# Challenging quantal calcium signaling in cardiac myocytes

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Local calcium release via RYRs, observed in the form of calcium sparks (Cheng et al., 1993), is generally accepted to be a principal mediator of calcium homeostasis and excitation–contraction coupling in cardiac myocytes. However, investigation of local calcium release signals is extremely difficult because these signals are near the spatial and temporal resolution of modern instrumentation. Therefore, experiments in cell-free systems, often combined with mathematical modeling, are used to explain in situ observations and verify their interpretation (Stern et al., 1999; Zahradníková et al., 2010). Concerning interpretation of calcium signaling in cardiac myocytes we have asked two questions. First, is the gating of RYRs in cell-free systems relevant to their gating in calcium release units? Second, can the local calcium release signal be quantal in nature?

In their recent Perspective, Xie et al. (2010) contemplate that, because of the formation of two-dimensional quasi-crystalline arrays, RYRs behave substantially different in cells than in bilayers. To the best of our knowledge, there are no major inconsistencies between RYR behavior in situ and in vitro. It can be documented that whether in situ or in vitro, the properties of RYRs, such as regulation by cytosolic and luminal calcium, pharmacology, metabolic regulation, functional association with accessory proteins, and close association between RYRs, are alike. In the model by Xie et al. (2010), the authors extend the concept of coupled RYR gating proposed by Marx et al. (2001) to all RYRs within a cluster, despite the fact that in bilayers the coupled activity of multiple RYRs is a rare phenomenon. The chance of observing coupled gating in individual incorporations of rat SR vesicles was 11, 1, 1, and 0% for two, three, four, and five or more channels, respectively (Gaburjakova and Gaburjakova, 2010), whereas the chance of observing independent RYR gating was 60, 14, 5, 4, and 3% for one, two, three, four, and five or more channels, respectively (unpublished data). The seminal study on RYR distribution in subsarcolemmal release sites (Baddeley et al., 2009) has shown that junctional RYRs occur both solo as well as in clusters of variable size from 2 to >100 RYRs (~14 RYRs per cluster on average), with smaller clusters being more frequent than the larger ones. This explains why fusion of isolated SR vesicles into bilayers provides a variable number of active RYRs, but not why only a small fraction of them is functionally coupled.

For lack of other experimental evidence, we tested the idea of Xie et al. (2010) by mathematical modeling. An appropriate model was designed using the experimental data on RYR distribution into clusters and release units (Soeller et al., 2007; Baddeley et al., 2009). Gating of RYRs was assumed to be coupled; that is, all RYRs in a cluster were either open (with probability $P_O$) or closed (with probability $1-P_O$). Individual clusters in a release unit were considered independent. As illustrated in Fig. 1, the model produced the probability distribution of the number of open RYRs, $n_O$, that was skewed toward lower $n_O$ but did not have the expected quantal structure that would correspond to in situ observations of release flux distribution (Wang et al., 2004). A similar result was obtained when the probability of cluster activation was reduced to simulate the effect of tetracaine (Wang et al., 2004). It should be noted that for release units composed of a small number of clusters (1–3) the probability distribution was not sensitive to the probability of cluster activation. Exploration of the parameter space of the model that included $P_O$, the size of clusters and of release units, revealed (not depicted) that there is no set of parameters that would provide the result predicted in Xie et al. (2010). Obviously, release units with exponentially distributed cluster size and with all RYRs in a cluster gating in concert cannot explain the observed quantal levels of calcium release flux (Wang et al., 2004).

According to the original proposal of Wang et al. (2004), the quantal distribution originates from the calcium release flux produced by the openings of a few (most frequently two) RYRs. This interpretation was tested again in the above model with the same composition of clusters and release units, in which individual RYRs were considered independent. No further assumptions were made about RYR gating, except that after the

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opening of the first RYR in the release unit, all remaining RYRs have the same probability $P_O$ of becoming open. The $P_O$ values used in Fig. 1 were set to approximate the observed shape of the release flux amplitude distribution (Wang et al., 2004) and therefore are independent of an RYR gating scheme. The model predicts, in agreement with Wang et al. (2004), that the calcium release flux of any release unit displays quantal character when activated repeatedly (Fig. 1); that the quantal character of any set of independent release units has similar characteristics (not depicted); and that decreasing RYR open probability (tetracaine) leads to a decrease in the number of quanta (Fig. 1). The strength of this model is in its coherence with the calcium dependence of diastolic spark frequency (Lukyanenko and Gyorke, 1999) and with RYR gating in bilayers (Zahradník et al., 2005), as shown in Zahradníková et al. (2010).

It should be noted, however, that in neither of the models treated here are reduction of luminal calcium concentration during release and related effects, such as decreased RYR open probability and decreased calcium flux, accounted for. This may contribute to smearing of the observed quantal distribution, but it also leaves space for alternative hypotheses, or even for refutation of the quantal theory of local calcium release.

