Locations of Calmodulin and FK506-binding Protein on the Three-dimensional Architecture of the Skeletal Muscle Ryanodine Receptor*

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Isolated skeletal muscle ryanodine receptors (RyRs) complexed with the modulatory ligands, calmodulin (CaM) or 12-kDa FK506-binding protein (FKBP12), have been characterized by electron cryomicroscopy and three-dimensional reconstruction. RyRs are composed of 4 large subunits (molecular mass 565 kDa) that assemble to form a 4-fold symmetric complex that, architecturally, comprises two major substructures, a large (~80% of the total mass) cytoplasmic assembly and a smaller transmembrane assembly. Both CaM and FKBP12 bind to the cytoplasmic assembly at sites that are 10 and 12 nm, respectively, from the putative entrance to the transmembrane ion channel. FKBP12 binds along the edge of the square-shaped cytoplasmic assembly near the face that interacts in vivo with the sarclemma/transverse tubule membrane system, whereas CaM binds within a cleft that faces the junctional face of the sarcoplasmic reticulum membrane at the triad junction. Both ligands interact with a domain that connects directly to a cytoplasmic extension of the transmembrane assembly of the receptor, and thus might cause structural changes in the domain which in turn modulate channel gating.

In striated muscle, the ryanodine receptor (RyR) mediates muscle contraction by functioning as the primary, intracellular calcium release channel of the sarcoplasmic reticulum (SR) (1–6). Additionally, in skeletal muscle, the RyR appears to play a signal-transducing role in excitation-contraction coupling by communicating with the voltage-sensing dihydropyridine receptor in the transverse tubule/plasmalemma.

RyRs are the largest ion channels known, having net molecular masses of 2.3 MDa. Both the skeletal muscle and heart RyRs comprise four identical large subunits (565 kDa) encoded by genes (designated Ryr1 and Ryr2, respectively). In addition to these very large subunits, a much smaller modulatory protein, identified as an FK506-binding protein (FKBP12 in skeletal and FKBP12.6 in cardiac muscle) copurifies with the receptor, and may also be considered as an integral component of RyRs (7–13). Up to four copies of FKBP12/FKBP12.6 can bind per molecule of RyR.

Electron microscopy is the only practical approach for elucidating morphological details of giant membrane-associated complexes such as RyRs (14–17). Recently, two independent three-dimensional reconstructions of the skeletal muscle RyR determined from electron micrographs of frozen-hydrated, detergent-solubilized receptors have been described (16, 17). Both reconstructions show the RyR to be a 4-fold symmetric molecule in which 10 or more distinct globular domains are apparent even at the limited resolution attained (~3 nm). About 80% of the mass of the receptor forms a cytoplasmic assembly (29 × 29 × 12 nm), and the remaining 20% projects as a baseplate from one face of the cytoplasmic assembly, and likely forms the transmembrane portion of the receptor. This interpretation agrees with predictions based upon the sequence of Ryr1 and Ryr2 which implicate the carboxyl-terminal fifth of Ryr1 as comprising the transmembrane segments (18–20). In skeletal muscle, RyRs are primarily localized at triad junctions, regions where the terminal cisternae of the SR form junctional contacts with the transverse tubules, invaginations of the plasmalemma (21–23). The transmembrane assembly of the RyR traverses the SR membrane, and the cytoplasmic assembly occupies the gap between the SR and sarclemma/transverse tubule membrane systems (24, 25). Upon depolarization of the myofiber, RyRs become activated when they receive a signal from voltage sensors, now known to be dihydropyridine receptors (DHPRs) (26, 27), located in the sarclemma/transverse tubules, by a process known as excitation-contraction coupling. Besides dihydropyridine receptors, several other proteins are localized to these junctional regions, some of which interact with the RyR to modulate its function.

