Synchronization of Intracellular Ca\textsuperscript{2+} Release in Multicellular Cardiac Preparations

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In myocardial tissue, Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) that occurs via the ryanodine receptor (RyR2) channel complex. Ca\textsuperscript{2+} release through RyR2 can be either stimulated by an action potential (AP) or spontaneous. The latter is often associated with triggered afterdepolarizations, which in turn may lead to sustained arrhythmias. It is believed that some synchronization mechanism exists for afterdepolarizations and APs in neighboring myocytes, possibly a similarly timed recovery of RyR2 from refractoriness, which enables RyR2s to reach the threshold for spontaneous Ca\textsuperscript{2+} release simultaneously. To investigate this synchronization mechanism in absence of genetic factors that predispose arrhythmia, we examined the generation of triggered activity in multicellular cardiac preparations. In myocardial trabeculae from the rat, we demonstrated that in the presence of both isoproterenol and caffeine, neighboring myocytes within the cardiac trabeculae were able to synchronize their diastolic spontaneous SR Ca\textsuperscript{2+} release. Using confocal Ca\textsuperscript{2+} imaging, we could visualize Ca\textsuperscript{2+} waves in the multicellular preparation, while these waves were not always present in every myocyte within the trabeculae, we observed that, over time, the Ca\textsuperscript{2+} waves can synchronize in multiple myocytes. This synchronized activity was sufficiently strong that it could trigger a synchronized, propagated contraction in the whole trabecula encompassing even previously quiescent myocytes. The detection of Ca\textsuperscript{2+} dynamics in individual myocytes in their \textit{in situ} setting at the multicellular level exposed a synchronization mechanism that could induce local triggered activity in the heart in the absence of global Ca\textsuperscript{2+} dysregulation.

\textbf{Keywords:} trabeculae, calcium, muscle, transient, EC coupling, rat

\section*{INTRODUCTION}

The sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release complex comprises more than a dozen proteins, with the ryanodine receptor (RyR2) as the core channel. The RyR2 has numerous binding sites for regulatory molecules and accessory proteins with the capability to modulate RyR2 function. Ca\textsuperscript{2+} release from the SR is a key determinant of cardiac contractility and both the cytoplasmic as well as the luminal Ca\textsuperscript{2+} concentration can regulate the RyR2. In the SR lumen, in particular, there is a substantial amount of Ca\textsuperscript{2+} buffering, and, according to several studies, approximately 50–90% of the total Ca\textsuperscript{2+} is bound to SR Ca\textsuperscript{2+} binding proteins, such as calsequestrin (CASQ2).
However, in diastole, it can cause an inadvertent SR Ca$^{2+}$ release (Gyorke and Terentyev, 2008). The probability can result in an increased Ca$^{2+}$ spontaneous Ca$^{2+}$ potential (Venetucci et al., 2006; Gyorke and Carnes, 2008). (EADs), and they are initiated by variations in the membrane afterdepolarizations (DADs) and early afterdepolarizations triggered arrhythmias.

The exact mechanism responsible for Ca$^{2+}$ release termination remains to be elucidated, a significant amount of evidence suggests that changes in luminal Ca$^{2+}$ play a key role in the termination process (Gyorke and Terentyev, 2008). RyR2 modulation by luminal Ca$^{2+}$ has been shown to involve several key proteins associated with the RyR2 in the lumen of the SR, most importantly CASQ2. CASQ2 not only acts as a Ca$^{2+}$ buffer, but it also mediates the responsiveness of the RyR2 channel to release that leaves the RyR2 hyperactive (Gyorke and Carnes, 2008). Both of these scenarios can lead to triggered arrhythmias.