A key argument for accepting the quantal spark structure would be direct evidence that it is possible to resolve single RYR openings in situ, similar as in the case of IP3Rs (Parker and Smith, 2010). Single sarcolemmal L-type calcium channel openings produce detectable signals smaller than the smallest calcium sparks (Wang et al., 2001). Therefore, it is reasonable to assume that single RYR openings lasting at least two sampling periods are detectable. Strong evidence that the smallest

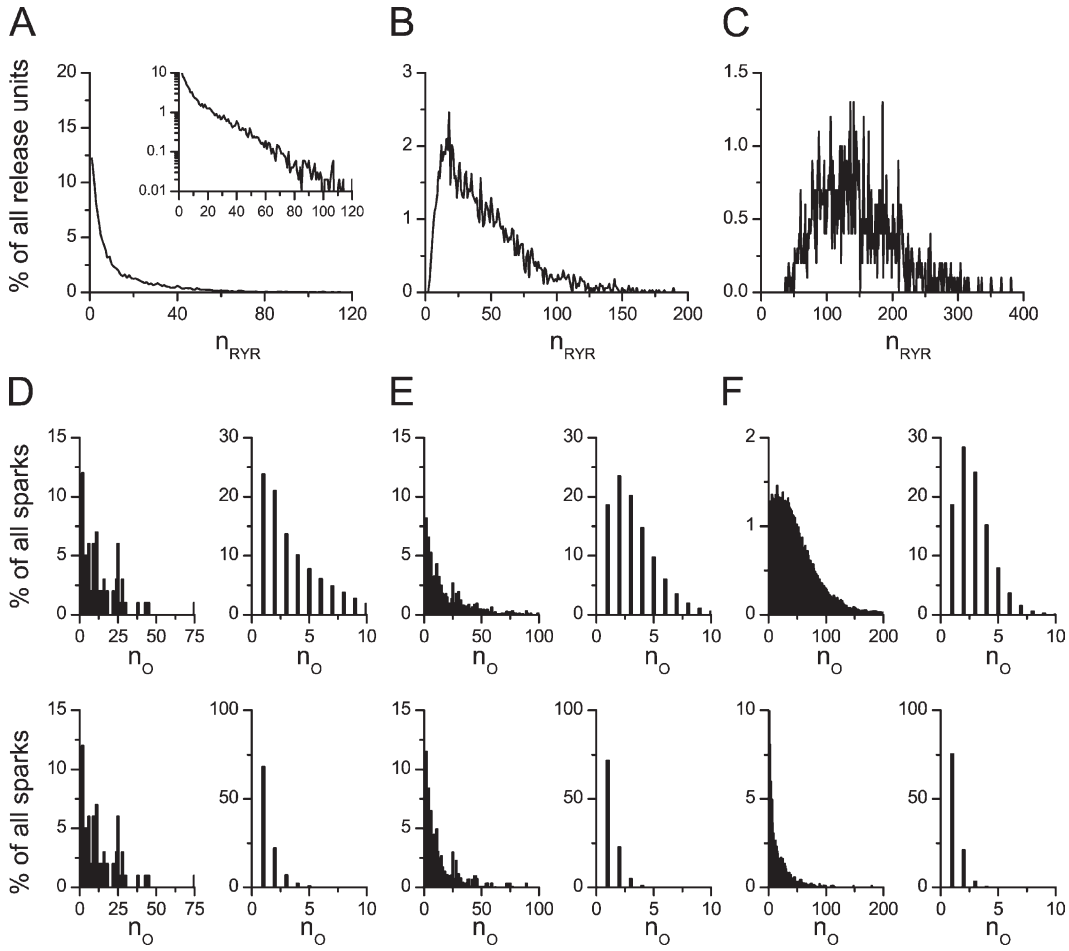


**Figure 1.** Simulation of RYR distribution into clusters and release units and their activation during calcium sparks. (A–C) Distribution of the size of release units (in number of RYRs per release unit, $n_{RYR}$) composed of 1 (A), 3 (B), and 10 (C) random clusters. The number of RYRs in clusters was distributed bi-exponentially according to Baddeley et al. (2009). The average number of RYRs per simulated release unit was 14 ± 18, 43 ± 31, and 145 ± 58 (mean ± SD). (D–F) Average of 100 simulations of the number of open RYRs ($n_O$) in release units formed of 1 (D), 3 (E), and 10 clusters (F), respectively. (Top row) Simulated controls. $P_O$ set to approximate the overall shape of the distribution observed by Wang et al. (2004). (Bottom row) Simulated effects of tetracaine ($P_O$ = 15% of control). (Left columns) Simulations of the release site model of Xie et al. (2010) with control $P_O$ = 0.34 for coupled RYRs. (Right columns) Simulations of the release site model with independent RYRs with control $P_O$ = 0.24, 0.06, and 0.002 for D, E, and F, respectively. For details of simulations see Zahradníková et al. (2010).

in-focus sparks represent single RYR openings comes from analysis of their times to peak, which approximate the open time of the underlying RYR openings, and which were distributed exponentially with a time constant of 11.6 ms (Wang et al., 2004). This value is similar to the mean RYR open time of ∼5 ms, as measured in bilayers in the presence of $Mg^{2+}$ and ATP (Laver and Honen, 2008). On the other hand, the coupling of RYR gating leads to open times prolonged by more than an order of magnitude from ∼10 to >100 ms (Gaburjakova and Gaburjakova, 2010); that is, much above the time to peak of the sparks. It has to be added, however, that the resolution of single RYR openings in focused sparks does not translate to their resolution in randomly positioned scan lines, when the majority of sparks are out of focus. Moreover, because the axial resolution of the confocal microscope is low (typically >600 nm), out-of-focus events can interfere with in-focus events. However, these difficulties can be obviated by cautious interpretation of the data and by experimental designs that reduce the probability of in-focus and out-of-focus events that occur simultaneously.

To summarize, we have provided arguments that both of the questions raised above can be answered positively because the observed quantal nature of calcium sparks could be reproduced by a model of release units with realistic distribution of RYRs into clusters and with RYRs opening independently, in accordance with their behavior in bilayers. On the other hand, a rigorous demonstration that single RYR openings can be resolved within an intact myocyte awaits future investigation.

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