Nonuniform and Variable Arrangements of Ryanodine Receptors Within Mammalian Ventricular Couplons


Rationale: Single-tilt tomograms of the dyads in rat ventricular myocytes indicated that type 2 ryanodine receptors (RYR2s) were not positioned in a well-ordered array. Furthermore, the orientation and packing strategy of purified type 1 ryanodine receptors in lipid bilayers is determined by the free Mg²⁺ concentration. These observations led us to test the hypothesis that RYR2s within the mammalian dyad have multiple and complex arrangements.

Objectives: To determine the arrangement of RYR2 tetramers in the dyads of mammalian cardiomyocytes and the effects of physiologically and pathologically relevant factors on this arrangement.

Methods and Results: We used dual-tilt electron tomography to produce en-face views of dyads, enabling a direct examination of RYR2 distribution and arrangement. Rat hearts fixed in situ; isolated rat cardiomyocytes permeabilized, incubated with 1 mmol/L Mg²⁺, and then fixed; and sections of human ventricle, all showed that the tetramer packing within a dyad was nonuniform containing a mix of checkerboard and side-by-side arrangements, as well as isolated tetramers. Both phosphorylation and 0.1 mmol/L Mg²⁺ moved the tetramers into a predominantly checkerboard configuration, whereas the 4 mmol/L Mg²⁺ induced a dense side-by-side arrangement. These changes occurred within 10 minutes of application of the stimuli.

Conclusions: The arrangement of RYR2 tetramers within the mammalian dyad is neither uniform nor static. We hypothesize that this is characteristic of the dyad in vivo and may provide a mechanism for modulating the open probabilities of the individual tetramers. (Circ Res. 2014;115:252-262.)

Key Words: calcium ■ electron microscope tomography ■ excitation contraction coupling ■ phosphorylation ■ ryanodine receptor calcium release channel ■ sarcoplasmic reticulum

The type 2 ryanodine receptor (RYR2) is an integral membrane protein of the cardiomyocyte sarcoplasmic reticulum (SR) that functions as a Ca²⁺-activated Ca²⁺ ion channel. Each receptor is a homotetramer, measuring roughly 29×29×12 nm, which can be readily identified in electron micrographs based on its location within the dyadic cleft and on its size and shape.1,2 Rotary shadowing studies of type 1 ryanodine receptors (RYR1) in skeletal muscle triads3 and numerous transmission electron micrographs of cardiac muscle4 left the impression that the tetramers filled the dyadic cleft, forming a defect-free crystalline array, often referred to as a checkerboard. The array’s formation is thought to be an intrinsic property of the protein reflecting the homotetramer’s 4-fold symmetry whereby adjacent tetramers were noncovalently connected through their adjacent clamp domains.5 This is also thought to provide the structural basis for interprotein allosteric interactions.6,7 Electron tomography and super-resolution fluorescence microscopy later revealed that the dyad contained subarrays that did not completely fill the cleft, and although neither technique had the resolution to determine the position and orientation of individual tetramers, the super resolution study assumed a regular checkerboard array when fitting their data.8,9 A single-tilt tomogram with higher resolution indicated that the subarrays were unlikely to be fitted with a simple checkerboard.10

RYR1 tetramers, purified from skeletal muscle and inserted in artificial bilayers, spontaneously formed 2 different types of array that depended on the free Mg²⁺ concentration. Using a nominally Mg²⁺-free buffer, the tetramers formed a checkerboard, but with the addition of 4 mmol/L Mg²⁺, the tetramers were more densely packed in a side-by-side orientation although there was no physical contact between them.11,12 The organization of the tetramers at the expected intracellular free Mg²⁺ concentration of ≈1 mmol/L was not investigated. Whether RYR2 behaves similarly, and if such changes can occur in vivo, is unknown.

In this study, we examined dual-tilt tomograms to visualize the position directly of individual RYR2 tetramers in adult rat ventricular myocytes. When fixed in situ, where the Mg²⁺...
is ≈1 mmol/L,13,14 en-face views of the dyads showed RYR2 in arrangements that were neither uniform nor regular. We obtained the same results from cells that were fixed after enzymatic dissociation or from cells that were fixed, permeabilized with the free Mg2+ set to 1.0 mmol/L, as well as from sections of juvenile human ventricle. The tetramer distributions could be moved into more regular arrays by lowering the free Mg2+ concentration to 0.1 mmol/L or by phosphorylation, both of which resulted in a largely checkerboard arrangement, whereas high Mg2+ (4 mmol/L) produced a more densely packed arrangement where the tetramers were largely side-by-side. Changes in tetramer positioning were visible at the earliest time point we examined, which was 10 minutes.

We conclude that the positioning of RYR2 tetramers within mammalian dyads is nonuniform, can change dependent on local factors, and is unlikely to be static.

Methods

Additional details are in the Online Data Supplement.

The experiments used ventricular myocytes from adult rats and left ventricular myocytes from humans. Animal handling was done in accordance with the guidelines of the Canadian Council on Animal Care and approved by the animal research committee of the University of British Columbia. Human tissue was acquired from informed subjects, and the study was approved by BC Children’s Hospital Research Ethics Board. All chemicals were purchased from Sigma-Aldrich (Oakville, ON) unless otherwise stated.

