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The FKBP12 subunit modifies the long-range allostereism of the ryanodine receptor

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Abstract

Ryanodine receptors (RyRs) are large conductance intracellular channels controlling intracellular calcium homeostasis in myocytes, neurons, and other cell types. Loss of RyR's constitutive cytoplasmic partner FKBP results in channel sensitization, dominant subconductance states, and increased cytoplasmic Ca^{2+} . FKBP12 binds to RyR1's cytoplasmic assembly 130 Å away from the ion gate at four equivalent sites in the RyR1 tetramer. To understand how FKBP12 binding alters RyR1's channel properties, we studied the 3D structure of RyR1 alone in the closed conformation in the context of the open and closed conformations of FKBP12-bound RyR1. We analyzed the metrics of conformational changes of existing structures, the structure of the ion gate, and carried out multivariate statistical analysis of thousands of individual cryoEM RyR1 particles. We find that under closed state conditions, in the presence of FKBP12, the cytoplasmic domain of RyR1 adopts an upward conformation, whereas absence of FKBP12 results in a relaxed conformation, while the ion gate remains closed. The relaxed conformation is intermediate between the RyR1-FKBP12 complex closed (upward) and open (downward) conformations. The closed-relaxed conformation of RyR1 appears to be consistent with a lower energy barrier separating the closed and open states of RyR1-FKBP12, and suggests that FKBP12 plays an important role by restricting conformations within RyR1's conformational landscape.

Keywords

Calcium; cryo-electron microscopy; excitation-contraction coupling; FKBP12; ryanodine receptor

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Author contributions

M.S. conceived the idea and wrote the manuscript, T.S. and M.S. developed the methodology and analyzed the data, T.S. reviewed the manuscript.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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1. Introduction

RyR is an intracellular calcium release channel that plays a fundamental role in muscle contraction. Upon stimulation by the adjacent dihydropyridine receptor (DHPR), RyR opens and releases large amounts of Ca^{2+} down its gradient from the endo/sarcoplasmic reticulum into the cytosol, which triggers excitation-contraction coupling in muscle (Eisner et al. 2017, Hernandez-Ochoa et al. 2015). Activation of RyR also generates downstream effects in neurons (Abu-Omar et al. 2018) and other cell types (Hosoi et al. 2001, Lewarchik et al. 2014). The three mammalian isoforms are RyR1, RyR2 and RyR3: skeletal muscle expresses RyR1, cardiac muscle expresses RyR2, and brain expresses all isoforms. They are also expressed in other cell types. The activity of RyR is exquisitely controlled mostly through its large, megadalton-sized cytoplasmic assembly (CytA) that contains multiple binding sites for different effectors (Samsó 2017). Among these, a main allosteric regulator is the FK506-binding protein (FKBP), which is tightly bound to RyR and considered an integral subunit (Timerman et al. 1993). Skeletal muscle isoform expresses a 12-kDa FKBP (FKBP12) and cardiac muscle additionally expresses a 12.6-kDa (FKBP12.6) (Gonano and Jones 2017). FKBP12 binds at four equivalent sites of the homotetrameric RyR1 and ~130 Å away from the ion gate (Samsó et al. 2006, Wagenknecht et al. 1997). A similar study indicated that FKBP12.6 binds at a similar position in RyR2 (Sharma et al. 2006). Single channel work showed that lack of FKBP results in higher sensitivity to Ca^{2+} , longer mean open times, and the appearance of subconductance states (Ahern et al. 1997, Ondrias et al. 1996). In vivo, deficiency or lack of FKBP12 or FKBP12.6 leads to aberrant function and heart disease (Shou et al. 1998, Sood et al. 2008, Yano et al. 2000).

The architecture of RyR is comprised of a large scaffold-shaped CytA and a transmembrane domain (TmD). The CytA has a peripheral region and a central domain that serves as interface with the TmD. The peripheral region of the CytA contains multiple subdomains, namely an N terminal domain (NTD) composed of the A-B-C domains, three SPRY domains, the P1 and P2 domains, a handle domain, and a long helical domain (HD) that connects to the SPRY2 domain of the adjacent subunit (reviewed in (Samsó 2017)). The TmD displays an S1–S6 alpha helical design typical of voltage-gated cation channels (Samsó et al. 2005, Yan et al. 2015, Zalk et al. 2015). CryoEM of the RyR1-FKBP12 complex revealed how cytoplasmic effectors induce long-range conformational changes that are coordinated with a change in diameter of the ion gate (Bai et al. 2016, des Georges et al. 2016, Samsó et al. 2009).

