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Recovery of cardiac calcium release is controlled by sarcoplasmic reticulum refilling and ryanodine receptor sensitivity

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Aims In heart cells, the mechanisms underlying refractoriness of the elementary units of sarcoplasmic reticulum (SR) Ca^{2+} release, Ca^{2+} sparks, remain unclear. We investigated local recovery of SR Ca^{2+} release using experimental measurements and mathematical modelling.

Methods and results Repeated Ca^{2+} sparks were induced from individual clusters of ryanodine receptors (RyRs) in quiescent rat ventricular myocytes, and we examined how changes in RyR gating influenced the time-dependent recovery of Ca^{2+} spark amplitude and triggering probability. Repeated Ca^{2+} sparks from individual sites were analysed in the presence of 50 nM ryanodine with: (i) no additional agents (control); (ii) 50 μM caffeine to sensitize RyRs; (iii) 50 μM tetracaine to inhibit RyRs; or (iv) 100 nM isoproterenol to activate β-adrenergic receptors. Sensitization and inhibition of RyR clusters shortened and lengthened, respectively, the median interval between consecutive Ca^{2+} sparks (caffeine 239 ms; control 280 ms; tetracaine 453 ms). Recovery of Ca^{2+} spark amplitude, however, was exponential with a time constant of ~100 ms in all cases. Isoproterenol both accelerated the recovery of Ca^{2+} spark amplitude ($\tau = 58$ ms) and shortened the median interval between Ca^{2+} sparks (192 ms). The results were recapitulated by a mathematical model in which SR [Ca^{2+}] depletion terminates Ca^{2+} sparks, but not by an alternative model based on limited depletion and Ca^{2+}-dependent inactivation of RyRs.

Conclusion Together, the results strongly suggest that: (i) local SR refilling controls Ca^{2+} spark amplitude recovery; (ii) Ca^{2+} spark triggering depends on both refilling and RyR sensitivity; and (iii) β-adrenergic stimulation influences both processes.

Keywords Ca^{2+} spark • Triggered activity • Calcium transient • Ventricular myocyte • Computer modelling

1. Introduction

In cardiac myocytes, release of Ca^{2+} from the sarcoplasmic reticulum (SR) is centrally involved in both normal heart function and dysfunction that can occur with pathology. SR Ca^{2+} release triggered by Ca^{2+} entry through L-type Ca^{2+} channels causes a large increase in intracellular [Ca^{2+}] that enables a strong heartbeat. Spontaneous release of SR Ca^{2+}, however, is potentially arrhythmogenic, and an increased risk of this deleterious release is associated with conditions such as heart failure and catecholaminergic polymorphic ventricular tachycardia (CPVT).

After SR Ca^{2+} release, time must elapse before a second release event of equal amplitude can occur.[1] The rate of recovery is considered potentially important in determining a cell's arrhythmogenic potential. Faster than normal recovery is associated with some forms of CPVT,[2,3] and Ca^{2+} release refractoriness has recently been predicted to influence the development of Ca^{2+} transient alternans.[4] At the cellular level, however, several factors can contribute to the recovery of SR Ca^{2+} release, including the L-type Ca^{2+} current trigger and beat-to-beat changes in SR Ca^{2+} content. To assess recovery of the release process itself, it is useful to examine Ca^{2+} 'sparks',[5] local release events caused by the opening of a cluster of SR Ca^{2+} release channels known as ryanodine receptors (RyRs). Because both spontaneous sparks and potentially arrhythmogenic cell-wide Ca^{2+} release are initially triggered by a spontaneous RyR opening, the

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factors that control the time-dependent probabilities of these events are obviously related.[6]

Mechanisms underlying recovery of Ca^{2+} sparks remain incompletely understood. Intuitively, however, these should be closely linked to the factors responsible for spark termination. In other words, whatever process shuts off release in a cluster of RyRs may reduce the probability that the channels will reopen during some subsequent interval. At present, the leading hypothesis for Ca^{2+} spark termination is that local depletion of [Ca^{2+}] in the junctional SR (JSR) plays the most important role. Simulations presented in 2002 with a mathematical model of the Ca^{2+} spark termed the 'sticky cluster' demonstrated the feasibility of this hypothesis,[7] and several subsequent studies have provided considerable experimental support. Local depletion of JSR [Ca^{2+}], originally just hypothesized based on computations, has been observed experimentally.[8,9] Increasing[10,11] or decreasing[11] the buffering capacity of the SR prolongs or abbreviates, respectively, the duration of SR Ca^{2+} release, as required by the hypothesis. Similarly, slowing the rate of JSR Ca^{2+} depletion by partially inhibiting RyRs also prolongs release.[12,13] Finally, SR [Ca^{2+}] refilling after depletion plays an important role in the recovery of SR Ca^{2+} release, at both the local[14] and cell-wide levels.[15]

Despite these results that are consistent with dynamic local changes in SR [Ca^{2+}] controlling release termination and recovery, several questions remain unresolved. For instance, in 2005, Sobie *et al.*[14] simultaneously estimated: (i) the local recovery of Ca^{2+} spark amplitude, and (ii) the recovery of the probability for a single RyR opening to trigger a Ca^{2+} spark. The latter was shown to lag the former by roughly 100 ms, but the authors could not determine the mechanisms responsible for the delay. Possibilities included: (i) that inactivation of RyRs by cytosolic Ca^{2+} contributed to refractoriness; and (ii) that after local JSR refilling, a rate-limiting conformational change in a protein such as calsequestrin (CSQ) had to occur before RyRs were again ready to release Ca^{2+}. At that time, it was unclear whether the minimal assumptions of the 2002 model[7] were sufficient to explain the experimental results.