Experimental Protocol

Isolation of rat ventricular myocytes was performed as previously described.15 In the case of human tissue, small sections of the left ventricle were obtained from patients undergoing coronary artery or valve replacement surgery. Within a minute of excision, the sections were cut into cubes roughly 1 mm on a side and then immersed in fixative (4% paraformaldehyde and 2.5% glutaraldehyde). The tissue blocks were then postfixed, dehydrated, embedded in resin, and stained for electron microscopic and tomographic analyses.16

Identification and Placement of Ryanodine Receptors

Dyadic clefts, regardless of their intracellular location, are complex 3-dimensional (3D) structures whose juxtaposed membranes are seldom, if ever, parallel. Even when they seem to be so, undulations in the membranes are commonplace and en-face images of the junctional sarcoplasmic reticulum (jSR), acquired from a vantage point within the cleft, invariably include elements of membrane that intersect the plane of view. We, therefore, viewed the tomograms using Amira (VSG, Burlington, MA) in all 3D to enable a positive identification of each structure. The en-face views obtained from the 3D tomogram were converted to a stack of TIFF images spaced 1 nm apart and passing through the entire width of the dyadic cleft. The stack was then read into a program written by one of us (D.R.L.S.) which allowed us to position a square, 29 nm on a side and outlined in red, over each RYR2 that had been identified; 29 nm is at the upper end of the range of sizes reported for the tetramers’ myoplasmic domain.11,17,18 Placement of the tetramer, to 0.5 nm, was by eye and the orientation could be adjusted in 1° increments. Nearest-neighbor distances (NNDs) were calculated from the tetramers’ centers. This approach allowed us to compensate for any curvature of the cleft because receptor clusters could be accurately visualized in whatever plane they were in focus. In addition, rocking the image back and forth through 1 or 2 planes of a tetramers’ plane of focus enabled us to position them precisely.

Statistical Analyses

Data were reported as mean±SD, and significance was evaluated by nonparametric Kruskal–Wallis test, while pairs of data were analyzed using the Mann–Whitney test with values of P<0.05 being considered significant.

Results

The image displayed in Figure 1Ai is a single plane extracted from the dual-tilt tomogram of a rat myocyte fixed in situ and shows a triad with characteristic jSR (arrows) and its ryanodine receptors on either side of a t-tubule (double arrow). Viewpoints within the volume of the tomogram are determined by the position of orthogonal planes which are outlined in different colors: XY in red, YZ in green, and XZ in blue. In Figure 1Ai, the XZ plane (blue line) has been positioned to parallel, as nearly as possible, the jSR membrane, but to be within the cleft and to bisect the ryanodine receptors on that side of the triad. The intersection point of all 3 planes has been positioned within a single ryanodine receptor identifiable by its characteristic shape (roughly square) and size (≈29 nm each side), which is visible in all 3 orthogonal views: XY (Figure 1Ai), YZ (Figure 1Aii), and XZ (Figure 1Aiii). The XY and YZ views demonstrate that the intersection point of the planes is not within the jSR or the t-tubule membranes. A second YZ plane (yellow line in Figure 1Ai and 1Aii) is within the t-tubule membrane, which in the en-face view (Figure 1Aiii) has a similar size and shape as a RYR2. We used this procedure to differentiate the ryanodine receptors from membranes and other structures in this and subsequent tomograms. The discontinuities that can be seen at the bottom and right of Figure 1Ai and in other figures in this article mark boundaries outside of which the dual planes used to synthesize the image did not overlap; regions beyond these boundaries were excluded from our analysis. Additional examples of identifying membrane and RYR2 are in Online Figure I, and the complete tomogram of this junction, in an XZ orientation, can be viewed in Online Movie I.

An enlarged en-face view of the junction is presented in Figure 1Bi, and in Figure 1Bii, red circles with diameters of 41 nm (equivalent to the diagonal of a 29-nm square tetramer) have been centered over the areas identified as RYR2. Areas that are stained but were not identified as RYR2 tetramers are sections of either SR or t-tubule membrane that were within the plane of view. It is apparent from these images that the RYR2s are not distributed in a well-ordered checkerboard array; an observation that agrees with our previously published single-tilt tomogram.19 The increased clarity and resolution of a dual-tilt tomogram19 enabled us to estimate the position and orientation of each of the tetramers, an example of which is shown in Figure 1C. Individual receptors were first outlined with a dashed yellow line (Figure 1Ci–1Ciii) and then fitted with squares (red), 29 nm on a side (Figure 1Civ and 1CV). The result was an en-face view of the junction in which the
position and orientation of the ryanodine receptors were identified (Figure 1Di), and the nearest-neighbor center-to-center distances were calculated (Figure 1Dii). We have acquired 11 tomograms from 6 hearts fixed in situ and examined 215 tetramers within dyads located on the cell surface and on both axial and transverse tubules. We have also measured the NND of...
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56 tetramers (3 tomograms) acquired from myocytes that were enzymatically dissociated then fixed, and another 30 tetramers (2 tomograms) from dissociated myocytes whose membrane was permeabilized with saponin and the cell was incubated in a solution containing 100 nmol/L Ca\(^{2+}\) and 1 mmol/L Mg\(^{2+}\) before being fixed. The histogram of the combined 301 tetramers’ NND (Figure 2A) shows a broad and bimodal distribution with modes at 32 and 38 nm. Separate histograms for each of the data sets are available in Online Figure II.

Although the tetramers’ positions and orientations were not uniform, an individual tetramer’s position relative to its neighbors could be broadly classified using the following set of criteria: We considered a tetramer to be in a checkerboard arrangement relative to its neighbor(s) if their sides were parallel and separated by ≤ 3 nm and overlapped by ≤ 19 nm (2/3 of its length). If those criteria were fulfilled but the overlap exceeded 19 nm, the tetramers were considered to be side-by-side. Some tetramers had neighbors in both configurations, whereas others had none and were considered isolated. These criteria accommodate the wide range over which neighboring tetramers can overlap, which, as is evident from the images, can vary from complete to just touching at the corners. The NNDs, sorted using the above criteria, are redisplayed in Figure 2B. For a direct comparison with our results, we calculated the NND using the Yin model for purified RYR111 but with a 29-nm square tetramer. This gave NND values of 30.3 nm for the side-by-side configuration (single arrow) and 32.4 nm for the checkerboard (double arrow).