Here we demonstrate that FKBP12 has a direct effect on the conformation of RyR1. We analyzed closed RyR1 with and without FKBP12 in the context of the closed-to-open motions of RyR1-FKBP12, and dissected the conformational changes in the cytoplasmic and transmembrane domains. To further evaluate this conformational change, we carried out multivariate statistical analysis on the original dataset of the raw images of the individual channels.

2. Materials and methods

2.1. Sample preparation, cryo-EM and 3D reconstruction

We analyzed the single-particle datasets corresponding to three 3D reconstructions previously obtained: rabbit RyR1 in closed-state conditions (20 mM Na-MOPS pH 7.4, 0.9 M NaCl, 0.5% CHAPS, 2 mM DTT, 2 mM EGTA, protease inhibitors (Samso et al. 2005), RyR1-FKBP12 in the same closed-state conditions (Samso et al. 2009), and RyR1-FKBP12 in open-state conditions (20 mM Na-MOPS pH 7.4, 0.9 M NaCl, 0.5% CHAPS, 2 mM DTT, 50 μ M CaCl₂, 10 μ M PCB95) (Samso et al. 2009). The EM DataBank (www.emdatabank.org) accession codes are emd_5014 for RyR1 closed, emd_1606 for RyR1-FKBP12 closed, and emd_1607 for RyR1-FKBP12 open. The probability of opening (P_o) of RyR1-FKBP12 in the closed state was 0 and the P_o for the open state was 0.96, as measured by single channel analysis (Samso et al. 2009). CryoEM was performed on a Tecnai F20 operated at 200 kV and at a magnification resulting in a pixel size of 2.8 Å. The 3D reconstructions correspond to the entire dataset and did not undergo further classification. The resolution of 10.3 Å was measured using the FSC cutoff of 0.143.

2.2. Difference mapping and docking

2D and 3D difference mapping were performed using the program SPIDER (Shaikh et al. 2008). A 2D difference map was obtained by direct subtraction of the 2D averages of the open and closed RyR1-FKBP12 datasets. For 3D difference mapping, the two 3D maps corresponding to RyR1-FKBP12 and RyR1 were aligned to each other, normalized and filtered to their common resolution of 10 Å prior to direct subtraction. 3D volumes are displayed with the program Chimera (Pettersen et al. 2004). Docking was performed using Situs (Wriggers et al. 1999) both with and without Laplacian filtration.

2.3. Measurement of the flexion angle

The NTD, SPRY, P1 domains, and part of helical domain (C' of HD1 and N' of HD2) form a rigid rhomboid structure that appears to tilt as a whole among the different RyR1 conformations. We define the flexion angle as the angle between the long diagonal of the rhomboid and the plane of the membrane (see Results section 3.2 and corresponding figure). The long diagonal crosses the center of the B subdomain in the NTD and the boundary between the P1 and helical domain. To set a diagonal line, we chose one residue that belongs to a structured region with secondary structure within each domain (residues 348 and 1052, respectively). The two residues were connected them with a line, and we then measured the angle between this line and the horizontal plane of the membrane for all the structures compared, using the Chimera program. For any pair of residues selected using this uniform criterion, the change in flexion angle can be compared among conformations. For the 10 Å resolution structures, the rhomboid structure corresponding to the 5tb0.pdb atomic map was docked into the 3D reconstructions prior to taking the measurement.

2.4. Creation of the Three Dimensional Vector Maps

A 3D vector map was created to illustrate the trajectory of individual domains within the CytA. We first segmented RyR1's cytoplasmic assembly into its constituent domains as

defined by regions of high density surrounded by lower density boundaries using a watershed algorithm. Refinement of the boundary regions through further grouping and ungrouping was required to attain the highest degree of similarity between the segmentations of the three volumes and to assure that corresponding fragments had similar volumes (see Results section 3.2 and corresponding figure). The three reconstructions, previously aligned to each other, were analyzed using a threshold corresponding to RyR1's molecular weight according to the average density of a protein ($0.735 \text{ \AA}^3/\text{Da}$, (Quillin and Matthews 2000)). Volume segmentation was carried out with the watershed segmentation algorithm Segger (Pintilie et al. 2010) implemented in Chimera (Pettersen et al. 2004). To quantify the movement of each segment, its center of mass was then determined and its Cartesian coordinates were recorded for all segmentations in all three maps. Then the vectors representing the change in location from the original to the final coordinates were computed for each pair of corresponding domains, multiplied by 2, and plotted in 3D.