Here we have investigated local recovery of SR Ca^{2+} release through a combination of mathematical modelling and experimental Ca^{2+} spark measurements. Simulations with the sticky cluster model revealed that the model can indeed account for the data, and these simulations generated testable predictions. Consistent with the model predictions, experimental results indicated that RyR sensitivity affects the recovery of Ca^{2+} spark triggering probability but not the recovery of spark amplitude. Moreover, we found that β-adrenergic stimulation increases both the rate of spark amplitude recovery and the apparent sensitivity of RyR clusters. The results provide new insight into the mechanisms controlling termination and recovery of SR Ca^{2+} release, and help us understand the increased propensity of potentially arrhythmogenic spontaneous Ca^{2+} release after β-adrenergic stimulation.

2. Methods

An expanded Methods section, describing cell isolation, confocal imaging, data analysis, and mathematical modelling is available in the Supplementary materials.

2.1 Ca^{2+} spark measurements and data analysis

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine. Ventricular myocytes from male rats weighing 200–300 g were prepared using standard enzymatic dissociation techniques.[16] Rats were given an intraperitoneal injection of a lethal dose of pentobarbital (250 mg/kg body weight), then hearts were removed and retrograde perfused with the following solutions: (i) Tyrode's solution containing 2 mM Ca^{2+} for 5 min; (ii) Ca^{2+}-free Tyrode's solution for 10 min; (iii) Ca^{2+}-free Tyrode's solution containing collagenase (141 U/mL) and protease (0.32 U/mL); (iv) Tyrode's solution containing 0.1 mM [Ca^{2+}] for 10 min. The ventricles were cut off from the heart, minced, and filtered through a 200 μm mesh, yielding individual cells. Isolated myocytes were loaded with fluo-3AM and imaged using a confocal microscope operated in line scan mode. Repetitive Ca^{2+} sparks originating from a single cluster of RyRs were obtained by applying 50 nM ryanodine as previously described.[14] For experiments in which cells were exposed to ryanodine along with caffeine, tetracaine, or isoproterenol, the timing of solution application was controlled precisely as described in the Supplementary material. Repetitive Ca^{2+} sparks were analysed using custom programs written in Matlab™ (Mathworks, Natick, MA, USA) and python (http://code.google.com/p/lsjuicer/). Criteria for selection and exclusion of data are described in the Supplementary material.

2.2 Mathematical modelling

Simulations were performed with a modified version of the sticky cluster model of Sobie *et al.*[7] In this representation, RyRs are triggered by local increases in cytosolic, 'subspace' [Ca^{2+}], and RyR gating depends on local JSR [Ca^{2+}] and allosteric coupling between RyRs. The model, however, contains no explicit inactivation process. Local depletion of JSR [Ca^{2+}] is responsible for the termination of Ca^{2+} sparks, and refilling of JSR [Ca^{2+}] therefore determines the recovery of the RyR cluster from refractoriness. Because this phenomenological model of RyR gating contains relatively few assumptions, it is useful for determining whether the assumptions are sufficient to explain experimentally observed features of Ca^{2+} sparks, or whether additional mechanisms must be considered. The Supplementary material describes the model in detail and lists parameters that have been altered, compared with the original,[7] to account for more recent experimental results.

The important output of the sticky cluster model is local SR Ca^{2+} release flux as a function of time. To compare these results to experimentally measured Ca^{2+} signals, we used release fluxes as inputs to a second model that computes Ca^{2+} buffering, diffusion, binding to the indicator fluo-3, and blurring by the confocal microscope, as in previous studies.[7,17]

Simulations of Ca^{2+} spark recovery were also performed with a model based on Ca^{2+}-dependent inactivation in the absence of JSR depletion. This stochastic model was derived from the sticky cluster model by fixing JSR [Ca^{2+}] at the diastolic value of 1 mM and introducing an inactivation state to RyR channel gating. Models details are provided in the Supplementary material.