One of these proteins is the FKBP12, mentioned above. Although RyRs still exhibit calcium channel activity in vitro following dissociation of FKBP12, their gating properties are altered (8, 10, 12, 28–32). Specifically, FKBP12 stabilizes the closed state of the receptor and decreases the frequency of subconductance states when the channel is induced to open. FKBP12 has also been found to be associated with an inositol-1,4,5-trisphosphate receptor/calcium release channel (33), whereas an isoform of FKBP12, FKBP12.6, is associated with the cardiac isoform of the RyR (9, 34, 35). Further underscoring the potential importance of FKBP12 in skeletal muscle, a recent study has reported that treatment of skinned skeletal muscle fibers with the drug FK506, which causes FKBP12 to dissociate from the RyR, disrupted excitation-contraction coupling (36).

Calmodulin (CaM) is another modulatory ligand of the RyR.
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In vitro studies have shown that CaM binds to the RyR with high (nanomolar) affinity and modulates its calcium channel activity in vitro without involvement of CaM-dependent kinases (37–39). Knowledge of the binding sites of FKBP12 and CaM on the three-dimensional architecture of the RyR, as well as their effects, if any, on the structure of the receptor are expected to aid in understanding the mechanisms of their modulation of RyR function. Previously, we showed by electron microscopy that both CaM and FKBP12 are bound to distinct locations at the peripheral regions of the cytoplasmic assembly of the RyR (40, 41). However, these localizations were deduced from averaged images that showed the RyR in projection from a single orientation (along the 4-fold symmetry axis) and, hence, did not show the three-dimensional locations of the CaM and FKBP12 relative to each other or to the distinctive structural domains comprising the RyR. Furthermore, for the CaM studies a gold cluster probe was covalently attached to the CaM to enhance its detectability in electron micrographs, but at the cost of compromising the accuracy of localization. Here we describe three-dimensional reconstructions of RyR-FKBP12 and RyR-CaM complexes in which FKBP12 and CaM are resolved directly, without attached high-density labels.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human FKBP12 was prepared as described previously (11). [3H]Ryanodine was purchased from NEN Life Science Products. CHAPS was from Sigma or Calbiochem (La Jolla, CA). Wheat germ and other biochemicals were from Sigma.

Preparation of RyR-CaM and RyR-FKBP for Cryo-EM—RyRs were isolated from terminal cisternae vesicles (42) from rabbit skeletal muscle by modifications (8, 41) of previously described procedures (25). RyR-CaM complexes were prepared using wheat germ calmodulin (43, 44) that was biotinylated at Cys-27 as described (40). Biotinylated CaM was used to provide an independent means of assaying binding to the RyR in the event that density arising from CaM was not detected in averaged images and three-dimensional reconstructions. The biotin modification did not contribute significantly to the images that were obtained by cryo-EM (described below). RyR-CaM complexes were formed by adding 0.5–1 μl of RyR (0.4 M) in buffer I (20 mM Tris-HCl (pH 7.4), 1.0 μl NaCl, 2 mM dithiothreitol, 0.5% CHAPS, 1 μg/ml leupeptin) to 20 μl of CaM (1 μM) in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.15 M KCl, 0.1 mM CaCl2 and incubating at room temperature. At between 10 and 20 min of incubation time, 5-μl aliquots were removed, applied to EM grids, and immediately frozen as described below. Since the final buffer conditions differed from those used in prior studies (16), grids were also prepared exactly as described except omitting the CaM for use as a control in the subsequent statistical analyses. The immobilization for storage of RyR on some of the holey carbon films, and therefore it was necessary to incubate the purified receptor with FKBP12 to assemble RyR-FKBP2 complexes, which was done as described by Wagenknecht et al. (41). The buffer (buffer I) used for this study was similar to that used previously to determine the three-dimensional architecture of the RyR (16), and therefore a new “control” reconstruction was not determined.

Electron Cryo-microscopy—Specimens were applied to 300 mesh molybdenum grids coated with a thin (∼5 nm) carbon film suspended over a thicker (>10 nm) holey carbon film (16). The thin carbon film was applied to the holey grid within a few hours of applying the specimen as follows. First, a thin layer of carbon was evaporated onto a piece of freshly cleaved mica which was then cut into ∼5 × 5-mm squares. The carbon was floated off of the mica pieces onto an air-water interface, a holey grid was placed onto the floating carbon with the holey-carbon side down, and then picked up with a forceps and allowed to dry. By this procedure, the surface of the thin carbon that faces away from the grid (and onto which the specimen is applied) is the same surface that had been in contact with the mica. The grids so prepared were glow-discharged in air using a Model PDC-3XG plasma cleaner/sterilizer (Harrick Scientific Corp., Ossining, NY) less than 1 h prior to sample application. Five-microliter portions of specimen were applied to the grids and blotted using a guillotine device (modification of the design of Cyrklaff et al., (45)) that clamps the grid between two pieces of filter paper (Whatman No. 1) until within only a few milliseconds of being plunged into the cryogen (liquid ethane that is supercooled by a liquid nitrogen bath).