2.5. Multivariate statistical analysis

We focused on the raw particle images corresponding to the side view of RyR1 (RyR1 seen perpendicular to the membrane), which most clearly reveal the opening-closing motion. The subset of side views was identified by cross-correlation to a side view projection of the 3D of RyR1, using a projection-matching algorithm. This identified 736, 710 and 2008 particles within the open RyR1-FKBP12, closed RyR1-FKBP12 and closed RyR1 datasets, respectively.

For multivariate statistical analysis, all three datasets were merged. A mask that excluded the outside of the particles was applied. Correspondence analysis was carried out on the 3,454-particle combined dataset, yielding a set of factors and their associated coefficient for each individual particle (see Results section 3.3 and corresponding figures). The next step was to identify a factor or factors associated with the dataset variability caused by different degrees of opening. By visually analyzing the eigenimages, we found that only one of the factors was associated with the degree conformational change in the CytA. This was further confirmed by cross-correlating every eigen-image with the 2D difference map between open and closed RyR1-FKBP12. For this specific factor associated with opening, the coefficient for each particle (the eigen-factor which reflects its degree of opening) was identified. All the above analysis was performed using the SPIDER software package.

The factor coefficients were then split into their original group (open RyR1-FKBP12, closed RyR1-FKBP12 and closed RyR1). Each coefficient was multiplied by a factor of 1000. The statistical parameters of the resulting three distributions, each approximating a Gaussian distribution, were calculated using the software Graphpad Prism.

3. Results

3.1. FKBP12 binds to RyR1 with high occupancy

The 3D reconstructions of RyR1 with and without FKBP12 bound obtained in identical closed buffer conditions were compared directly. First, the 3D reconstruction of RyR1 was subtracted directly from that of RyR1-FKBP12, yielding a $\sim 10 \text{ \AA}$ resolution 3D difference

map corresponding to FKBP12 (Fig. 1A-B). FKBP12 binds to RyR1 at four equivalent sites formed by the concave region delimited by the SPRY1 and the handle domain, and possibly SPRY3 (Fig. 1A) within the CytA. Automated docking of FKBP12's atomic coordinates into the cryoEM map yielded a top-scoring solution, followed by solutions with significantly lower cross-correlation values (Fig. 1C), which is in full agreement with a 16 Å-resolution earlier determination carried out under different buffer conditions (Samso et al. 2006), and with the near-atomic structure of RyR1-FKBP12 (Yan et al. 2015, Zalk et al. 2015). The FKBP12 density map has a teardrop shape with maximum dimensions of $48 \times 36 \times 30 \text{ Å}^3$ and matches the shape and dimensions of the FKBP12 crystal structure, which is formed by an alpha helix surrounded by a beta sheet, protruding loops, and a hydrophobic cavity (Fig. 1D).

The spatial agreement between FKBP12's crystal structure and the boundaries of FKBP12's difference map when displayed at the optimal RyR1 density threshold indicates high FKBP12 occupancy, a necessary condition for our statistical analysis, and suggests that the FKBP12 subunit is rigidly attached to the RyR1 complex.

3.2. Removal of FKBP12 under closed state conditions sets the CytA of RyR1 in a distinct conformation

A comparison of RyR1 with and without FKBP12 in closed state conditions reveals that the absence of FKBP12 results in a concerted repositioning of domains within RyR1. A noticeable conformational change takes place in the CytA, which has four radially arranged rhomboid structures on its top surface (Samso et al. 2005), each constituted by the NTD, SPRY, P1, and part of the HD domains (Fig. 2A-B). We analyzed the structural change of the rhomboids in the context of the conformational change of RyR1-FKBP12 upon gating. When the RyR1-FKBP12 complex shifts from the closed to the open state, each of the rhomboids swivel as if prying open the central part of the RyR1 structure (Fig. 2C-D) (Samso et al. 2009). Specifically, the proximal region of the rhomboids moves away from the TmD and away from the fourfold axis, which relaxes the vestibular ring and opens the central part of the structure, while the distal portions of the rhomboids move towards the TmD. Considering the large dimensions of the rhomboid structure and the position of its pivot point, this results in the change of position of the tip of the rhomboid of 8 Å towards the membrane upon opening as described earlier (Samso et al. 2009). We denote the closed and open conformations of RyR1-FKBP12 “upward” and “downward”, respectively. The conformational change of closed RyR1 upon removal of FKBP12 is quite similar to the transition observed for RyR1-FKBP12 in going from the closed to the open state, although with lower magnitude, as seen in Fig. 2E and described in more detail below. We denote the closed conformation of RyR1 without FKBP12 as “relaxed”.