Of the 301 tetramers that were identified (15 tomograms), 140 (46.5%) were in a checkerboard arrangement, 117 (38.9%) were side-by-side, 24 (8.0%) were isolated, and 20 (6.6%) had neighbors in both configurations (Table 1). The mean NND of the tetramers in a checkerboard arrangement was 36.9±2.2 nm (median, 37.0 nm), whereas those in a side-by-side configuration was 30.7±1.2 nm (median, 30.7 nm), and those that were isolated was 42.1±9.3 nm (median, 40.3 nm; Table 2).

To determine whether the RYR2 distribution in human dyads was comparable, we obtained left ventricular tissue from patients undergoing cardiac surgery to repair valve or coronary artery defects; none of the patients were in heart failure or displayed any evidence of hypertrophy. The tomogram of a dyad, from the left ventricle of a 9-year-old boy, and the analysis of its RYR2 distributions are displayed in Figure 3. Orthogonal views are displayed in Figure 3A, with the intersecting planes positioned over a single ryanodine receptor. The mitochondrial cristae are clearly visible and demonstrate that the cell was well preserved. It is apparent from Figure 3Ai and 3Aii that the distance between the t-tubule and jSR membranes is variable, with the result that a single XZ plane (blue line in Figure 3Ai) cannot transect all of the visible ryanodine receptors. In these cases, multiple XZ planes were needed to determine the position and orientation of the receptors, and
in this instance, we display 2, separated by 8 nm. The en-face views of those planes are displayed in Figure 3Bi and 3Bii, and the ryanodine receptors are identified by the 41-nm-diameter red circles in Figure 3Ci and 3Cii. The fitted receptors are shown in the en-face images in Figure 3Di and 3Dii and their summed distribution is in Figure 3Eii. Of the 5 human dyads examined, we identified 84 RYR2 tetramers, 35 (41.7%) of which had neighboring tetramers in a checkerboard configuration, 38 (45.2%) were side-by-side, 5 (6.0%) had a neighboring tetramers in both configurations, and 6 (7.1%) of the tetramers were isolated (Table 1). The mean NND of those with a checkerboard configuration was 37.2±1.8 nm (median, 37.2), whereas the mean NND of the tetramers considered side-by-side was 30.2±1.0 nm (median, 30.2 nm; Table 2). A histogram of the NND is displayed in Figure 3Eii. The results are virtually identical to those obtained from rat ventricular myocytes (Figures 1 and 2).

We hypothesized that the bimodal NND could be explained, in part, by the cells’ expected free Mg2+ concentration, 1 mmol/L, which is between those used to produce the side-by-side and checkerboard arrays of purified RYR1 in vitro. We, therefore, permeabilized isolated rat myocytes with saponin and incubated them for 10 minutes with solutions containing a free Mg2+ concentration of either 0.1 or 4 mmol/L, whereas the free Ca2+ concentration was 100 mmol/L. Although these Mg2+ concentrations are well outside the physiological range, they allowed a direct comparison of our results with those of Yin et al.11,12 Ca2+ spark frequency and caffeine transients were recorded before fixing the myocytes in place, on the coverslips, for electron tomography. Changing the free Mg2+ concentration had the expected effects on the frequency of Ca2+ sparks, which was significantly increased by 0.1 mmol/L Mg2+ and significantly decreased by 4.0 mmol/L Mg2+.20 The caffeine transients were not significantly affected by the different free Mg2+ concentrations (Online Figure III).

The en-face images acquired from cells incubated in 0.1 mmol/L Mg2+ (Figure 4) are remarkable in that the RYR2s are almost all in the checkerboard arrangement. This pattern was obvious even in the raw data (Figure 4A) before the tetramers were positioned (Figure 4B), and their NND was calculated (Figure 4C) and was a consistent observation for the 78 tetramers (5 tomograms, 5 cells, and 4 rats) that we examined. Of those tetramers, 64 (82.1%) were in a checkerboard configuration with a mean NND of 37.2±1.8 nm, whereas only 4 (5.1%) were side-by-side with a mean NND of 31.0±0.4 nm (median, 31.0 nm). Nine (11.5%) of the tetramers were isolated and 1 (1.3%) had neighbors in both configurations. The configurations and their NND are listed in Table 1, and a histogram of the combined NND is displayed in Figure 4D. Additional images from this and subsequent tomograms and movie sequences through their en-face views are available in Online Figures IV to VI and Online Movies II to IV.

In contrast, images acquired from cells incubated in 4.0 mmol/L Mg2+ produced a different result. Two en-face images of a dyadic cleft (Figure 5Ai and 5Bi), separated by 6 nm, demonstrate that the tetramers were more densely packed (Figure 5Ai and 5Bi). The NNDs for this cell are displayed in Figure 5Ci, and comparable results were obtained from the 5 tomograms we examined (5 cells and 5 rats). In these tomograms, we identified 97 tetramers, 67 (69.1%) were side-by-side with a mean NND of 29.4±1.0 nm (median, 29.3 nm) and 16 (16.5%) were in a checkerboard configuration with a mean NND of 34.7±3.0 nm (median, 34.3 nm). The results are listed in Table 1, and a histogram of their combined NND is displayed in Figure 5Cii.

We then assessed the effect of phosphorylation on tetramer distribution in permeabilized myocytes in the presence of 1 mmol/L free Mg2+ and 100 mmol/L free Ca2+. This increased the calcium spark frequency (Online Figure III), and a Western blot demonstrated that the receptor had been phosphorylated on S2814 (Online Figure VIIA and VIIB). The change in phosphorylation status and function was paralleled by changes in the tetramer distribution, as shown in Figure 6. Three en-face planes of the tomogram are displayed both as raw data (Figure 6Ai, 6Bi, and 6Ci) and with their tetramers positioned (Figure 6Aii, 6Bii, and 6Cii). Figure 6Di displays the distribution and NND of these tetramers. The 56 tetramers identified in 5 different tomograms demonstrated that 50 (89.3%) were in a checkerboard arrangement with a mean NND of 37.3±1.8 nm, with only 3 (5.4%) that were side-by-side. The data are listed in Table 1, and a histogram of their NND is shown in Figure 6Dii.