Specimen grids were loaded into a Gatan (Warrendale, PA) model 626 cryoholder and examined in a Philips EM420 transmission electron microscope operated at 100 kV and a nominal magnification of ×38,000. The temperature of the specimen grid was maintained at −180 ± 2 °C. Three exposures were collected for each specimen field: two with the grid tilted to 50° and one untitled. The objective lens was underfocused by 1.5–2.0 μm, and the electron dose for each exposure was estimated at 800–1000 electrons/μm². Two exposures were made with the specimen tilted to improve the probability that a useable micrograph would be obtained, since most micrographs of tilted specimens suffered from specimen drift or charging; for unknown reasons, often these effects were reduced in the second micrograph.

Image Processing—Analysis of the micrographs was done using the SPIDER software package (46, 47). Three-dimensional reconstructions were determined by the random conical tilt method from images of individual RyRs extracted from micrographs recorded with the specimen tilted (16, 48, 49). The corresponding images obtained for untitled specimens were identified by the 2–4-fold symmetric orientations (i.e. “right-side-up” and “upside-down”) by multivariate statistical analysis of RyR images that had been brought into mutual alignment. The relative azimuthal angles of the 4-fold symmetric particles are used by the three-dimensional reconstruction algorithm, but the images themselves are not used to compute the reconstruction. Details of the procedure were essentially the same as were used in our previous study of RyRs without bound CaM or FKBP12 (16).

Before comparing two reconstructions quantitatively they were scaled with respect to density distribution and magnification using the SPIDER program DRIFF and the SPIDER procedure, SIZEMINUS, respectively (documentation is available at the World Wide Web site http://www.wadsorth.org/spider_doc/docs/spider.html). The resolutions of all of the reconstructions, as assayed by the differential phase residual and Fourier ring correlation (16), were 35–40 Å, which is somewhat poorer than was attained (31–33 Å) in our previously described reconstructions of the unliganded RyR (16), but is, nevertheless, sufficient to resolve the RyR-bound CaM and FKBP12.

RESULTS

Two-dimensional Localization of RyR-bound CaM—Complexes of skeletal muscle RyR and CaM were prepared by incubating purified RyR with a molar excess of wheat germ CaM under conditions that have been shown to result in 4 mol of bound CaM/mol of RyR (see “Experimental Procedures”). Owing to the low signal-to-noise ratio in micrographs of frozen-hydrated macromolecules and the fact that the molecular mass of CaM is only 2.8% of the Ryr1 protein, we did not expect to observe any differences between unprocessed images of RyR-CaM complexes and RyR, and indeed none were observed (data not shown, for examples of electron micrographs of frozen-hydrated RyRs, see Refs. 16, 40, 41, and 50).

To determine whether bound CaM was present and detectable by cryoelectron microscopy of RyR-CaM complexes, we first analyzed micrographs of receptors from untitled specimens, in which the RyRs were usually found oriented with their 4-fold symmetry axes normal to the grid support surface (“4-fold views”). This two-dimensional analysis can be done much faster than a three-dimensional one, and the statistical significance of structural features is easier to test. Correlation alignment, multivariate statistical analysis, and image averaging were done as described under “Experimental Procedures.” Averaged images of RyRs (which show the receptor’s protein density projected along its 4-fold symmetry axis) that had been incubated in the presence and absence of CaM are shown in Fig. 1, A and B, respectively. The two averaged images appear nearly indistinguishable, but the image formed by subtracting the two (Fig. 1C) shows four symmetrically related differences corresponding to additional mass in the CaM-treated RyR (indicated by asterisk). These regions are highly significant statistically (confidence level greater than 99.9% by two-factor f test), and we attribute these density maxima to the four molecules of CaM that are known to bind to the RyR under the experimental conditions used. That these differences indeed correspond to CaMs is confirmed by similar
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Fig. 1. Two-dimensional analysis of RyR-CaM complexes. A, averaged (projection) image of RyR-CaM in the 4-fold symmetric orientation (n = 712). B, averaged image of RyR (control), no CaM present (n = 456). C, difference formed by subtracting the image in B from that in A. One of the four symmetrically related major positive differences is indicated with an “*”. D, the control image from B with the location of the main positive differences from C superimposed (white regions). Width of each frame is 470 Å.