In the three conformations analyzed, rhomboids move practically as a rigid unit, a feature common among other structural studies of RyR1 gating. For example, the rhomboid structures in open (5tal.pdb) and closed (5tb0.pdb) RyR1 (des Georges et al. 2016) have an RMSD of 0.955 Å, a small value considering its ~170 Å long diagonal. Given the rigidity of the rhomboid structure, it is then possible to define a “flexion angle” as the angle between the horizontal plane of the membrane and a line defined by the long axis of the rhomboid,

which crosses the center of the NTD-B subdomain and the boundary between P1 and the helical domain (Fig. 2A-E). Measuring an angle offers higher accuracy, is more portable to different structures than measuring absolute distances, and circumvents potential magnification errors. The flexion angle was measured in our three structures (see Methods), resulting in values of -2.9° for open RyR1-FKBP12 (downward), 1.9° for closed RyR1-FKBP12 (upward), and -1.4° for closed RyR1 (relaxed) (squares in Fig. 2F). Thus, the flexion angle changes -4.8° (downward motion) in going from RyR1-FKBP12 closed to open, and -3.3° in going from RyR1-FKBP12 to RyR1 under closed state conditions. This suggests that in the absence of FKBP12, the CytA of RyR1 in the closed state has a conformation more akin to the open-state conformation. We measured the change in flexion angle in other deposited atomic structures using the uniform criterion described in the Methods section, and found the same trend (non-squares in Fig. 2F). In these other deposited structures, RyR1-FKBP12/12.6 has an average flexion angle of $-2.6^\circ \pm 1.6$ under open state conditions and $1.4^\circ \pm 0.4$ under closed state conditions. In the absence of FKBP12, RyR1 under closed state conditions has a flexion angle of -0.9° (Efremov et al. 2015). In the open state conditions and in the absence of FKBP12, RyR1 has a flexion angle of -3.5° (Willegems and Efremov 2018). Note that additionally, RyR1-FKBP12/12.6 channels prepared in the open state conditions may present a sub-population with a closed ion gate (Bai et al. 2016, des Georges et al. 2016); and that RyR1-FKBP12/12.6 channels prepared in closed state may have a sub-population presenting the CytA in a relaxed-like conformation (des Georges et al. 2016). These structures are considered in the Discussion section.

To study the FKBP12-induced allosteric change in further detail we compared two sets of conformational changes, those in the transition from RyR1-FKBP12 to RyR1 (equivalent to FKBP12 removal) under closed-state conditions, and those undergone by RyR1-FKBP12 in the transition from the closed to the open state. Thus, both conformational changes start from the RyR1-FKBP12 closed conformation. In a first step, the CytA of RyR1 was segmented automatically into its morphological domains using a watershed algorithm (Pintilie et al. 2010), resulting in an almost equivalent fragmentation for all three reconstructions except for the segment corresponding to FKBP12 itself (Fig. 3A). Most of the algorithmically determined 3D segments approximate functional RyR1 domains (Radermacher et al. 1994, Samsó 2017). In a second step, a 3D vector map was constructed wherein a vector was drawn from the center of mass of each individual segment within the closed RyR1-FKBP12 complex to the corresponding segment's center of mass in the closed RyR1 without FKBP12 (blue arrows in Fig. 3B-C). A similar 3D vector map was built from the closed to the open RyR1-FKBP12 segmentation (red arrows in Fig. 3B-C, Movie S1). In this manner, the two three-dimensional vector maps could be overlaid and viewed simultaneously.

In both conformational changes, the directions of the 3D vectors agree for most pairs of corresponding vectors. However, the magnitudes for the FKBP12 removal are, on average, half of those observed for RyR1-FKBP12 opening (Fig. 3B-C). In other words, removal of FKBP12 appears to set RyR1's CytA in a conformation halfway between those of the open and the closed states of RyR1-FKBP12, which we call "relaxed".