### Discussion

The current view of RYR2 disposition within the cardiac dyad is derived largely from scanning electron micrographs of skeletal muscle RYR1, coupled with 2D images of cardiac muscle dyads.3,4 Our results, which use the 3D capability of electron tomography, have provided views of the dyad and its associated RYR2 that could not have been previously obtained. We have shown that under physiological conditions, the distribution of RYR2 within a dyad is neither homogenous nor well structured (Figure 1) and that when the environment is altered, the arrangement of the tetramers changes quickly.

The pseudocrystalline array of RYR1 observed in lipid bilayers subjected to various concentrations of Mg2+ implies that the NND between the tetramer centers within a dyad should (assuming the 29-nm square tetramer that we used in our calculations) be either 30.3 nm (side-by-side array) or 32.4 nm.
The latter value imposes an overlap of 14.5 nm between the sides of the RYR1, allowing interaction between adjacent tetramers’ clamp domains, should it occur. The NND of the combined rat and human data (Figure 2; Online Figure IIA–IIC) shows that even under normal physiological conditions, the arrangement of the tetramers cannot be characterized using a simple 2-state model. In addition, the distribution of the NND of those tetramers that could form a checkerboard arrangement had a roughly bell shape with a peak at 38 nm, a value far greater than that of the RYR1 model. Furthermore, such a broad distribution implies that under normal physiological conditions, the tetramers are not in any fixed position, and although they often abut, their degree of overlap is highly variable, suggesting that the positioning of the tetramers...
is dynamic and that fixing the cell is providing a snapshot of this process. This contention is supported by our observations of the effects of phosphorylation and varying the free Mg\textsuperscript{2+} concentration, all of which led to a dramatic change in the positioning and the NND distributions compared with the controls (cells fixed in situ; cells isolated and fixed; cells isolated, permeabilized with the free Mg\textsuperscript{2+} set to 1 mmol/L and the free Ca\textsuperscript{2+} to 100 mmol/L, and then fixed). Notably, saponizing the sarcolemma had no effect on the results (Online Figure IIC).

These findings are controversial and we, therefore, analyzed our methodology to determine whether the large value for the NND and the broad distribution could be artifacts arising from the identification, placement, and measurement process.

Identifying the tetramers and differentiating them from membrane and other structures, although laborious, are straightforward when using the 3 orthogonal images as a guide, provide a general location for the position of a tetramer, and have a low error rate. The final positioning of the tetramer, by fitting the 29-nm square box on the en-face (XZ) view of the dyad, has an inherent error of \(\approx 1\) nm because of the thickness of the line drawn on the screen. The positioning and orientation were often helped by the tetramers having a clear edge or corner and being adjacent to other tetramers. The latter situation was particularly useful because a single well-defined tetramer was used to seed the position of the adjacent tetramers given that they cannot overlap. Although our judgment of best fit was done by eye, it was constrained by these conditions. In addition, we have individually reviewed the positioning of the tetramers and found that differences rarely exceeded 3 nm. Even when the position was in dispute, the large number of tetramers examined (<600) meant that such errors would have little or no effect on the NND distributions. Heavy metal staining, a requirement for generating the electron tomographic image, sometimes gave the tetramer unclear boundaries, and if there were no adjacent tetramers, we positioned the tetramer in the center of the blob and guessed as to the orientation. Such situations were also rare and had little influence on the final result given the large number of tetramers that we identified. The determination of the exact orientation of the receptor is the weakest part of this process, but because we used the center-to-center distances for calculating the NND, any uncertainty in the orientation has no effect on the values in the histograms or on the conclusions we reached.

We also analyzed the effect that curvature or the undulating nature of the dyad would have on these results. Our technique maps the tetramer positions onto a horizontal plane above the dyad which is where we calculate NND, so the effect of a curvature would be to decrease the distances calculated, leading to an underestimate. However, the effect is minimized because tetramer clusters tend not to straddle a curve (Figure 6), and even if they do, the error is a few percent at most because the change in depth over the length of a dyad (\(\geq 250\) nm) was never >25 nm, less than the width of a single tetramer.

Another possibility is that we have a scaling error and that the real size of boxes fitted to the tetramers is <29 nm. There are many arguments against this hypothesis: first the boxes fit the tetramers well and in many cases tetramers abut (see Figure 1 for example); any larger and they would overlap which is clearly impossible. Second, the error would have to be consistent in each of the 52 tomograms collected as well as quite large (\(\approx 20\%\)). Last, the NND for the side-by-side tetramers is close to that predicted by Yin et al,\textsuperscript{11} suggesting that our scaling is reasonably accurate.

There was a dramatic widening of the SR lumen in low Mg\textsuperscript{2+} (Online Figure IVD). This was not because of low osmotic pressure and simple cellular swelling as the mitochondrial cristae were clearly visible and well preserved. Because the peak...
NND (39 nm) was no different from that seen for tetramers in a checkerboard configuration in both the control (38 nm) and phosphorylated cells (38 nm), the phenomenon cannot be due to an overall expansion of the SR because it would be expected to shift the peak NND to larger values. We hypothesize that structural elements holding the junctional SR membranes together require bound Mg$^{2+}$, and in its absence, the membranes separate and the lumen of the junctional SR widens.