In our previous study in which the CaMs were labeled with a gold cluster (40). The locations of the gold clusters in that study correspond precisely (within ~1 nanometer) to the major differences seen in Fig. 1, which are at the periphery of the cytoplasmic assembly about 10 nm from the center of the RyR (Fig. 1D).

Other density differences are also present in Fig. 1C, but they are smaller in area and none have densities greater than 50% of that of the main differences. The minor differences might be due to structural rearrangements of the RyR that accompany CaM binding (see “Discussion”). We conclude from this two-dimensional analysis that the RyR-bound CaM can be detected by averaging images of non-stained, frozen-hydrated complexes without the need for labeling the CaM with heavy metal compounds.

Three-dimensional Reconstruction of RyR-CaM—To more precisely map the locations of the RyR-bound CaMs we determined three-dimensional reconstructions of RyR-CaM and RyR (control) from micrographs that were obtained by tilting the specimen. Selected slices that are normal to the 4-fold symmetry axis are shown for the reconstructions of RyR-CaM and RyR in Fig. 2, A and B, respectively. The slices appear essentially identical for the two reconstructions, but when corresponding slices are subtracted, a prominent difference, repeated four times as required by the symmetry, appears in two successive slices that pass through the cytoplasmic assembly of the receptor (third and fourth panels from the left in Fig. 2C). The only other differences apparent in Fig. 2C are smaller, both in size and density. Projecting the three-dimensional reconstructions along the 4-fold axes, followed by subtraction, also shows this same set of symmetrically related differences as the dominant ones (Fig. 2D), and superposition of these regions onto the projected structure shows their locations to be identical to that found in the two-dimensional averaging experiment discussed in the previous section (cf. Figs. 1 and D). Thus the major difference between RyR-CaM and RyR detected by the three-dimensional analysis (which was determined from the images of tilted specimen) agrees with that found from the two-dimensional analysis (determined from images of untitled specimens).

In Fig. 3, A and B, the RyR-CaM and RyR reconstructions are displayed as surface representations in several orientations. The two reconstructions appear nearly identical. A single, symmetrically-disposed set of differences is apparent when the structure is viewed from the SR-facing side (leftmost panel in Fig. 3) or from the side (rightmost panel, one of these regions is indicated by a “+”). The locations of these differences correspond to those of the main differences observed in the selected slices in Fig. 2C. Subtraction of the two reconstructions confirms that these differences are indeed the largest in terms of volume (Fig. 3C). Other than the main density differences, which we attribute to bound CaM (see below), the reconstructions of RyR-CaM and RyR are remarkably similar, implying that any conformational differences that might accompany binding of CaM are small, and to characterize them will require higher resolution than was achieved in this study (~3.7 nm).

We are confident that the four main differences between the RyR-CaM and RyR reconstructions are directly attributable to the excess mass contributed by the bound CaM, primarily because their locations in the 4-fold symmetric projection (Fig. 2D) agree with those determined for CaM that was labeled with a gold cluster probe in our earlier study (40). Also, the finding that FKBP12, a protein of comparable mass to CaM, binds to a different location than does CaM (Ref. 41, and see below) further underscores the specificity of binding for these two ligands. It should be noted that in the earlier study of RyR-CaM (40), we attempted to infer the three-dimensional location of the CaM, and that the results of the current study show that our estimate was inaccurate by several nanometers (see “Discussion”).