Trabeculae Isolation
Male, Brown Norway rats (approximately 200–300 g) were anesthetized using an intraperitoneal injection of eutanasol (392 µg pentobarbital and 40 µg phentoyin). Heparin (1000 U) was injected at the apex of the left ventricle after the chest had been opened by bilateral thoracotomy. The heart was quickly removed and placed in a Krebs–Henseleit (KH) buffer containing (in mM) 137 NaCl, 5 KCl, 1.2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 20 NaHCO$_3$, 0.25 CaCl$_2$, and 10 Glucose. 2,3-Butanedione monoxime (BDM, 20 mM) was added to the KH buffer to minimize the cutting injury (Mulieri et al., 1989), as exposure to BDM for a short period of time has been shown to be reversible (Zimmermann et al., 1996; Janssen et al., 2002). Hearts were cannulated via the ascending aorta and perfused using KH buffer with the addition of BDM. This buffer was kept in equilibrium with 95% O$_2$ and 5% CO$_2$ and constant pH of 7.4. The right ventricle was opened, and thin, uniform, non-branched trabeculae were extracted and mounted as previously described (Slabaugh et al., 2012). The muscle was bathed in fresh, oxygenated KH solution without BDM and containing 1.5 mM Ca$^{2+}$, and stimulated at a frequency of 1 Hz at room temperature until contractile parameters had stabilized.

Force Measurements
The trabeculae were stretched to a length where further stretching raised diastolic and systolic force proportionally (Monasky et al., 2010). It has been shown that this muscle length corresponds with a sarcomere length of around 2.2–2.3 µm (Allen and Kentish,
Intracellular Ca\(^{2+}\) Measurements
Isolated rat trabeculae were loaded with 40 \(\mu\)M Rhod-2 AM, which was dissolved in 2% pluronic (20% w/v) in 1 mL KH buffer with the addition of TPEN (4.3 mg/L) and cremophor (5 mg/L), in a bath for 45 min at room temperature (Brunello et al., 2013). The electrical stimulation was turned on at 1 Hz during the last 5 min of incubation. After this loading period, the muscle was slackened and perfused with a KH solution containing 5 mM BDM and 20 \(\mu\)M blebbistatin to stop cell contraction and allow for Ca\(^{2+}\) imaging (Kovacs et al., 2004). In order to optimize the resolution and minimize motion-induced artifacts, a modified glass capillary tube connected to a micromanipulator was utilized to gently push the trabecula against the coverslip. All data were collected using a 60\(\times\) water objective, and the line-scan images were acquired at the rate of 8 \(\mu\)s/pixel. The fluorescence emitted was expressed as \(\frac{F}{F_0} \left[ \frac{F - F_0}{F_0} \right] \), where \(F\) is the fluorescence at time \(t\) and \(F_0\) represents the background signal.

In separate experiments, we performed 2D scanning of the entire muscle at the same depth the Ca\(^{2+}\) transients are typically measured. In order to confirm that the line-scan represented five to seven myocytes in the center part of the preparation, trabeculae were loaded with di-4-ANEPPS to identify myocytes borders, as shown in Figure 1.

Data Analysis
Data were collected and analyzed using LabView, ImageJ, and Origin software. All data are presented as mean \(\pm\) SEM, unless otherwise stated. All experiments were performed using protocols approved by The Ohio State University Laboratory Animal Care and Use Committee.

RESULTS
Effect of Isoproterenol and Caffeine on Contraction
To investigate the effect of both isoproterenol and caffeine on the incidence of ESCs in trabeculae, we monitored the contractile activity in these muscles while electrically pacing at 1 Hz. As expected, the control, untreated muscle showed no signs of ESCs following relaxation (Figure 2A); however, after 1 min of perfusion with 100 nM isoproterenol and 0.5 mM caffeine the trabeculae displayed ESCs between successive twitches (Figure 2B). Upon application of both isoproterenol and caffeine, the developed force remained unchanged in the muscles as compared with control, most likely due to the diastolic Ca\(^{2+}\) release diminishing the SR Ca\(^{2+}\) store. Thus, ESCs in the trabeculae occurred in a highly synchronized manner leading to a spontaneous contraction with characteristics similar to those of the stimulated twitch in both amplitude and relaxation timing (Biesiadecki et al., 2014). Over time, the incidence of ESCs increased, reaching a maximum of three ESCs per cycle, with typically two ESCs occurring per cycle (Figure 2C). We found that not only does the frequency of ESCs increase with time but that the amplitude is altered as well.