The changes that we observed in the distribution and its associated NND when phosphorylating the receptors or changing the free Mg$^{2+}$ concentration also support our contention that the results in Figure 2 are not an artifact. Both phosphorylation (Figure 6Di) and low Mg$^{2+}$ (Figure 4D) reoriented the tetramers into a largely checkerboard formation with a peak NND of 39 nm, close to the 38 nm found in our controls, whereas high Mg$^{2+}$ packed the tetramers into a mostly side-by-side formation with a peak of 30 nm (Figure 5Cii), close to the Yin model for side-to-side tetramers. The distribution of the tetramers under control conditions would seem to be a mix of these 2 extremes that could be explained by an intermediate Mg$^{2+}$ concentration (1 mmol/L) and basal phosphorylation of RYR2.

All of these considerations led us to conclude that the large values and broad distribution of the NND of tetramers in a checkerboard arrangement, and the dramatic alterations in the distribution following changes in the environment, were real and evidence for the tetramers being both mobile and able to respond to changes in physiological and pathological stimuli by repositioning within the dyadic cleft. Importantly, tomograms of human myocytes produced qualitative and quantitative results that were indistinguishable from those obtained from the rat under control conditions (Figure 3; Online Figure III). It is, therefore, likely that human RYR2s are equally mobile and would respond to local changes in phosphorylation and Mg$^{2+}$ concentration in a similar manner.

Figure 5. Type 2 ryanodine receptor distribution in a permeabilized rat cardiomyocyte with a free Mg$^{2+}$ concentration of 4.0 mmol/L. Scale bars are 30 nm. Ai, XZ image of the dyadic cleft. ii, Final position and orientation of the tetramers that were well defined in Ai. Bi, XZ image of the dyadic cleft 6 nm deeper in Y. ii, Final position and orientation of the tetramers that were well defined in Bi. Ci. The distribution and orientation of all of the tetramers along with their nearest-neighbor distances (NNDs) in nm. ii, Histogram showing the NND of all 87 tetramers identified in 5 similarly treated cells.
Comparing the tetramer organization with the spark frequencies, it is notable that stimuli which organized the tetramers into a largely checkerboard arrangement (low Mg\textsuperscript{2+} and phosphorylation) were associated with marked increases in the spark frequency compared with the control where the arrangement was mixed (Figures 1Bi and 2). In contrast, a high Mg\textsuperscript{2+} concentration organized the tetramers into a side-by-side configuration (Figure 5; Online Figure V) and was associated with a significant decrease in the spark frequency. The relationship is nonlinear because phosphorylation or low Mg\textsuperscript{2+} doubles the proportion of tetramers in a checkerboard configuration compared with control, whereas the spark frequency increases ≈6-fold. These results suggest that a correlation exists between the tetramers’ arrangement and their open probability, which would fit well with our observation in control cells of the

Figure 6. Type 2 ryanodine receptor distribution in a permeabilized rat cardiomyocyte dyad after phosphorylation. Scale bars are 30 nm. Because of the unevenness and curvature of the dyad, 3 separate planes A, B (6 nm deeper in Y than A), and C (20 nm deeper in Y than A) were required to identify all of the tetramers. Ai, Bi, and Ci are raw images; Aii, Bii, and Cii are the final orientation and position of the tetramers. Di, The distribution and orientation of all of the tetramers along with their nearest-neighbor distances (NNDs) in nm. ii, Histogram showing the NND of all 55 tetramers identified in 5 similarly treated cells.
tetrads being in a mixed configuration and giving rise to an intermediate spark frequency.

Although the 0.1 and 4 mmol/L concentrations of Mg\textsuperscript{2+} are unlikely to occur in vivo, we have shown that the tetrads move in response to changing the Mg\textsuperscript{2+} concentration and to changes in the phosphorylation levels. A question that arises is why both phosphorylation and a low Mg\textsuperscript{2+} produce the same tetramer arrangement and are associated with an increase in the spark frequency. Li et al\textsuperscript{22} observed that RYR2 phosphorylation produced a rightward shift in the Mg\textsuperscript{2+} concentration dependency of RYR2 inhibition, which they interpreted as a decrease in the tetrads’ affinity for Mg\textsuperscript{2+}. If true, phosphorylation might reduce the amount of bound Mg\textsuperscript{2+}, shifting the tetrads into a checkerboard arrangement.

There is no obvious reason why the tetrads would shift their relative positions as they alter their open probability, but a possible explanation is the experimental observations of, and the theoretical models proposing, interprotein allosteric interaction. Positive allosteric interaction has been reported between adjacent RYR\textsuperscript{26} and RYR\textsuperscript{17} tetrads in vitro, although the former used no Mg\textsuperscript{2+} in their solutions, the latter observed an inverse relationship between the free Mg\textsuperscript{2+} concentration and the degree of allosteric interaction. RYR2 phosphorylation has also been reported to increase the synchrony of Ca\textsuperscript{2+} release, among other actions.\textsuperscript{23} These results, coupled to our own, imply that the checkerboard configuration is associated with more tightly co-ordinated channel openings and possibly with positive allosteric interaction. However, if there is positive allosteric interaction, it is unlikely to be explained by a simple model involving a fixed position on the tetramer’s clamp domain because the degree of overlap between adjacent tetrads is highly variable. The correlation between low spark frequency and the side-by-side configuration may represent evidence for the theoretically proposed negative allosteric interaction\textsuperscript{24} but remains speculative in the absence of any other confirming data.

In conclusion, our results provide a new framework for investigating, understanding, and modeling the function of the dyad.

Acknowledgments

We thank the University of British Columbia BioImaging Facility, Saira Mohammed, and the staff in the Departments of Pediatrics and Surgery at BC Children’s Hospital for their assistance. P. Asghari performed all the experimental procedures, including electron tomography, Western blot, spark analysis, and tetramer fitting. Human ethics approval, patient contact and consent, and surgical samples were provided by S.K. Gandhi, A.I.M. Campbell, and S. Sanatani. P. Asghari, D.R.L. Scriven, and E.D.W. Moore wrote the article and analyzed the data. D.R.L. Scriven wrote the tetramer fitting program.