3. Results

The sticky cluster model of the Ca^{2+} spark[7] assumes that local depletion of JSR [Ca^{2+}] is responsible for termination of Ca^{2+} release, and recovery therefore depends on JSR refilling. Simulations of consecutive Ca^{2+} sparks were performed with this model to generate predictions of how the delay between individual RyR openings (Δt) influenced: (i) the probability that the second opening would trigger a Ca^{2+} spark (P_{trig}), and (ii) when events were triggered, the amplitude of the second relative to the first. Examples presented in *Figure 1A* show that at a relatively short interval (e.g. $\Delta t = 150$ ms), few sparks were triggered, and these were smaller than their

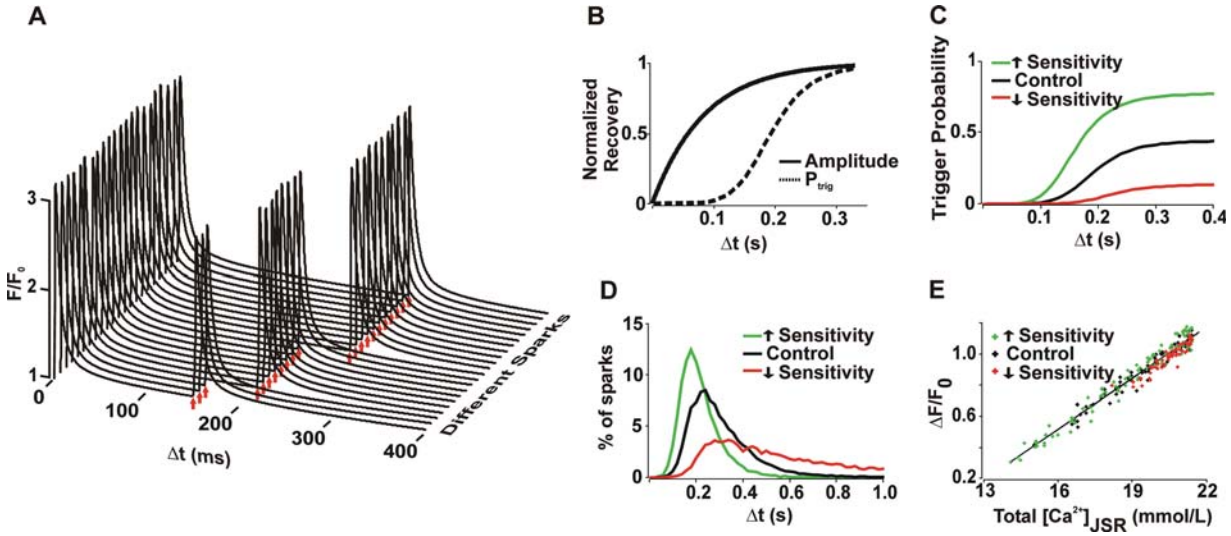

Figure 1 Simulation results. (*A*) Ca^{2+} sparks triggered by a single RyR opening at different times after the initial spark. The simulated examples shown are representative of 30 trials at each interval; i.e. fewer events at shorter intervals reflect an increase in spark triggering probability (P_{trig}) with time. (*B*) Normalized Ca^{2+} spark amplitude and P_{trig} recovery curves. (*C*) P_{trig} vs. Δt curves under three conditions: default parameters, increasing maximum RyR opening rate by a factor of 3 (green), decreasing maximum RyR opening rate by a factor of 3 (red). Curves displayed were obtained by fitting simulation results to a Hill function. Half-times of the three curves are as follows: increased sensitivity: $t_{1/2}$= 170 ms; control: $t_{1/2}$= 200 ms; decreased sensitivity: $t_{1/2}$= 230 ms. Original results and fit parameters are shown in Supplementary material online, *Figure S8*. (*D*) Simulated spark-to-spark delay histograms assuming that a single, autonomous RyR within the cluster is opening orders of magnitude more frequently than the others. Each histogram is of 10 000 simulated trials, and bin size is 20 ms. (*E*) Second Ca^{2+} spark amplitude (expressed as normalized increase in fluorescence $\Delta F/F_0$) vs. total JSR [Ca^{2+}] (free plus bound to buffers) immediately prior to the spark.

predecessors. At a longer interval (e.g. $\Delta t = 250$ ms), a greater number of Ca^{2+} sparks were triggered, and these were larger than those at $\Delta t = 150$ ms. Running repeated ($n = 500$) simulations at each Δt allowed us to calculate how Ca^{2+} spark amplitude (solid line) and P_{trig} (dashed line) recovered as a function of Δt (*Figure 1B*). Consistent with published experimental data,[14] the simulations predict that Ca^{2+} spark amplitude recovery is approximately exponential with a time constant of ~90 ms, and the sigmoidal recovery of P_{trig} lags the recovery of spark amplitude.

Next we explored how the sensitivity of the RyRs influenced the simulation results (*Figure 1C–E*). An increase in the maximal opening rate of the RyRs raises the plateau of P_{trig} vs. Δt and shifts this relationship to the left, whereas a decrease has opposite effects (*Figure 1C*). To more easily compare these results with experimental data (see below), we implemented a model to account for the fact that RyR openings in cells occur stochastically (see Supplementary material). This model allowed us to generate simulated histograms of spark-to-spark delays, and these predicted that RyR sensitivity influenced the histogram shape (*Figure 1D*). The amplitude of the second Ca^{2+} spark, however, did not depend on RyR sensitivity but instead was linearly related to total JSR [Ca^{2+}] immediately prior to the spark (*Figure 1E*). The simulations therefore generate two predictions that can be tested experimentally. One is that sensitizing or inhibiting RyRs will shift spark-to-spark histograms to the left or the right, respectively. Equally important, the simulations predict that altering RyR sensitivity will not affect the recovery of Ca^{2+} spark amplitude.