3.3. Multivariate statistical analysis uncovers the conformation of individual RyR1s

We analyzed the conformation of the individual RyR1 particles within the dataset. We focused on the raw cryoEM images of RyR1 corresponding to the side view (membrane perpendicular to the viewer), where the difference between the open and closed conformations is most apparent. Indeed, when these raw images were aligned and averaged, the 2D averages of open/closed RyR1-FKBP12 reveal the downward/upward conformation of the CytA, while the RyR1 closed without FKBP12 reveals the relaxed conformation (Fig. 4A, Movies S2 and S3). When the closed RyR1-FKBP12 is subtracted from the open RyR1-FKBP12, the 2D difference map shows positive (white) and negative (black) differences especially noticeable at the position of two protruding domains, P1 and P2, which reflect the conformational change (Fig 4B). A mass deficiency in the center of the CytA near the TmD in the open state reflects the relaxation around the ion gate. At this resolution, no further structural differences were discernable within the TmD region.

After establishing that the side views recapitulate the conformational changes observed for the 3D structure upon opening, we carried out a multivariate statistical analysis of the dataset (Frank and van Heel 1982, van Heel and Frank 1981). Correspondence analysis was performed on the combined dataset of raw cryoEM images (3,454 particles) comprised of all the side views of RyR1-FKBP12-open (736 particles), RyR1-FKBP12-closed (710 particles), and RyR1-closed (2008 particles) (see Methods). Among the different orthogonal factors and their corresponding visual representations (eigen images) describing the dataset variance, eigen image 4 closely matched the 2D difference map between open and closed RyR1-FkBP12 (Fig. 4B and Fig. 1S). This was confirmed by cross-correlating the 2D difference map with each eigen image, which yields a normalized cross-correlation of 0.62 for factor 4, significantly (5-fold) higher than the next best (Fig. 4D), and the complementary opposite-sign eigen image has the highest cross-correlation to the 2D difference map with subtraction performed in the opposite direction (not shown). This indicated that the factor associated with this eigen image 4 embodied the variation due to the different degree of opening for each RyR1 particle within the combined dataset.

The coefficients of each particle for this factor were further analyzed by splitting them back into the three original datasets, i.e., the three datasets were combined prior to correspondence analysis and were then separated a posteriori according to biochemical condition. This yielded three distributions of factor coefficients, which each approximated a normal distribution (Fig. 4E). Their averages were -1.67 (RyR1-FKBP12 open), 3.60 (RyR1-FKBP12 closed) and -0.66 (RyR1 closed), with homogeneity of variances (Fig. 4F). A one-way ANOVA conducted on the three distributions indicated a statistically significant difference. A post-hoc comparison using the Tukey test indicated that the differences among all three distributions were statistically significant ($P < 0.001$). Equivalent results were obtained when using low-pass filtered images and when using principal component analysis instead of correspondence analysis (not shown). The fact that displaying the data according to original biochemical condition yielded distinct distributions confirmed that the main features of this conformational change were largely captured by the factor identified by correspondence analysis.

3.4. Lack of FKBP12 alters RyR1's CytA but the ion gate remains closed

We analyzed if the presence or absence of FKBP12 affects RyR1's ion gate. The ion gate is defined by the inner S6 helices, which span the transmembrane region and extend into the cytoplasm. In the closed state, the S6 helices form a constriction (ion gate) at the membrane/cytoplasm boundary, whereas in the open state they separate, allowing ion permeation. A direct comparison highlights the position of the long helix S6 in the three conformations and indicates that under closed state conditions the ion gate of closed RyR1 is indeed in the closed conformation regardless of whether FKBP12 is present or not (Fig. 5). Thus, although the CytA of closed RyR1 in the absence FKBP12 has a conformation intermediate between that of closed and open RyR1-FKBP12, in the TmD the S6 helices define a closed ion gate that is nearly equivalent to that of closed RyR1-FKBP12.

4. Discussion

4.1. FKBP12 alters the conformation of RyR1

We sought to understand the role that FKBP12 plays on the conformational dynamics of RyR1 using cryoEM and image processing. Sub-classification is frequently performed as a way to address the heterogeneity within a dataset, and in this case, different particle sub-groups result in different 3D reconstructions. Here no sub-classification was carried out, therefore the three 3D reconstructions presented represent the entire dataset.

The buffer conditions used for the closed RyR1 and RyR1-FKBP12 was identical, including the detergent (0.5% CHAPS). Thus, the differences observed between these two reconstructions are solely due to the presence or absence of FKBP12. Comparison of the 3D reconstructions indicates that in the absence of FKBP12, closed state conditions set the CytA of the RyR1 in a relaxed conformation, approximately half-way between the open and closed conformations, while the ion gate remains in a closed conformation.