To illustrate more clearly the location and geometry of the RyR-bound CaMs, we show in Fig. 5 the four main differences between the reconstructions determined in the presence and absence of CaM (Fig. 3C) superimposed (yellow regions) on a reconstruction of the RyR determined in the absence of CaM. The CaM is located in clefts formed by structural domains labeled 3, 4, and 7, which had been identified and assigned numbers previously (16). The density attributed to the CaM has an elongate shape with major axis about 6 nm and width about 3 nm, values that are in fair agreement with the dimensions of a CaM-peptide complex determined by x-ray crystallography and NMR (51, 52). Most of the exposed surface of the CaM is visible from the RyR face that inserts into the junctional face membrane of the SR (Fig. 5B), whereas very little is visible from the side which would face the t-tubule in situ (Fig. 5A).

Three-dimensional Reconstruction of RyR Containing FKBP12—Previously, we determined averaged images from electron micrographs of frozen-hydrated RyRs in the 4-fold symmetric orientation to which FKBP12 had been added (41). Comparison of these images with comparable images obtained from purified RyRs not treated with FKBP12 (or treated with the drug, FK590, to remove any remaining endogenous FKBP12) showed that FKBP12 binds at the periphery of the cytoplasmic assembly of the RyR. Apparently much of the endogenous FKBP12 dissociates during the purification or storage of the receptor, hence, the requirement to add FKBP12 to purified receptors. In these two-dimensional studies the location of the bound FKBP12 in the direction parallel to the 4-fold symmetry axis was undetermined, and so we have determined a three-dimensional reconstruction of RyR-FKBP12.
**DISCUSSION**

In this study we have localized in three-dimensions the binding sites of CaM and FKBP12 on the surface of the skeletal muscle RyR. We find one binding location per RyR subunit for both ligands. FKBP12 binds along each of the edges of the receptor’s 4-fold symmetric cytoplasmic assembly, near the face that interacts with the transverse tubule *in situ*. CaM also binds to peripheral sites on the cytoplasmic assembly, but on the face that is closest to the junctional face membrane of the SR.

**CaM Binding Location on RyR**—The conditions that we used for forming the CaM-RyR complexes were adapted from an earlier study Yang *et al.* (38), of CaM binding to RyR-containing SR-derived vesicles. Yang *et al.* (38) found that, in the presence of 0.1 mM Ca$^{2+}$, 4 mol of CaM bound per mole of RyR with high ($K_d \approx 50$ nM) affinity, and that additional lower affinity sites ($K_d \approx 200$ nM) were also present. Recently, in an independent study Tripathy *et al.* (39), using similar conditions, also reported 4 high-affinity ($K_d \approx 20$ nM) CaM-binding sites on the RyR, but the low affinity sites were not detected. Of particular relevance to our studies, which employed purified, detergent-solubilized RyRs, Tripathy *et al.* (39) demonstrated essentially the same binding behavior for solubilized RyRs as for RyRs incorporated into a lipid bilayer. Both groups (38, 39) found that severalfold more CaM binds with high affinity to the RyR when Ca$^{2+}$ levels are submicromolar. Intriguingly, RyR-bound CaM has opposing effects depending on the concentration of Ca$^{2+}$; at micromolar levels and higher, CaM inhibits channel opening, whereas at submicromolar levels RyR channel activity is activated (39, 53).

In the study described here we have focused on conditions (0.1 mM Ca$^{2+}$) where four high-affinity CaM sites per molecule...
of RyR are expected, and, indeed, have been identified in the three-dimensional reconstruction (studies at submicromolar levels, where more than 4 mol of CaM bind per mol of RyR, are currently under way in our laboratory).2 Previously, we prepared a 1.4-nm diameter gold cluster-CaM conjugate as an electron dense probe for cryo-electron microscopic characterization of the CaM-binding site on the RyR. We found that gold-CaM complex bound to the cytoplasmic assembly of the RyR, but the precision of the localization was limited to about 4 nm because only the gold cluster was detected (i.e. not the CaM itself). In this study the gold cluster was omitted, and the density contributed by CaM was detected directly by image averaging. Also, the localization has been extended from two to three dimensions.