Amplitude and Latency Period Distribution
We calculated both the ESC amplitude and the time from the stimulated contraction to the start of the ESC, or latency period from recordings from \(n = 16\) rats, 10-20 s of recordings per rat. During each diastolic period, we observed up to three ESCs, and these ESCs were divided into separate groups for analysis. Figure 3 illustrates the distribution of the amplitude of the ESCs as a percentage of the stimulated contraction’s amplitude. The
majority of the ESCs (62%) have an amplitude of less than 40% of the developed force of stimulated contractions. However, 24% of the ESCs showed an amplitude between 40 and 80%, while 14% of ESCs showed an amplitude 80% or higher. Notably, the majority of the ESCs with the highest amplitude occurred within the first ESC group. Although we were not able to simultaneously measure the membrane potential, we hypothesize that many of these high-amplitude ESCs would synchronize and then could result in extra-systolic APs triggered in the trabeculae. In a previously published study by our group (Brunello et al., 2013), performed in parallel to this study, we indeed showed that in a genetic model of disease, some of these ESC’s generated APs. Therefore, these results suggest that the addition of both isoproterenol and caffeine can lead to ESCs in rat myocardium. The ESCs displayed a high probability of occurrence around two or sometimes even three specific time points (Figure 4). The latency distribution shows a period of refractoriness after the stimulated twitch, when no ESC occurred, followed by the presence of the first ESC at 310 ± 4 ms (Figure 4A). The second ESC occurred at 578 ± 10 ms (Figure 4B) while the third ESC appeared at 702 ± 10 ms (Figure 4C). Therefore, these results show that the ESCs were highly temporally aligned, which is consistent with a synchronized recruitment of myocytes in the multicellular preparation. The number of waves, and amplitude, varied from muscle-to-muscle, likely due to the various sizes of cross-sectional areas of muscle used, and also increased with time of exposure.

Effect of Isoproterenol and Caffeine on [Ca\(^{2+}\)]_i

To further examine the synchronization of Ca\(^{2+}\) release, we loaded cardiac trabeculae with the Ca\(^{2+}\) indicator Rhod-2. Figure 5 shows a line-scan image of Ca\(^{2+}\) changes in a single trabecula. As expected, the electrical stimulation triggers SR Ca\(^{2+}\) release simultaneously across each of the myocytes in the field of view. Following the addition of isoproterenol and caffeine, the trabecula shows spontaneous Ca\(^{2+}\) waves that occur between the stimulated Ca\(^{2+}\) release. These Ca\(^{2+}\) waves have a lower amplitude than the induced Ca\(^{2+}\) transients and, while they occur in several of the cells, they do not occur simultaneously across each of the myocytes. However, after 5 min the Ca\(^{2+}\) waves synchronize as the Ca\(^{2+}\) release occurs much more uniformly in the myocytes. Figure 5 also shows the line-scans and corresponding Ca\(^{2+}\) fluorescence plot profiles for each of the individual myocytes in the field of view. The fluorescent plot profiles in the top panel demonstrate that each of the diastolic Ca\(^{2+}\) release events in the cells behaves differently, as the fluorescence signal from the Ca\(^{2+}\) waves peaks throughout the entire diastolic period. As the Ca\(^{2+}\) release becomes synchronized, the fluorescence peaks at the same time in each of the myocytes. Consistent with our force experiments, these results indicate that there is a synchronized diastolic Ca\(^{2+}\) release that occurs across each of the myocytes in the field of view (Figure 5). In Figure 6A, we show a typical example of
the time of onset of the largest ESC at 1 min, and after 5 min in six adjacent cells. This shows that over time, the adjacent cells seem to synchronize their ESC, i.e., onset of ESC grows closer and closer in all adjacent cells. Still, after 5 min, there is remains some little variation in time of onset (up to 32 ms in this example) in the adjacent cells, indicating synchronization that is not directly due to an AP. In Figure 6B, it can be seen that a non-externally stimulated AP causes the adjacent cells (four in this example), to all start the non-externally stimulated event at identical timepoint (0–1 ms variation among the four cells).