Sources of Funding

E.D.W. Moore received Canadian Institutes of Health Research Grant (MOP 115158) and a Natural Sciences and Engineering Research Council (NSERC) Discovery Grant. P. Asghari received NSERC Canada Graduate Scholarship and University of British Columbia College of Interdisciplinary Studies Graduate Awards.

Disclosures

None.

References

What Is Known?

- Based largely on 2-dimensional electron micrographs, it is thought that type 2 ryanodine receptors (RYR2s) within the mammalian couplon are positioned in a regular checkerboard array.
- It is tacitly assumed that the position of the tetramers is fixed.
- Ryanodine receptors that have been purified from skeletal muscle (type 1 ryanodine receptors) and examined in artificial bilayers form different arrays depending on the free Mg²⁺ concentration; a regular checkerboard array in a nominally Mg²⁺-free solution and a more densely packed side-by-side array in 4 mmol/L Mg²⁺.

What New Information Does This Article Contribute?

- The distribution of RYR2 in couplings of cardiomyocytes is irregular and contains a mix of both checkerboard and side-by-side arrangements; rat hearts and sections of human heart give identical results.
- The tetramer arrangements depend on the Mg²⁺ concentration or on their phosphorylation status; in low Mg²⁺ and after phosphorylation, RYR2s are largely positioned in a checkerboard arrangement, whereas in response to high Mg²⁺, the tetramers are positioned largely side-by-side.
- The 3 observed tetramer arrangements, side-by-side, mixed, and checkerboard, are associated with progressively increasing spark frequencies.

Novelty and Significance

The timing and magnitude of Ca²⁺ sparks are determined in part by the mechanisms that control RYR2 gating and by the tetramers’ positions relative to each other. The latter is important because the efficacy of interprotein calcium-induced calcium release is affected by the distance between tetramers and because of the possibility of allosteric interactions between them. Applying electron tomographic techniques to rat and human hearts, we have demonstrated that the tetramers are not positioned in a regular checkerboard array but in an arrangement that is neither regular nor uniform. We hypothesize that this mixed, irregular distribution likely reflects the resting state because the tetramers’ relative positions could be changed either by altering the free Mg²⁺ concentration or by phosphorylation. Tetramers in a checkerboard arrangement have center-to-center nearest-neighbor distances that vary considerably, with a mean that was significantly greater than that recorded for type 1 ryanodine receptor, implying that allosteric interactions in RYR2 are unlikely to occur through previously suggested mechanisms. The correlation between tetramer arrangement and spark frequency suggests that rearrangements of the tetramers may be another mechanism whereby physiological processes operate. These findings provide potential new mechanisms by which the activity of RYR2 tetramers, the dyad, and cardiac contractility may be regulated.
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Circ Res. 2014;115:252-262; originally published online April 30, 2014;
doi: 10.1161/CIRCRESAHA.115.303897

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Data Supplement (unedited) at:
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Asghari et al. – RYR2 Tetramer arrays are non-uniform and variable.
Online Figure II

A

B

C
Online Figure III

A

![Graph showing sparks/s/mm² vs mmol/l free Mg²⁺](image)

B

![Graph showing I/I₀ vs mmol/l free Mg²⁺](image)
Online Figure VII

(A) 

MW | Control, Intact | Saponin | Phosphorylation Cocktail
---|-----------------|---------|--------------------------
360- | 360- | 360- |
268- | 268- | 268- |
163- | 163- | 163- |
117- | 117- | 117- |

RyR2-S2814

(B) 

MW | Control, Intact | Saponin | Phosphorylation Cocktail
---|-----------------|---------|--------------------------
55- | 55- | 55- |
18- | 18- | 18- |

CSQ | Cav3

Relative RyR2-S2814 Phosphorylation

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<th>Saponin</th>
<th>Phosphorylation Cocktail</th>
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<td>0.4</td>
<td>0.6</td>
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<td>Phosphorylation Cocktail</td>
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<td>1.2</td>
<td>1.4</td>
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</table>

Normalized Intensity

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<th>Phosphorylation Cocktail</th>
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<tr>
<td>Phosphorylation Cocktail</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

CSQ | Cav3
Supplemental Material

Materials and Methods

Rat Ventricular Cell isolation
We used male Wistar rats, 200 – 300 grams, (Charles River Laboratories, Wilmington, MA) that were given an intraperitoneal injection of 2 ml of 1000 units of heparin (Hepalean; Organon, Mississauga, ON) followed 30 minutes later with an intraperitoneal injection of 2 ml of sodium pentobarbital (240 mg/ml; MTC Pharmaceuticals, Cambridge, ON). The experiments proceeded only after hard pressure on the footpad failed to produce a withdrawal reflex.

The isolation technique is based on the method of Rodrigues and Severson1: The hearts were excised, hung on a Langendorff apparatus and perfused for 5 min. at 37°C with a nominally Ca²⁺-free physiological saline solution, PSS, (in mmol/l): 138 NaCl, 5 KCl, 0.3 KH₂PO₄, 0.3 Na₂HPO₄, 10 HEPES, 15 D-glucose, 1 creatine, 1 carnitine, pH 7.4) that had been equilibrated with 95% O₂/5% CO₂. Cell dissociation was initiated by switching to a perfusate of PSS containing 0.5 mg/ml Type II Collagenase (Worthington Biochemical, Lakewood, NJ) and 1 mg/ml bovine serum albumin. When the heart began to soften, the ventricles were cut free and sliced into small chunks, which were gently shaken to dislodge cells. These were filtered through a 200 µm nylon mesh (Nitex) into fresh PSS. There were typically greater than 90% quiescent, rod-shaped, cells.