To test these predictions, we established experimental conditions under which Ca^{2+} sparks occurred repeatedly at only a few RyR clusters within the cell. Quiescent rat ventricular myocytes were perfused with 50 nM ryanodine to produce, at a limited number of locations, repeated Ca^{2+} sparks from individual RyR clusters.[14] Experiments in which nifedipine was applied demonstrated that these sparks originated from spontaneous openings of RyRs rather than Ca^{2+} flux through L-type Ca^{2+} channels (Supplementary material online, *Figure S13*). In addition to the control group exposed to only ryanodine, groups of cells were also treated with either 50 μM caffeine or 50 μM tetracaine to sensitize or inhibit RyRs, respectively. These interventions caused no change in diastolic [Ca^{2+}] or the variability in spark amplitude when events were produced repeatedly at individual sites (Supplementary material online, *Figures S11* and *S12*).

Figure 2 shows example confocal line scan recordings obtained under the three conditions and a quantification of delays between repeated Ca^{2+} sparks. The spark-to-spark delay histogram was shifted to the left by caffeine (*Figure 2A*) and to the right by tetracaine (*Figure 2C*) compared with control (*Figure 2B*). Thus, sensitization of RyRs caused more second Ca^{2+} sparks to occur soon after the initial events. *Figure 2D* illustrates that caffeine caused an increase in the percentage of second sparks occurring within 100 ms of the initial event, and, conversely, tetracaine caused an increase in the percentage occurring after 1 s. In contrast to the effects on spark-to-spark delay histograms, however, caffeine and tetracaine had no effect on the recovery of Ca^{2+} spark amplitude, as a time constant of approximately 95 ms was obtained under all three conditions (*Figure 3*). These two results, that RyR sensitivity alters spark-to-spark delays, but that sensitivity does not affect spark amplitude restitution, are consistent with the sticky cluster model predictions shown in *Figure 1*.

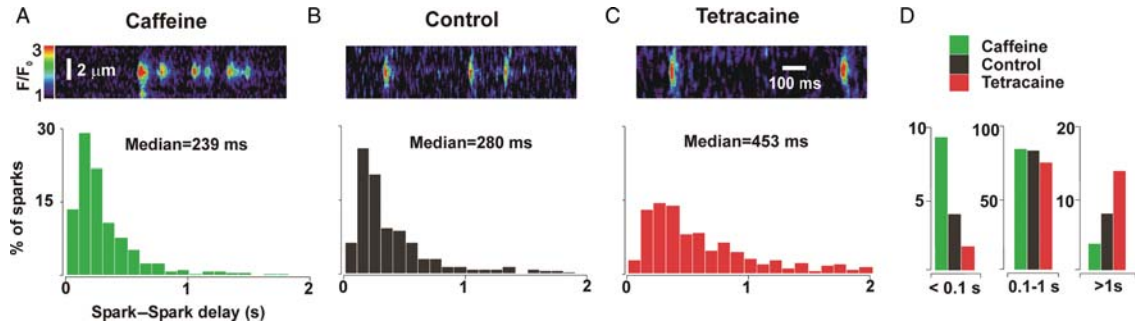

Figure 2 Sample recordings and spark-to-spark delay statistics. (*A*) Ryanodine plus caffeine (354 spark pairs from 8 cells). (*B*) Ryanodine only (445 spark pairs from 9 cells). (*C*) Ryanodine plus tetracaine (429 spark pairs from 14 cells). Each panel shows line-scan image (top) and histogram of spark-to-spark delays (bottom). (*D*) For the three experimental conditions, percentages of spark-to-spark delays either less than 100 ms (left), between 100 ms and 1 s (middle) or greater than 1 s (right).

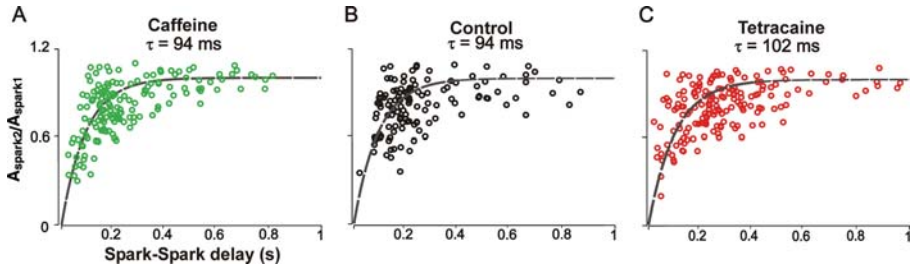

Figure 3 Spark amplitude restitution. From analysis of Ca^{2+} spark pairs, each plot shows the normalized amplitude of the second spark (A_{spark2}/A_{spark1}) vs. the delay between sparks. (*A*) Ryanodine plus caffeine (157 spark pairs from 8 cells). (*B*) Ryanodine only (127 pairs from 9 cells). (*C*) Ryanodine plus tetracaine (174 pairs from 14 cells). Dashed lines show fits to the data of exponential recovery curves with indicated time constants.