Since the rhomboid structures in the CytA move largely as a rigid body we defined a flexion angle, the degree to which the rhomboid structures swivel with respect to the membrane plane. When comparing the flexion angle using a uniform criterion, all existing 3D structures follow the same pattern, with an upward conformation for closed RyR1-FKBP12/12.6, a downward conformation for open RyR1 either with or without FKBP12/12.6, and a relaxed conformation for closed RyR1 without FKBP12/12.6. Thus, a downward flexion angle, which is caused by maximally activating Ca^{2+} , appears to emerge as a common trend that correlates with increased open probability. On the other hand, under closed state conditions (submicromolar Ca^{2+}), the upward conformation is only achieved in the presence of FKBP12. Finally, under closed state conditions, lack of FKBP12 results in the "relaxed" conformation, which is intermediate between the upward and downward conformations.

For the deposited structures obtained by others under open-state biochemical conditions, we considered only those with an open ion gate. Our 3D reconstruction of the overall dataset obtained with Ca^{2+} with PCB95 showed an open ion gate. However, sub-classification of RyR1-FKBP12/12.6 prepared in open state conditions resulted in a mix of 3D reconstructions with an open ion gate and 3D reconstructions with a closed ion gate (Bai et

al. 2016, des Georges et al. 2016), despite that both the use of Ca^{2+} with PCB95, or the mixture of Ca^{2+} , ATP and caffeine, yield near 100% probability of ion gate opening. A report that detergent can reduce the open probability (Willegems and Efremov 2018) could account, at least in part, for this discrepancy. The closed-channel structures prepared under open state conditions have a wide range in flexion angle with a downward tendency (average flexion angle -2.1°), and were qualified as “primed”. Our multivariate statistical analysis illustrates that, at least for the CytA, there is a certain degree of variability and even overlap of conformations between datasets prepared under different biochemical conditions. In any given dataset, it is likely that sub-classification is heavily influenced by the flexion angle, which is associated with large mass translocations, thus choosing different parts of a histogram in Fig. 4E could recreate different flexion angles in the resulting 3D structures.

To see the full extent of the conformational change induced by FKBP12 removal, we segmented the CytA of each 3D reconstruction into its different subdomains. This enabled us to build a 3D vector map that measured the conformational changes undergone by the CytA. The conformational change undergone by RyR1 upon removal of FKBP12 under closed state conditions was compared to the conformational change of RyR1-FKBP12 in going from closed to open. Overall, the vector maps are similar in terms of vector directionality, implying that removal of FKBP12 induces an overall conformational change in the CytA that is predominantly propagated in a fashion similar to that of the opening motion. In agreement with the more simplified flexion angle measurement, the magnitude of the conformational change induced by FKBP12 removal is smaller than that of opening.

As single-particle cryoEM affords the opportunity to see the conformation of each individual particle, we carried out correspondence analysis of the three datasets, in order to further understand the differences observed among them. Our analysis captured one factor that described most of the conformational variability accounted for by channel opening and closing. The distribution of coefficients for this factor for each particle forms three normal distributions, which are each significantly different from the others. In summary, correspondence analysis enabled us to visualize how FKBP12 binding shifts the conformation of the RyR1 particles, both in the 3D reconstruction, which corresponds to an average, and in the context of their population ensemble.

4.2 The relaxed conformation of RyR1 in the physiological context

Ryanodine binding and single-channel studies showed that dissociation of FKBP12 induces a leftward shift in the activation dependence of RyR1 by Ca^{2+} such that RyR1 becomes more sensitive to Ca^{2+} activation (Ahern et al. 1997, Mayrleitner et al. 1994, Ondrias et al. 1996), resulting in leaky RyR1 channels. Similarly, in the heart, dissociation or lack of FKBP12/FKBP12.6 results in leaky RyR2 that can result in heart failure (Shou et al. 1998, Sood et al. 2008). In addition, single channel studies show that under activating conditions, RyR1 without FKBP12 can present long-lived subconductance states whereby RyR1's conductance is $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$, intermingled with the typical full conductance state (Ahern et al. 1997, Brillantes et al. 1994). Nevertheless, at the submicromolar concentrations of Ca^{2+} used in our study, RyR1 channels with or without FKBP12 are silent both by single channel as well as by ryanodine binding assays, i.e. their open probabilities as measured by single

