cells are stimulated for 2 min from -60 mV to +10 mV at 0.5 and 2 Hz in presence of lidocaine, TTX and cesium. The amplitude of the last I_{CaL} is measured.

Middle, examples of I_{CaL} at 0.5 and 2 Hz stimulation are shown with mean data on the right (n_{cells}=6; N_{pigs}=3). (C) The amplitude of the last I_{CaL} of the train and the I_{CaL} after 1s of recovery are compared; an example and mean values are shown (n_{cells}=5; N_{pigs}=3) (D). Ca^{2+} transients after preconditioning at 0.5 and 2 Hz but with a 1 s pause to allow decline of diastolic Ca^{2+} and partial recovery of the LTCC. The Ca^{2+} transients after 2 Hz conditioning have larger amplitude than after 0.5 Hz conditioning (n_{cells}=8; N_{pigs}=3). There is trend to reduction with CaMKII but this is not significant (n_{cells}=7, N_{pigs}=3).

References
Online Figure I

A  NOX2 inhibition with gp91 ds-tat peptide

<table>
<thead>
<tr>
<th>Coupled RyR</th>
<th>Non-coupled RyR</th>
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<tr>
<td>Spark frequency (Sparks / 100 µm / s)</td>
<td>Spark frequency (Sparks / 100 µm / s)</td>
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<tr>
<td>0.5 Hz</td>
<td>0.5 Hz</td>
</tr>
<tr>
<td>2 Hz</td>
<td>2 Hz</td>
</tr>
<tr>
<td>Control</td>
<td>gp91 SCRAMBLED</td>
</tr>
<tr>
<td>gp91 ds-tat</td>
<td>gp91 SCRAMBLED</td>
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B  Mitochondrial ROS inhibition with rotenone

<table>
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<th>Non-coupled RyR at 2 Hz stimulation</th>
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<tbody>
<tr>
<td>Spark frequency (Sparks / 100 µm / s)</td>
<td>Spark frequency (Sparks / 100 µm / s)</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Rotenone</td>
</tr>
</tbody>
</table>
Online Figure II

A

Integrated NCX current

B

Local caffeine-induced transients

C

(NCa2+ content)  (NCa2+ content)

(F/F0)  (pC/pF)

0.5 Hz  2 Hz  2 Hz  0.5 Hz

(Coupled)  (Non-coupled)

(F/F0)  0.5 Hz  2 Hz
Online Figure III

**A**
- 0.5 Hz and 2 Hz stimulation
- Ca transient amplitude during stimulation
- Peak value of the Ca transient

**B**
- 5 Hz and 2 Hz stimulation
- Ca^{2+} current amplitude

**C**
- 5 Hz and 2 Hz stimulation
- Ca^{2+} current amplitude after 1s

**D**
- Ca transient amplitude after 1s pause

- Ca transient amplitude
- Peak value of the Ca transient

Mathematical expressions:
- $\Delta F_S = F_{peak}$
- Ca transient amplitude during stimulation
- Peak value of the Ca transient