Next we treated cells with ryanodine and isoproterenol (100 nM) to investigate how β-adrenergic stimulation affects refractoriness and recovery of Ca^{2+} sparks. β-Adrenergic stimulation caused a more rapid recovery of Ca^{2+} spark amplitude ($\tau = 58$ ms vs. 94 ms with ryanodine only, *Figure 4A*) as well as shorter spark-to-spark delays on average (*Figure 4B*). *Figure 4C* directly compares the delay histograms obtained under the four conditions. Isoproterenol caused a leftward shift in the histogram, even greater than that produced by 50 μM caffeine.

Figure 4 suggests that β-adrenergic stimulation influences both local refilling of JSR Ca^{2+} stores, likely through stimulation of SERCA pumps, and the sensitivity of RyRs, possibly through PKA or CAMKII-mediated phosphorylation. For mechanistic insights into the relative contributions of these factors, we performed additional simulations with the sticky cluster model. In the model, Ca^{2+} movement from NSR to JSR is controlled by the parameter τ_{refill}. The faster Ca^{2+} spark amplitude recovery with isoproterenol can be reproduced by reducing τ_{refill} (*Figure 5A*). One effect of this more rapid refilling is that an early spontaneous opening of a single RyR is more likely to trigger a spark due to the greater flux of Ca^{2+} through the open channel. This factor by itself causes a leftward shift in the predicted spark-to-spark histogram (*Figure 5B*), with a decrease in the median value from 286 to 217 ms. The median value of the experimental data, however, is 192 ms, so we considered more rapid refilling alone insufficient to account for the spark-to-spark histogram seen experimentally with isoproterenol. A computed spark-to-spark histogram more similar to that seen experimentally (median = 187 ms) could be produced if we assumed that β-adrenergic stimulation increased both the rate of JSR refilling and the sensitivity of the RyRs to activation by cytosolic $[Ca^{2+}]$ (*Figure 5B*).

The simulation results (*Figures 1* and *5*) show that the minimal assumptions of the sticky cluster model are sufficient to explain the experimental results, but these say nothing about competing hypotheses. We performed additional simulations to address potential alternative explanations. Specifically, we considered a situation such as that seen in amphibian skeletal muscle, where JSR depletion during Ca^{2+} sparks is minimal,[18] and spark termination results from Ca^{2+}-dependent inactivation.[19] With this model, the shifts in the spark-to-spark histograms could be reproduced by assuming that caffeine accelerates, whereas tetracaine retards recovery from inactivation (*Figure 6A*). In these simulations, however, Ca^{2+} spark amplitude is roughly proportional to the number of channels that open during the spark. Thus, faster recovery of RyRs from inactivation causes a leftward shift in both the histogram (*Figure 6A*) and the spark amplitude recovery curve (*Figure 6B*). Because this is contrary to the experimental data, the results provide evidence against such a mechanism being responsible for Ca^{2+} spark refractoriness and recovery.

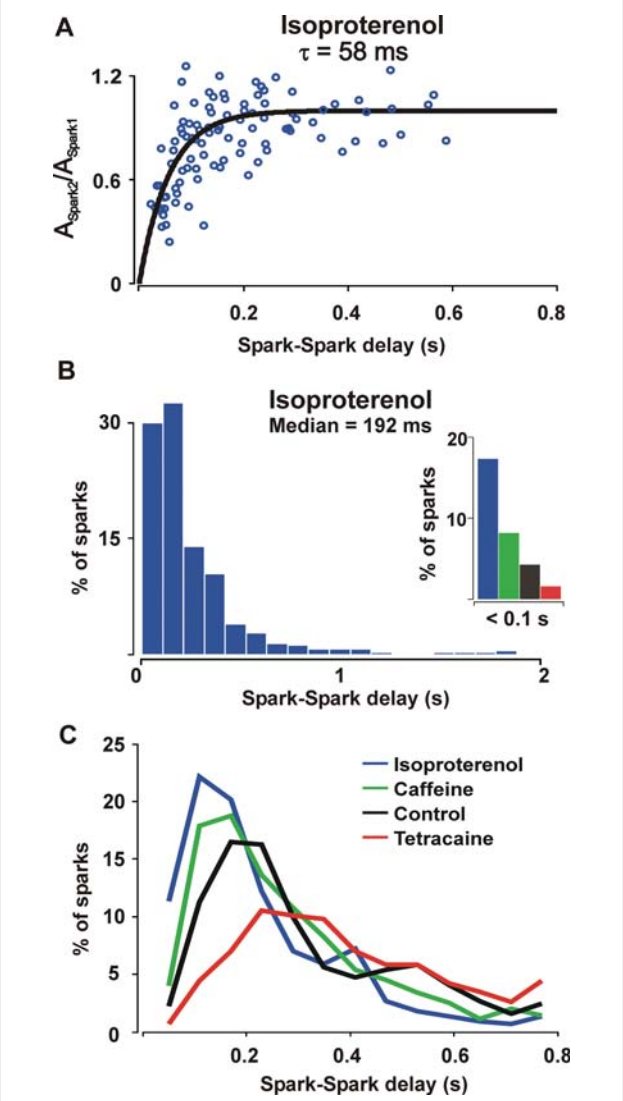

Figure 4 Ca^{2+} spark restitution after β-adrenergic stimulation. (*A*) Amplitude restitution (107 spark pairs from 9 cells) is faster ($\tau = 58$ ms) in the presence of isoproterenol (100 nM) compared with control conditions ($\tau = 94$ ms). (*B*) Spark-to-spark delay histogram. Inset compares percentage of delays <100 ms under different experimental conditions. (*C*) Comparison of spark-to-spark delay histograms. β-Adrenergic stimulation induces the most dramatic leftward shift.