We next investigated the myocytes response to a lower dose of caffeine (Figure 7). At 0.2 mM caffeine, the distribution of the latency of the first Ca$^{2+}$ wave was shifted slightly rightward, compared to the distribution at 0.5 mM caffeine. The distribution of all waves had a similar behavior. In addition, the number of waves per cycle decreased significantly at the 0.2 mM caffeine concentration.

**Role of Systolic Ca$^{2+}$ Release in Synchronization**

To investigate the role of systolic Ca$^{2+}$ release in the synchronization of Ca$^{2+}$ waves in a multicellular trabecula, we examined the effects of several stimulated pulses on the timing of diastolic Ca$^{2+}$ release. Figure 8A shows the last three stimulated Ca$^{2+}$ transients before the stimulation was
FIGURE 5 | (Left) Confocal line-scan images of a cardiac trabecula loaded with the Ca²⁺ dye Rhod-2 and paced at 1 Hz (stimulation indicated by the arrows). The black dotted lines represent myocyte borders, and they were manually drawn based on the deflection of the Ca²⁺ waves. The electrical stimulation triggers SR Ca²⁺ release simultaneously across each of the myocytes in the field of view (control). Following the addition of both 0.5 mM caffeine and 100 nM isoproterenol, the trabecula shows Ca²⁺ waves that occur between stimulated Ca²⁺ release. The Ca²⁺ waves occur only in four out of six cells. However, after 5 min, the Ca²⁺ waves synchronize as the Ca²⁺ release occurs simultaneously across all six myocytes. (Right) Confocal line-scan images with the corresponding Ca²⁺ fluorescence plot profiles for each of the six myocytes in the field of view. Top: the Ca²⁺ release is not simultaneous across the myocytes, as the fluorescence peaks occur at different time points and not in all cells. Lower: the fluorescence peaks at the same time in each one of the six cells in the field of view, showing synchronization.

FIGURE 6 | (A) Typical example of time of onset of largest ESC at 1 and 5 min after addition of isoproterenol and caffeine. Time of onset is highly variable in six adjacent myocytes after 1 min (spread of onset 100’s of ms), but becomes more synchronized at 5 min (spread of onset of ~30 ms). (B) A non-externally stimulated action potential causes the spread of onset to virtually disappear, i.e., the spread of onset is 0–1 ms between adjacent cells (four in this example) in a triggered event.
stopped (0.5 mM Caffeine). Immediately following the last stimulated Ca\(^{2+}\) transient, there was a non-externally triggered Ca\(^{2+}\) release event that occurred uniformly throughout the myocytes; however, over time, this Ca\(^{2+}\) release decreased in both amplitude and temporal homogeneity among myocytes until there were only sporadic and spontaneous Ca\(^{2+}\) waves. Conversely, on occasion, there was a synchronized SR Ca\(^{2+}\) release event long after the stimulation had been removed (Figure 8B). Before this synchronized release, the myocytes in this line-scan were completely quiescent, but the amplitude of this Ca\(^{2+}\) transient was as large as in stimulated ones. This indicated that the Ca\(^{2+}\)-transients in these myocytes were likely triggered by a propagating AP that originated elsewhere in this preparation.

**DISCUSSION**

The present study demonstrated that under conditions of Ca\(^{2+}\) dysregulation, i.e., in the presence of both isoproterenol and caffeine, diastolic SR Ca\(^{2+}\) release occurs with increasing synchronization among the myocytes in situ in isolated cardiac
FIGURE 8 | Role of systolic Ca\textsuperscript{2+} release in the synchronization of Ca\textsuperscript{2+} waves in the multicellular trabeculae (0.5 mM caffeine); effects of several pulses on the timing of the Ca\textsuperscript{2+} waves. (A) Last three stimulated Ca\textsuperscript{2+} transients before the stimulation stopped. A synchronized Ca\textsuperscript{2+} release event occurred immediately following the last stimulated Ca\textsuperscript{2+} release; however, over time the amplitude and temporal homogeneity of Ca\textsuperscript{2+} waves decreased. (B) A synchronized SR Ca\textsuperscript{2+} release occurred long after the last stimulated Ca\textsuperscript{2+} release in an area of the muscle that was previously completely quiescent, strongly suggesting of triggered activity elsewhere on the preparation that propagated via AP to this quiescent area. SC, stimulated contraction.