channel analysis or ryanodine binding are zero (Damiani et al. 1997, Samsó et al. 2009, Xu et al. 1998). Our 3D reconstruction of RyR1 without FKBP12 performed at submicromolar Ca^{2+} displays a closed conformation for the ion gate in agreement with single channel studies. On the other hand, the CytA has an intermediate conformation between the RyR1-FKBP12 closed and open conformations. This reveals two distinct closed states of this ion channel that depend on the presence or absence of the FKBP12 subunit (Fig. 6A-B), a fact that is unascertainable using functional methods that rely on channel activity. In view of our data, the higher sensitivity to Ca^{2+} activation of RyR1 without FKBP12 could be explained by the smaller transformation needed for the CytA to reach the open state conformation (Fig. 6, compare transition B to D with transition A to C).

By acting as a wedge at the hollow formed between the SPRY1 and handle domains, FKBP12 might play a role in RyR1's long-range allostereism. The positioning of FKBP12 at four symmetrically located concave regions could promote the coordination of the four subunits of RyR1, in the same way that all of the interconnected leaves in a steamer basket are necessary for its proper function (see schematics in Fig. 6E). This could limit the flexibility of the CytA, and buttress the upward position. Lower subunit coordination could also account for the observed subconductance states.

In terms of the conformational landscape, RyR1-FKBP12 under Ca^{2+} activation would flicker between the “fully open” (Fig. 6C-D) and “fully closed” (Fig. 6A) energy wells. Without FKBP12, RyR1 closed in the relaxed conformation (Fig. 6B) would have a shallower energy well and require less energy to transition to the open state upon Ca^{2+} activation. In addition, loss of intra-subunit coordination would enable access to the subconductance states.

4.3. Different effects of FKBP12 at the cardiac and skeletal RyR isoforms

Removal of FKBP12 produces different effects on RyR1 and RyR2 despite their sequence similarity. These seem to be rooted in a different conformation of the ~20 nm long helical domain. This domain is fastened to the SPRY2 domain of the adjacent subunit in RyR1, but appears more flexible in the case of the RyR2 isoform (Dhindwal et al. 2017). Lack of FKBP12.6 correlated with a further increase in flexibility of the helical domain (Dhindwal et al. 2017, Peng et al. 2016), and could imply that removal of FKBP induces an additional conformational destabilization in RyR2 with respect to RyR1.

4.4. FKBP12 in the context of skeletal type excitation-contraction coupling.

Skeletal muscle type excitation-contraction coupling involves direct contact between the RyR1 and the DHPR (Rios and Brum 1987, Tanabe et al. 1988). Although the structural basis of this protein communication remains elusive, important components include the cytoplasmic portions of the α_1 subunit of the DHPR, and the regions of RyR1 most proximal to the DHPR.

It was found that FKBP12 binding to RyR1 enhances the gain of skeletal muscle excitation-contraction coupling (Avila et al. 2003), suggesting that in the presence of FKBP12, the communication between RyR1 and skeletal type DHPR is more reliable. In this context, the upward conformation of RyR1-FKBP12 in the closed state places the peripheral regions of

RyR1's CytA in a conformation where they protrude more noticeably towards the DHPR (Fig. 6A). In principle, the upward conformation brought about by the FKBP12 subunit would facilitate the coupling between the two proteins across the triadic cleft under resting conditions. In addition, the long range conformational changes involved in gating would be of greater amplitude. Thus, our findings provide a plausible structural basis for the higher gain of skeletal type excitation-contraction coupling in the presence of FKBP12.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CryoEM	cryo electron microscopy
CytA	cytoplasmic assembly
DHPR	dihydropyridine receptor
FKBP12	FK506-binding protein of 12 kDa
FKBP12	FK506-binding protein of 12 kDa
FKBP12.6	FK506-binding protein of 12.6 kDa
HD	helical domain
NTD	N-terminal domain
RyR1	ryanodine receptor isoform 1
RyR2	ryanodine receptor isoform 2
TmD	transmembrane domain

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Highlights:

- Lack of RyR1's main subunit FKBP12 results in higher sensitivity to activation
- RyR1 has two closed-state conformations depending on presence or absence of FKBP12
- FKBP12 is necessary to set RyR1's cytoplasmic assembly in the upward conformation
- Without FKBP12, closed RyR1 adopts a relaxed conformation

Fig. 1.