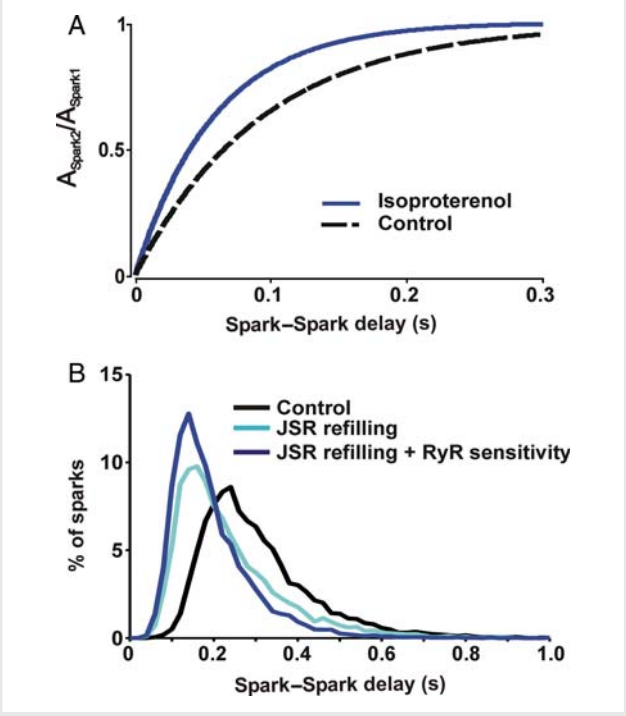

Figure 5 Simulations of β-adrenergic stimulation. (*A*) Predicted Ca^{2+} spark amplitude restitution curves with normal NSR to JSR refilling (dashed line), and with refilling time constant reduced from 6.5 to 4.0 ms (solid line). (*B*) Predicted spark-to-spark histograms with the control model, with faster NSR to JSR refilling, and with faster refilling plus increased (by a factor of 1.5) RyR sensitivity. Each histogram is of 10 000 simulated trials, and bin size is 20 ms.

4. Discussion

In this study, we have obtained new insight into the factors controlling refractoriness of cardiac SR Ca^{2+} release. Using an experimental protocol that elicits repeated Ca^{2+} sparks at a few RyR clusters within the myocyte,[14] we have examined the recovery with time of: (i) Ca^{2+} spark triggering probability; and (ii) Ca^{2+} spark amplitude. Sensitizing RyRs with caffeine, or inhibiting RyRs with tetracaine, causes an increase or decrease, respectively, in the rate of triggering probability recovery (*Figure 2*). RyR sensitivity, however, has no effect on spark amplitude recovery (*Figure 3*). Since these results can be recapitulated by the sticky cluster model of the Ca^{2+} spark[7] but not by an alternative model based on Ca^{2+}-dependent inactivation (*Figure 6*), the data strongly suggest that: (i) refilling of JSR [Ca^{2+}] after local depletion controls Ca^{2+} spark amplitude recovery, and (ii) Ca^{2+} spark triggering depends on both JSR refilling and RyR gating properties. Isoproterenol speeds the recovery rates of both spark amplitude and triggering probability, thus offering new insight into the changes caused by β-adrenergic stimulation.

Three lines of evidence support the hypothesis that spark amplitude recovery depends on JSR refilling but not on time-dependent changes in RyR gating. First and most important, low doses of caffeine and tetracaine, which influence Ca^{2+} spark frequency, do not affect the amplitude recovery rate. Secondly, the sticky cluster model, in which spark amplitude depends primarily on pre-spark JSR [Ca^{2+}] (*Figure 1E*), generates simulation results consistent with the data. Third, the time constants of amplitude recovery measured here are similar to the time constants of local SR Ca^{2+} depletion signals, Ca^{2+} 'blinks,' seen by Zima *et al.*[13] in their comprehensive study. It should be noted, however, that Zima *et al.*[13] observed considerable site-to-site heterogeneity in blink time constants. Our data currently do not allow us to determine whether Ca^{2+} spark amplitude recovery also exhibits such heterogeneity because we record from each cell for a limited period (<8 min). We would predict, however, that sites exhibiting fast refilling would generate second sparks earlier than sites that refill more slowly.