Under our experimental conditions of Ca\textsuperscript{2+} overload, that also cause human ventricular tissue to display ESCs (Elnakish et al., 2017), the relatively low dose of caffeine allows for increases in the open probability of the RyR2 without depleting the SR, and it decreases the threshold for SR Ca\textsuperscript{2+} release (Nieman and Eisner, 1985; Trafford et al., 2000). On the other hand, isoproterenol increases the SR Ca\textsuperscript{2+} content to a high enough level to reach the threshold for SR Ca\textsuperscript{2+} release (Venetucci et al., 2007). Therefore, when both a low concentration of caffeine and high concentrations of isoproterenol are present, diastolic Ca\textsuperscript{2+} waves occur in the majority of myocytes. This leaves less Ca\textsuperscript{2+} to be released during systole and effectively activate the myofilaments. The analysis of the amplitude of the force measurements and the Ca\textsuperscript{2+} imaging experiments support the hypothesis that intracellular diastolic SR Ca\textsuperscript{2+} release can become synchronized in a multicellular cardiac preparation to the extent that it can involve many or all adjacent myocytes and evoke a triggered AP. Thus, this synchronization mechanism can occur in the absence of genetic mutations as shown by our previous study (Brunello et al., 2013) that was conducted in parallel to this study.

Muscles in otherwise healthy rat myocardium. The Ca\textsuperscript{2+} dysregulation causes ESCs, and the amplitude of these ESCs ranged up to 100% of the amplitude of the developed force in electrically stimulated contractions. This latter finding suggests that all the myocytes were activated during some of these ESCs, which is in close agreement with the findings of our previous study regarding synchronization leading to non-externally stimulated APs in genetically manipulated murine myocardium (Brunello et al., 2013), and that synchronization depends on high SR calcium load (Astrup et al., 2006; Wasserstrom et al., 2010). While we were not able to measure membrane potential simultaneously, the data suggest that many of these ESCs ultimately have the capability to stimulate extra-systolic APs. Using line-scan mode imaging in different locations within a muscle, we observed that the simultaneous Ca\textsuperscript{2+} release in a group of myocytes can initiate an non-externally triggered ESC, even well after repetitive electrical stimulation has stopped, and even in quiescent areas of the muscle. Taken together, the force measurements and the Ca\textsuperscript{2+} imaging experiments suggest that this mechanism of ESCs could become synchronized in a multicellular cardiac preparation to the extent that it can involve many or all adjacent myocytes and evoke a triggered AP. Thus, this synchronization mechanism can occur in the absence of genetic mutations as shown by our previous study (Brunello et al., 2013) that was conducted in parallel to this study.
RyR2 restitution determines the timing of the Ca\(^{2+}\) waves (Bers et al., 1993; Satoh et al., 1997).

To further examine the synchronization of Ca\(^{2+}\) release, a separate group of muscles were loaded with the Ca\(^{2+}\) indicator Rhod-2. In the control, non-Ca\(^{2+}\)-overloaded muscles, we found simultaneous stimulated SR Ca\(^{2+}\) release in all six of the myocytes in the field of view, while in diastole no Ca\(^{2+}\) waves were observed. However, when both isoproterenol and caffeine were added Ca\(^{2+}\) waves were detected between the electrically stimulated Ca\(^{2+}\) release. Early after the addition of the drugs, not all of the myocytes demonstrated spontaneous Ca\(^{2+}\) release. After 5 min, however, the Ca\(^{2+}\) release typically occurred uniformly in each of the myocytes (four to seven) within the line-scan, indicating it was (non-externally) triggered rather than spontaneous. As the fluorescence profiles of the individual cells show, when the (spontaneous) Ca\(^{2+}\) release was asynchronous, the corresponding fluorescence signal peaked at different time points throughout diastole. However, when there was a synchronous Ca\(^{2+}\) release across all of the myocytes, the fluorescence peaked around the same time point in each cell. Consistent with the force experiments, this finding shows that with the addition of isoproterenol and caffeine diastolic Ca\(^{2+}\) release events do, indeed, occur, and these Ca\(^{2+}\) release events were able to synchronize across multiple myocytes in all the trabeculae examined. Gap junctions connect myocytes, and possibly calcium diffusion across these intercellular junctions can help contribute to a reduction in the threshold for a diastolic calcium release event. When a sufficient number of adjacent, coupled myocytes experience this reduction in threshold, it would increase the chance for the generation of a propagating AP.