From the three-dimensional reconstructions it is clear that each of the four bound CaMs binds in a cleft formed between domains 3 and 7 on the SR-facing side of the receptor (Fig. 5B). Previously we had proposed, on the basis of a limited number of images of gold cluster-labeled RyR-CaM complexes in which the RyR was oriented with its 4-fold symmetry axis approximately parallel to the grid support (“side views”), that the CaM was located on the opposite face of the RyR, between domains 4 and 6 (Fig. 5C). This error could be attributed to the imprecision in inferring the CaM-binding site indirectly, i.e. based upon the location of the gold clusters.

Another advantage of direct detection of the CaM is that information on the orientation of the CaM relative to the RyR is obtained. Even at the rather limited resolution attained (3.7 nm), the direction of the major axis of the CaM is apparent in Fig. 5. The overall apparent dimensions of the CaM, $\sim 3 \times 6 \text{ nm}$, are in fair agreement with that expected from the known high resolution structures of CaM bound to peptide (51, 52).

**FKBP12 Localization on RyR**—The binding locations of FKBP12 and CaM are shown together in Fig. 5, which shows that FKBP12 binds even farther than does CaM from the presumed location of the entrance to the transmembrane ion-conducting channel of the RyR on the cytoplasmic side of the transmembrane assembly (at or near the center of RyR as oriented in Fig. 5A). If the entrance to the ion channel is indeed at this location, then the distance between the center-of-mass

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2 M. Samso, J. Berkowitz, and T. Wagenknecht, unpublished data.
of the bound FKBP12 and the ion channel entrance is 12 nm, whereas the distance for CaM is 10 nm. FKBP12 and CaM bind at opposite ends of the domains labeled 3 (also referred to as “handles” (17)) and are 9 nm apart.

Although knowledge of the location of FKBP12 on the surface of the RyR does not lead directly to hypotheses regarding the mechanisms of its modulator effects on the receptor, it is, we believe, worth mentioning some correlations of the mode of binding and some of these effects. For example, it is perhaps significant that the FKBP12-binding sites are near the face of the RyR that would interact with the transverse-tubule/sarclemma membrane system \textit{in situ}. The FKBP12s could therefore be positioned to exert direct effects on excitation-contraction coupling. Interestingly, Lamb and Stephenson (36) have reported that depletion of FKBP12 from skinned myofibers causes a disruption of excitation-contraction coupling. We and others have discussed evidence which implicates the regions formed by the “clamps,” the domains that form the corners of the cytoplasmic assembly (domains 5–10 in Fig. 3), as being involved in interacting with the DHPRs associated with the transverse tubule (54–56). The DHPRs function as voltage sensors in skeletal muscle excitation-contraction coupling (26, 27), and they are believed to physically interact with the RyRs so as to control their gating by a conformational coupling mechanism (57–59). FKBP12 appears to interact directly with domain 9 and is also near domain 5, both of which are found in the clamps. Thus, FKBP12 appears to be positioned so that it could mediate communication between the DHPRs and the RyRs during excitation-contraction coupling.

The locations of the RyR-bound FKBP12s appear also to be consistent with a role in mediating communication between neighboring RyRs in the t-tubule/sarclemma. RyRs in vertebrate skeletal muscle interact to form ordered arrays consisting of two or three rows of receptors in which adjacent RyRs interact with each other isologously near their corners, but with some overlap (~12 nm) of their edges (14, 42, 60). Electron microscopy studies indicate that not all of the RyRs are associated with voltage sensors (23, 59), and it has been postulated that the gating of these non-DHPR-ligated RyRs is controlled by interacting with adjacent RyRs that are associated with voltage sensors. In support of this proposal, Marks (13, 61) has reported that when two FKBP12-containing RyRs are present in a bilayer, they exhibit cooperative gating transitions, but only in the presence of FKBP12.