If recovery of SR Ca^{2+} release from refractoriness does not involve Ca^{2+}-dependent inactivation of RyRs, as these results suggest, what accounts for the delay between amplitude recovery and triggering recovery? The mathematical modelling allows us to make inferences

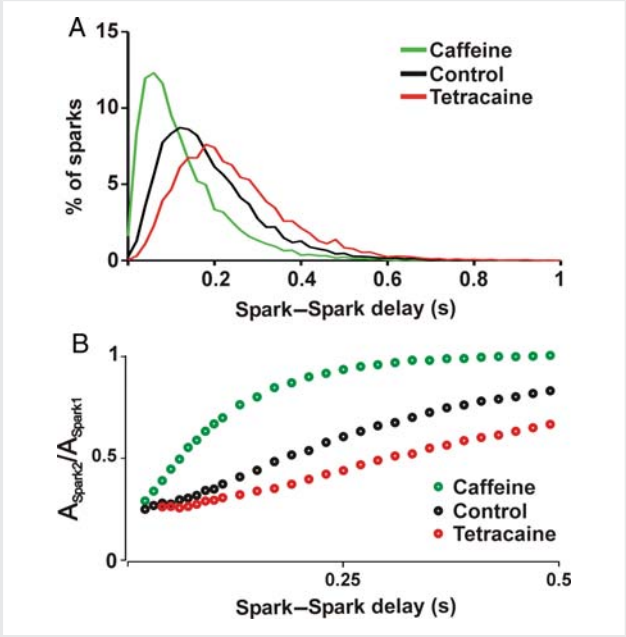

Figure 6 Simulation results to explore alternative explanations. (*A*) Predicted spark-to-spark histograms in a model based on Ca^{2+}-dependent inactivation without JSR depletion. Histograms computed from Hill equation fits of P_{trig} vs. Δt simulation results (see Supplementary material online, *Figure S8*). (*B*) Ca^{2+} spark amplitude restitution in the model with no JSR depletion. Contrary to the experimental data, these simulations predict that RyR sensitivity influences both the recovery of P_{trig} and spark amplitude restitution.

about the factors that may be responsible. It is important to note that in these experiments, the trigger for SR Ca^{2+} release is Ca^{2+} flux through an RyR that opens spontaneously. Thus, as local JSR refills with Ca^{2+}, two changes occur simultaneously: (i) RyRs recover their sensitivity due to the modulation of RyR gating by SR $[Ca^{2+}]$;[20–22] and (ii) each spontaneous RyR opening becomes a more effective trigger due to the increased Ca^{2+} flux. In the sticky cluster model, it is the combination of these two factors, along with the non-linear dependence of RyR opening on dyadic $[Ca^{2+}]$, that accounts for the delay between the amplitude and triggering probability recovery functions.

A possible alternative hypothesis is that after JSR $[Ca^{2+}]$ refilling, a conformational change in an SR protein such as CSQ must occur before RyRs are primed to release Ca^{2+}.[20,23] While our data cannot directly address such conformational changes, it appears unlikely that such a step is rate-limiting. If a conformational change in CSQ were rate-limiting, one would not expect caffeine and isoproterenol to induce leftward shifts in the spark-to-spark delay histograms, as was observed (*Figure 4*).

Over the past several years, evidence has accumulated that dynamic local changes in SR $[Ca^{2+}]$ are primarily responsible for termination and recovery of SR Ca^{2+} release.[10–15,24,25] Several compelling experimental results fit extremely well into the framework established by the sticky cluster model.[7] The general hypothesis is that local depletion of JSR $[Ca^{2+}]$ desensitizes RyRs and allows for release termination, and refilling of JSR stores subsequently controls the recovery of RyRs from refractoriness. Early results, such as Ca^{2+} spark prolongation caused by exogenous SR Ca^{2+} buffers,[10] could not directly address whether a process such as Ca^{2+}-dependent inactivation of RyRs might also contribute to termination. More recent studies, however, have provided evidence against such a mechanism. For instance, Zima *et al.*[12] found that 700 μM tetracaine (compared with 50 μM used here) caused both an increase in SR Ca^{2+} load and an increase in spark duration, presumably due to slower JSR depletion. The latter observation was contrary to the shorter durations predicted by a model based on Ca^{2+}-dependent inactivation. Similarly, in permeabilized cells, Stevens *et al.*[25] found that 100 μM cytosolic $[Ca^{2+}]$, the extreme concentration required to prevent spontaneous Ca^{2+} waves, led to a decrease in SR $[Ca^{2+}]$, rather than the increase that would have occurred if high cytosolic $[Ca^{2+}]$ had inactivated RyRs. Our results also suggest that Ca^{2+}-dependent inactivation of RyRs is not responsible for terminating SR Ca^{2+} release. A computational model based on this mechanism could not reproduce the data (*Figure 6*), since in these simulations caffeine and tetracaine altered both the histograms and the amplitude recovery curves.