When investigating the role of systolic Ca\(^{2+}\) release in the synchronization of Ca\(^{2+}\) waves, we observed that after the electrical stimulation had been stopped, a Ca\(^{2+}\) release event occurred uniformly throughout the myocytes. Over time, Ca\(^{2+}\) release decreased in both amplitude and temporal homogeneity until only sporadic Ca\(^{2+}\) waves occurred. Occasionally, a non-externally triggered whole-preparation synchronized SR Ca\(^{2+}\) release happened long after the stimulation had stopped. Since in the monitored area of the muscle there were no Ca\(^{2+}\) waves leading up to this uniform Ca\(^{2+}\) release, we conclude that it was the result of a triggering event that occurred from a distant cluster of myocytes and propagated to the field of view.

The vast majority of research in cardiomyocyte Ca\(^{2+}\) handling is primarily focused on individual myocytes; however, DADs and triggered arrhythmias in myocytes cannot directly be extrapolated to the intact heart, where the electrical coupling of myocytes acts as a “sink” for depolarizing currents (Joyner et al., 1983; Rohr et al., 1997; Spach and Boineau, 1997). On average, a ventricular myocyte is coupled with 11 other myocytes ( Hoyt et al., 1989; Peters and Wit, 1998); when the membrane potential in a myocyte is altered by DADs, the depolarizing current will flow to the surrounding myocytes to minimize the voltage difference. DADs will be suppressed unless a sufficient number of neighboring myocytes synchronously develop a DAD. Several studies have used computer simulations and mathematical models to calculate the number of myocytes that are required for a triggered arrhythmia. It has been proposed that a DAD would have to arise in approximately 1000 myocytes for an ectopic beat to take place (Winslow et al., 1993), while other studies have calculated that a minimum of 700,000 to 800,000 myocytes are required for triggered arrhythmias to take place in the whole heart (Plotnikov et al., 2007; Xie et al., 2010). This number is derived from computer models that typically view the ventricular structure as a single connected entity. However, the endocardial surface of the mammalian heart has a highly trabeculated structure, with many hundreds of small linear muscle preparations, varying from 10 s of microns to a few millimeters in diameter, and from 1 mm to a few cm in length. In our experiments, the trabeculae investigated contained on average only 500–1000 myocytes. Within such a linear preparation, we observed that synchronized Ca\(^{2+}\) release was able to trigger a full-sized ESC in the entire preparation, including in parts of the muscle that were virtually completely quiescent, and were thus at least partially acting as an electrical sink. Anatomically, these trabeculae typically insert into the more solid ventricular mid-myocardium in wafer-like shapes, where the number of myocytes in the cross-section slowly increases as the trabecula inserts into the free wall. Many of the smaller muscles insert first into larger ones that, in turn, insert into the ventricular wall. We postulate that it is possible that this gradual change in the electrical sink capacity is not sufficient to quench the propagation of DADs, and may thus allow the trigger of an ESC. Future investigations will be needed to address this hypothesis.

**CONCLUSION**

The present study demonstrates that diastolic SR Ca\(^{2+}\) release can become synchronized in multicellular trabeculae. Assessment of the developed force and intracellular Ca\(^{2+}\) suggests that the addition of both isoproterenol and caffeine can result in ESCs, and these events were able to spatially and temporally synchronize across multiple myocytes. Given the anatomy of the endocardial surface, it is feasible that triggered ventricular ESCs can be caused by a small, specifically localized group of myocytes, and may not necessarily require the much larger amounts of myocytes predicted by previously conducted modeling studies.

**AUTHOR CONTRIBUTIONS**

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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