The binding sites for FKBP12 as resolved by three-dimensional reconstruction appear to be near the regions where RyRs...
interaction could be influencing the activity of the adjacent RyR either directly or through conformational coupling. It is therefore of interest to determine which of the two possible ways of constructing the arrays is used in vivo, that is, which side of a given edge of the RyR interacts with a neighboring RyR in the array (alternatives I and II in Fig. 6). For alternative II, the FKBP12 nearest to the site of interaction between adjacent RyRs would be on the edge adjacent to the one involved in RyR-RyR interaction (indicated by an asterisk in Fig. 6). For alternative I, two molecules of FKBP12 would be at the site of RyR-RyR interaction, perhaps directly contributing to it. A preliminary answer to this question is readily obtained by analysis of the ultrastructure of RyRs comprising the limited arrays that sometimes occur in micrographs of isolated RyRs (14). We find, for the negatively stained arrays shown by Saito et al. (14), that alternative II occurs, implying that any allosteric effects of FKBP12 on inter-receptor cooperativity involve rather long-range conformational effects. A caveat of this analysis is the implicit assumption that the limited arrays formed by the isolated RyRs are the same as those formed in vivo.

Control of RyR Activity by Long-range Conformational Changes—Both CaM and FKBP12 are bound greater than 10 nm from the putative entrance of the ion channel on the cytoplasmic surface of the transmembrane assembly. Since both of these ligands can affect channel activity, it seems that long-range conformational changes are involved in the modulatory mechanism. Consistent with this interpretation are the results of a structural study by Orlova et al. (56), in which three-dimensional reconstructions were determined under conditions that, based upon functional studies, should favor either the closed or open states of the receptor. Structural differences between RyR under the two conditions were found in the transmembrane regions as well as in the cytoplasmic assembly, including the clamp regions that are most distal to the transmembrane assembly. Studies by Ikemoto and co-workers (62, 63), using RyR labeled with fluorescent probes, have also been interpreted as indicating that conformational changes of a global nature occur when the RyR switches between open and closed states.

We have not detected any of the structural changes that were documented by Orlova et al. (56) in the studies described here, but quite likely this is because the RyRs are predominantly in the closed configuration under the experimental conditions used. In the studies to localize FKBP12, the buffer contained no activators other than endogenous levels of Ca^{2+}, but the buffer did contain 2 mM dithiothreitol, which strongly inhibits (IC_{50} = 0.1 mM) channel activity (64) as assessed by binding of ryanodine. For the CaM localization study, the RyRs were diluted into a buffer lacking dithiothreitol and containing 0.1 mM Ca^{2+}, an activator of RyRs, and therefore the receptors might be expected to switch to an open state, but we did not observe evidence of this; apparently Ca^{2+} alone is not a sufficiently strong activator under these conditions (e.g. Tripathy et al. (39) find an open probability of 0.06 under similar conditions in single-channel conductance studies), or perhaps the residual dithiothreitol (~0.1 mM) contributes to inhibiting the receptor. Addition of CaM to the receptor under these conditions is expected to further inactivate the channels. A proviso to the preceding arguments regarding the functional states of the RyR is that they require extrapolations of findings on functional, membrane-associated channels to the solubilized receptors used in the structural studies.

Both RyR-bound CaM and FKBP12 appear to contact directly domain 3 (handle domain) of the RyR, albeit on opposite sides (Fig. 5). Perhaps this common structural motif is a clue to how these ligands modulate RyR activity. RyR domain 3 is attached at two locations to domain 1, which emanates directly from the transmembrane assembly (rightmost panels in Figs. 3A and 4A), and may, in fact, be a cytoplasmic extension of a transmembrane domain of the receptor. We suggest that the sequence of structural transitions responsible for the modulatory actions of FKBP12 and CaM begins with a direct influence of the ligand on the conformation or orientation of domain 3,
which in turn induces a change in the disposition of domain 1, and finally the change in domain 1 couples to the ion channel gating apparatus associated with the transmembrane assembly. Further structural studies are in progress that hopefully will clarify the intriguing structural transitions that underlie the calcium release mechanism in SR.

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FIG. 6. The two possible motifs for forming arrays of RyRs such as have been observed in electron micrographs of skeletal muscle and SR terminal cisternae vesicles (23, 42, 60). The RyRs shown contain 4 copies each of FKBP12, those of which are nearest the RyR-RyR interaction surfaces being indicated with asterisks.
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