Our measurements in the presence of isoproterenol (*Figure 4*) show that β-adrenergic stimulation speeds recovery of both spark amplitude and triggering probability. The more rapid spark amplitude recovery, although expected given the well-established increase in SERCA activity caused by β-adrenergic stimulation,[26] provides new quantitative information. We can obtain a rough estimate of the relative contributions to JSR refilling of intra-SR diffusion and SERCA pumping if we assume that: (i) the overall refilling rate (reciprocal of the time constant) is the sum of the two contributions; and (ii) β-adrenergic stimulation increases SERCA's contribution by a certain percentage but does not influence diffusion. The analysis suggests, for instance, that if β-adrenergic stimulation triples the rate of SERCA pumping,[27] then under baseline conditions, diffusion contributes 75%, and SERCA contributes 25%, of the refilling flux. After β-adrenergic stimulation, the contributions to refilling from diffusion and SERCA are roughly equal. Estimates such as these, which can be made more precise in future experiments, will be important for building spatially specified models of cardiac Ca^{2+} release[28] (Supplementary material online, *Figure S7*).

The results also suggest that β-adrenergic stimulation increases RyR sensitivity. Although biochemical studies have unambiguously shown that PKA and CAMKII phosphorylate the RyR and modify its activity,[29] β-adrenergic stimulation affects many proteins involved in Ca^{2+} regulation, including L-type Ca^{2+} channels and SERCA pumps. It has therefore proven difficult to determine directly how changes in RyR properties caused by β-adrenergic stimulation influence SR Ca^{2+} release. A study by Li *et al.*[30] suggested that, at constant SR Ca^{2+} load, β-adrenergic stimulation did not increase the frequency of spontaneous Ca^{2+} sparks. More recent studies, however, have suggested that RyRs become more sensitive after β-adrenergic stimulation, even after correcting for changes in SR load,[31–33] and our results are consistent with these. However, our data cannot address whether changes in RyR function are due to PKA or CAMKII phosphorylation.

The results have implications for our understanding of arrhythmias initiated by triggered activity, which are believed to originate with spontaneous SR Ca^{2+} release, and are considered the predominant ventricular arrhythmias in both heart failure[34] and CPVT.[35] The results with caffeine show that although sensitizing RyRs does not accelerate recovery of Ca^{2+} spark amplitude, it does increase the probability that an early spontaneous RyR opening will trigger the

other RyRs in the cluster. By extension, sensitization of RyRs due to mutations or sustained hyperphosphorylation should also increase the probability that a spontaneous early Ca^{2+} spark will initiate a regenerative Ca^{2+} wave. The results additionally suggest that isoproterenol accelerates Ca^{2+} release recovery due to both increased SERCA activity and RyR sensitization. In addition to increases in SR load, these factors may together act to predispose the myocardium to triggered activity after β-adrenergic stimulation. Faster recovery of Ca^{2+} release is currently thought to be one of the mechanisms underlying arrhythmia risk in CPVT caused by mutations in CSQ.[2,3] It has proven challenging, however, to differentiate the effects of reduced Ca^{2+} buffering in the JSR from the consequences of altered CSQ regulation of the RyR. An advantage of the present experimental protocol, particularly when simulations aid data interpretation, is that Ca^{2+} spark amplitude and triggering probability recoveries are measured separately (*Figures 2* and *3*). Thus, new insight into mechanisms controlling pathological SR Ca^{2+} release can be gained by applying this protocol to mouse models of CPVT,[36,37] as well as to experimental models of the more complex condition of heart failure.

Several limitations of the protocol and the analysis should be considered. Spark restitution was assessed after applying ryanodine, an agent that can induce RyR subconductance states and extremely long Ca^{2+} sparks,[5] which would confound the analysis. We mitigated against this possibility by: (i) using only 50 nM ryanodine; (ii) applying pharmacological agents with precise timing (see Supplementary material); and (iii) acquiring all Ca^{2+} spark pairs within the first 10 min after ryanodine exposure. On the occasions that a Ca^{2+} spark lasting longer than 200 ms was observed, we excluded all subsequent data from that cell (Supplementary material online, *Figure S1*). Although these strategies helped to avoid artefacts due to the pharmacological interventions, a disadvantage is that we could only record a limited number of Ca^{2+} spark pairs from each repetitively active site, and we can therefore not draw conclusions about possible heterogeneity in SR refilling rates at different RyR clusters.[13] We should also note that after several minutes of exposure, caffeine will cause a decrease,[38] and tetracaine and isoproterenol will cause increases,[30,38] in the SR Ca^{2+} load in quiescent myocytes. In the cases of caffeine and tetracaine, these alterations will counteract the changes in spark rate due to modified RyR sensitivity. Thus, the fact that we still observed earlier second sparks with caffeine, and later second sparks with tetracaine, indicates that the effects of RyR sensitivity predominate over the secondary changes in SR load.

In summary, our combined experimental and computational study helps define the mechanisms controlling recovery of SR Ca^{2+} release in ventricular myocytes. RyR sensitivity influences the time-dependent recovery of spark triggering probability, but has no effect on the recovery of spark amplitude. β-Adrenergic stimulation appears to influence both the rate of recovery of Ca^{2+} spark amplitude and RyR sensitivity. Since these observations can be recapitulated by the sticky cluster model of the Ca^{2+} spark,[7] the results: (i) imply that local JSR refilling controls the recovery of spark amplitude; and (ii) further strengthen the hypothesis that Ca^{2+} release termination and refractoriness depends primarily on changes in SR $[Ca^{2+}]$ rather than on a process such as Ca^{2+}-dependent inactivation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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