Ca²⁺ Sparks in Permeabilized Myocytes
Isolated cells were suspended in a solution (final volume 50 μl) containing (mmol/l): potassium aspartate 100; KCl 20; EGTA 0.5; MgCl₂ 0.75; and HEPES 10; (adjusted to pH 7.2 with KOH), and placed in the experimental chamber for 15 min. Laminin-coated (5 μg/cm²; Roche Diagnostics, Laval, QC) glass-bottom grid-50 μ-dishes (ibidi, Verona, WI) were used as the experimental chamber to relocate cells for electron microscopic examination later. The cell membrane was permeabilized by adding 0.005 % (w/v) saponin for 30 s. After 30 s the bath solution was exchanged to a saponin-free internal solution composed of (mmol/l): potassium aspartate 100; KCl 15; KH₂PO₄ 5; NaATP 5; EGTA 0.5; CaCl₂ 0.2; phosphocreatine 10; HEPES 10; fluo-4 potassium salt 0.03 (Molecular Probes, Eugene, OR); creatine phosphokinase 5 U ml⁻¹; 40000 MW dextran 8 %; pH 7.2 (adjusted with KOH). In addition, the solution contained MgCl₂ at one of the following concentrations (in mmol/l): 2.4, 5.5 or 8.0, giving a free [Mg²⁺] in solution of 0.1, 1, or 4 mmol/l respectively. In all cases the free [Ca²⁺] was 100 nmol/l. The concentrations of MgCl₂ and CaCl₂ were determined using WEBMAXC Extended (http://maxchelator.stanford.edu/webmaxc/webmaxcE.htm) with a temperature of 22°C, 0.1N ionic strength and pH of 7.2. After a 10 min incubation period Ca²⁺ sparks were recorded using a Zeiss AxioObserver inverted microscope with a 20X/1.4 NA water immersion objective and a narrow bandpass filter (Semrock, Rochester, NY) optimized
for fluo-4. Images were captured on an Evolve 512 CCD camera (Photometrics, Langley, BC) and the sparks were analyzed using Fiji and spark frequencies were expressed as sparks/s/mm². The SR Ca²⁺ content was evaluated by the addition of 20 mmol/l caffeine. The cells were immediately fixed in a solution containing 4% paraformaldehyde and 2.5% glutaraldehyde for 2 hours at room temperature and then prepared for electron-microscopy (EM) as described below.

**Phosphorylation**

After baseline Ca²⁺ sparks were recorded for 60 s in permeabilized myocytes exposed to a free Mg²⁺ concentration of 1 mmol/l, and a free Ca²⁺ concentration of 100 nmol/L, a saponin-free internal solution containing 1 μmol/l thapsigargin, was added to the myocyte suspension to prevent Ca²⁺ uptake into the SR. After incubating for 10 min the cells were washed two times with the saponin-free internal solution, after which the cells were bathed with internal solution to which we added a phosphorylation cocktail consisting of (μmol/l): 10 c-AMP, 10 3-isobutyl-1-methylxanthine, 10 okadaic acid, 0.5 calyculin A and 1 thapsigargin, pH 7.2 (KOH). This cocktail activates kinases including protein kinase A and calcium-calmodulin-dependent protein kinase II; it deactivates phosphodiesterases and inhibits phosphatases PP1 and PP2. After a 10 min incubation period Ca²⁺ sparks were recorded for 60 seconds after which 20 mmol/l caffeine was applied, then the cells were fixed in a solution containing 4% paraformaldehyde and 2.5% glutaraldehyde for 2 hours at room temperature and prepared for EM. The addition of thapsigargin in this protocol prevented the Ca²⁺ content of the SR from rising, which after the addition of caffeine, just before fixation, was lower than in the control cells (1 mmol/l Mg²⁺) (Online Figure IIIB) due to the accelerated spark rate (Online Figure IIIA).

The cells for the phosphorylation, isolated fixed, and 1 mmol/l Mg²⁺ protocols were obtained at the same time from each animal, then split into these three groups and processed accordingly. The results obtained from the isolated fixed, and the 1 mmol/l Mg²⁺ experiments (Tables 1 and 2) served as controls for the phosphorylation.

**Confirmation of RYR2 phosphorylation**

We used a standard Western Blot protocol to confirm phosphorylation of RyR2 by the phosphorylation cocktail. The 6X sample buffer included: 350 mmol/l Tris-Cl (pH 6.8), 30% glycerol, 10% SDS, 600 mmol/l dithiothreitol, NaF (6 mmol/l) and a protease inhibitor (P-8340, Sigma) was directly added to the cells before and after treatment. The samples were frozen in liquid N2 for storage at -80°C until use. Protein (quantified via a Bradford Protein assay, Bio-Rad 500-0001, Hercules, CA) was separated on 6% SDS-polyacrylamide gels and transferred to nitrocellulose membranes overnight at 4°C (BioRad, Hercules, CA). A polyclonal antibody to the Phospho Serine-2814 (A010-31 AP; Badrilla Ltd., Leeds, UK) was used to detect whether RyR2 was phosphorylated. To establish the loading control, each membrane probed with phospho-antibody was washed, duplicates were split and one was reprobed with an antibody to calsequestrin (rabbit polyclonal; PA1-913, Affinity BioReagents) while the other was reprobed with an antibody to caveolin-3 (mouse monoclonal; 610420, BD Biosciences). Protein bands
were visualized using the SuperSignal West Femto Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL).

**In Situ Fixation**

In rats, after the foot pad reflex failed to produce a response, the chest was opened, the aorta and vena cava were cannulated and the hearts were perfused for 10 min with physiological saline solution (PSS) followed by a fixative containing 4% paraformaldehyde, 2.5% glutaraldehyde and 4 mmol/l CaCl₂ in a 0.1 mol/l cacodylate buffer (pH 7.4; Canemco & Marivac Inc, Lakefield, PQ), for 10 min. The left ventricle was then removed, cut into small blocks and the samples immersed in fixative for about 2 hours, after which we proceeded as described below.