Docking the FKBP12 atomic coordinates into the cryoEM envelope. (A) 3D reconstruction of RyR1 in the fourfold and side views with superimposed FKBP12 difference map in blue. The side view is semitransparent to show the FKBP12 on all binding sites. (B) 3D difference map of FKBP12. (C) Plot of the cross correlation scores showing the unambiguity of the first docking solution. (D) Atomic coordinates of FKBP12 (1d06.pdb) in the orientation corresponding to the first docking solution. The hollow FK506 binding cavity is indicated with (c). Scale bar for the FKBP12 difference map, 1 nm.

Fig. 2.

The flexion angle summarizes the downward, upward and relaxed conformations of the cytoplasmic assembly and corresponds to distinct functional states. (A) The rhomboid-like structures (dashed lines) swivel with respect plane of the membrane upon Ca^{2+} addition as well as upon FKBP12 removal. (B) Detail of the domains forming the rhomboid structure with highlighted long diagonal in blue. (C-E) The flexion angle between the membrane plane (horizontal dashed line) and the long diagonal of the rhomboid (blue line) is displayed on the side view under the conditions indicated. (F) Flexion angle measured for the three functional states described in this work (squares) compared to flexion angles measured on other 3D structures of RyR1 or RyR1-FKBP12 obtained under comparable conditions. Open symbols correspond to open RyR1, full symbols correspond to closed RyR1, and gray symbols correspond to closed RyR1 without FKBP12. FKBP12.6 was used instead of FKBP12 in (des Georges et al. 2016). The EMD or pdb deposition codes are indicated below each point.

Fig. 3. The cytoplasmic 3D vector maps associated to opening and removal of FKBP12 share common trends. (A) Segmentation of the RyR1 3D reconstructions into reproducible globular domains for the open RyR1-FKBP12 (magenta), closed RyR1-FKBP12 (green), and closed RyR1 (blue) (asterisk indicates empty FKBP12 binding site). The top and the side orthogonal views are represented. (B) Stereo view with the 3D Vector representation of the translocation for each specific domain for the closed to open transition (red arrows), and for the removal of FKBP12 in closed state conditions (blue arrows). For clarity, only the vectors within a central section are shown. (C) 3D vector map after a 90-degree rotation with respect to the y-axis showing the central section delimited with dashed lines. Scale bar, 5 nm.

Fig. 4. Multivariate statistical analysis of the raw images of the RyR1 combined dataset. The analysis is done on the side view, which is the best representation of the upward/downward motion. (A) 2D average of the side view of RyR1-FKBP12 in open state conditions. (B) 2D difference map between the 2D averages of RyR1-FKBP12 open (upward) and closed (downward) in the same orientation reveals translocation of the peripheral domains P1 and P2, and a change in density in the central region. (C) Eigen image 4 has the highest cross-correlation with the 2D difference map and represents the dataset variability due to the upward/downward motion. (D) Normalized cross-correlation between the 2D difference map

and each of the first six eigen images. (E) Histograms of the distributions of coefficients for the RyR1-FKBP12 open (R-F op), RyR1-FKBP12 closed (R-F cl), and RyR1 closed (R cl) datasets. The vertical dashed line facilitates the comparison. (F) Mean and S.E. corresponding to each dataset. A one-way ANOVA followed by post-hoc comparison using the Tukey test indicates that all three distributions are significantly different among them ($P < 0.001$).

Fig. 5.

Under closed-state biochemical conditions, absence of FKBP12 does not open the channel. Top panels: side view of the TmD and part of the central domain. Bottom panels: ion gate seen in the orthogonal direction from the cytoplasmic side. The section displayed corresponds to the inset in A, top left panel. (A) RyR1-FKBP12 open with docked open-state coordinates (5tal.pdb). (B) RyR1-FKBP12 closed with docked closed-state coordinates (5tb0.pdb) (C) RyR1 without FKBP12 closed docked with closed-state atomic coordinates (5tb0.pdb). Dashed lines indicate the approximate boundaries of the sarcoplasmic reticulum membrane. The S6 helices are in dark red and the S4-S5 linker in magenta. Scale bar, 1 nm.

Fig. 6. Conceptual schematics illustrating the allosteric effect of FKBP12 and of maximally activating Ca^{2+} on RyR1. RyR1 is represented in green, FKBP12 in blue, Ca^{2+} in yellow. (A-D) Side view of RyR1 under different conditions. (A) RyR1-FKBP12 closed with FKBP12 buttressing the upward conformation. (B) RyR1 closed. (C) RyR1-FKBP12 open. (D) RyR1 open. (E) Fourfold view illustrating how FKBP12 promotes the coordination of the four subunits.