**Preparation for Tomography**

Tissue or cells immersed in fixative were cyclically microwaved (2 min on, 2 min off, 2 min on) in a vacuum using a Pelco 3450 laboratory microwave (Ted Pela Inc., Redding, CA), at power 5, then rinsed and microwaved twice in 0.1 mmol/l cacodylate buffer for 40 seconds at power 1, then post-fixed with 1% OsO₄ solution (EMS, Hatfield, PA), at power 1, then cyclically microwaved twice. En bloc staining of samples was done with 2% aqueous uranyl acetate (Ted Pela Inc.), cyclically microwaved twice at power 1, then rinsed three times with distilled water followed by dehydration in ethanol (50-100% in steps of 10%; microwaved 1 min at each dilution on power 3), then embedded in a mixture of Epon and Spur's resin².

From blocks demonstrating well-preserved ultrastructure, serial semi-thick (200 nm - 300 nm) sections were cut using a Leica Ultracut T with a Pelco Ultra 45⁰ diamond knife (Ted Pela Inc) and collected on 0.5% w/v Formvar coated slot grids. Post staining with 2% uranyl acetate for 30 min was followed by Sato’s lead citrate for 15 min³.

**Image Acquisition and Tomography**

Grids were placed in a rotating, dual high-tilt stage and observed in the 200 kV Tecnai G2 transmission electron microscope (FEI, Hillsboro, OR). A suitable junction was imaged twice from orthogonal directions with serial tilt views ranging from +65° to -65° at 1° increments using TIA (FEI), an automated acquisition program. The 3D volumes were reconstructed using either real-space back-weighted projection or the simultaneous iterative reconstruction technique (SIRT) implemented in Inspect 3D (FEI). Dual-tilt alignment and visualization was done using Amira 5.3 (VSG, Burlington, MA). Dual data sets examined using the Multi Planner Viewer, a sub-application in Amira 5.3. No image processing steps beyond the contrast stretch were employed.
**Supplemental Results**

Online fig. I shows two different sets of orthogonal views of the tomogram presented in Figure 1 (main manuscript). Fig. IAi is in the XY orientation and shows that the XZ (blue) and YZ (green) planes intersect within the jSR membrane; the jSR is indicated by the white arrow. This is also apparent in the YZ orientation (Fig. IAii) showing the intersection point of the XY (red) and XZ (blue) planes. In the en face orientation, Fig. IAiii, it is not possible to identify that point as a RYR2 or a section of membrane without the orthogonal views in Figs. IAi and IAii. Fig. IB shows a comparable set of images where the intersection point of the planes is now centered within a RYR2, and well above the jSR membrane.

Online fig. II displays histograms of the tetramer's nearest neighbor distances for myocytes fixed in situ (IIA), for myocytes that were enzymatically isolated and then fixed (IIB), and for myocytes that were enzymatically isolated, permeabilized and incubated for 10 minutes in a solution with free Ca$^{2+}$ and Mg$^{2+}$ concentrations of 100 nmol/L and 1 mmol/L respectively (IIC), and then fixed.

Online fig. III shows the spark frequency (A) and the SR Ca$^{2+}$ content (B) for cells that were permeabilized and exposed to the indicated solution plus 100 nmol/L free Ca$^{2+}$ for 10 minutes prior to recording. Incubating the cells for ten minutes in the phosphorylation cocktail or in 0.1 mmol/L Mg$^{2+}$ both produced significant increases in spark frequency compared to 1 mmol/L Mg$^{2+}$. The 4.0 mmol/L Mg$^{2+}$ produced a significant decrease and the different solutions produced no significant differences in SR Ca$^{2+}$ content. The effect of Mg$^{2+}$ on spark frequency agrees with previously published analyses$^4$. The phosphorylation cocktail ensured that the SR Ca$^{2+}$ content was roughly comparable between the different treatments, and would have activated protein kinase A, Epac and CamKII.

Online figs. IV, V and VI provide additional images of the tomograms for cells that were permeabilized and exposed to 100 nmol/L Ca$^{2+}$ and to one of: (IV) 0.1 mmol/L Mg$^{2+}$; (V) 4 mmol/L Mg$^{2+}$; (VI) cAMP with phosphodiesterase and phosphatase inhibitors. In each of the figures (A) shows the tomogram in XY (i), YZ (ii) and XZ (iii) orientations with the planes intersecting within an RYR2 tetramer. Single arrow – jSR; double arrow – t-tubule. (B) (odd numbers) displays an XZ view, or multiple XZ views separated in Y by the indicated values (6 nm between Vi and Viii; 6 nm between Vi and iii; 20 nm between Vi and v), and (even numbers) highlights the RYR2 with a red circle 41 nm diameter. (C) (i) shows magnified views of one of the XZ planes where the insets highlight several RYR2 (ii-v). The magnified RYR2 are outlined by hand and fitted with appropriately positioned and oriented tetramers. We noted that the SR was enlarged when incubating the cells in 0.1mmol/L Mg$^{2+}$, but as Fig IVD demonstrates, the mitochondrial (M) cristae are well preserved indicating that the effect is not due to osmotic stress. The cause of this effect is unknown. T, t-tubule; SR, sarcoplasmic reticulum.
Online fig. VIIA displays the Western blots probed with anti-S2814. An average of three western blots demonstrated that the phosphorylation cocktail produced a significant increase in S2814 phosphorylation, an epitope phosphorylated by both CAM KII and PKA. Fig VIIB displays Western blots of the proteins selected as controls, calsequestrin and caveolin-3, and the normalized intensity of the bands.

References