Further Characterization of the Type 3 Ryanodine Receptor (RyR3) Purified from Rabbit Diaphragm*

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We characterized type 3 ryanodine receptor (RyR3) purified from rabbit diaphragm by immunoaffinity chromatography using a specific antibody. The purified receptor was free from 12-kDa FK506-binding protein, although it retained the ability to bind 12-kDa FK506-binding protein. Negatively stained images of RyR3 showed a characteristic rectangular structure that was indistinguishable from RyR1. The location of the D2 segment, which exists uniquely in the RyR1 isoform, was determined as the region around domain 9 close to the corner of the square-shaped assembly, with use of D2-directed antibody as a probe. The RyR3 homotetramer had a single class of high affinity [3H]ryanodine-binding sites with a stoichiometry of 1 mol/mol. In planar lipid bilayers, RyR3 displayed cation channel activity that was modulated by several ligands including Ca2+, Mg2+, caffeine, and ATP, which is consistent with [3H]ryanodine binding activity. RyR3 showed a slightly larger unit conductance and a longer mean open time than RyR1. Whereas RyR1 showed two classes of channel activity with distinct open probabilities (P0), RyR3 displayed a homogeneous and steeply Ca2+-dependent activity with P0 ~1. RyR3 was more steeply affected in the channel activity by sulfhydryl-oxidizing and -reducing reagents than RyR1, suggesting that the channel activity of RyR3 may be transformed more precipitously by redox state. This is also a likely explanation for the difference in the Ca2+-dependence of RyR3 between [3H]ryanodine binding and channel activity.

Intracellular Ca2+ stores play a critical role in regulation of the cytosolic Ca2+ concentration in various cells. Ryanodine receptors (RyRs) belong to a family of Ca2+ release channels of the Ca2+-stores (1–7). Two isoforms of RyRs (RyR1 and RyR2) have been purified from skeletal and cardiac muscles and play a crucial role in excitation-contraction (E-C) coupling, a process whereby depolarization in the sarcotendinous triggers Ca2+ release from sarcoplasmic reticulum (SR) resulting in muscle contraction (6, 8, 9). Type 2 RyR (RyR2) is the primary isoform in heart and is involved in the E-C coupling in cardiac muscle. cDNA and mRNA for type 3 RyR (RyR3) was first identified in mink lung epithelial cells and in specific regions (hippocampus, thalamus, and corpus striatum) of rabbit brain (3, 4). Recent studies revealed that mRNAs for all these isoforms can be widely detected in various tissues, suggesting potential coexpression of multiple isoforms (5).

RyR1 and RyR2 have been purified from skeletal and cardiac muscles, respectively, and are well characterized (1–3, 5, 7). A homotetramer of the ~560-kDa subunit is a functional unit and shows a characteristic structure with 4-fold symmetry that is identical to feet which span the gap between the transverse tubule and terminal cisterna of SR (6, 7). A homotetramer can specifically bind one [3H]ryanodine molecule with a Kd of nanomolar order, after which it is named RyR (1–3). When incorporated into planar lipid bilayers, the receptor demonstrates cation channel activity with a large conductance showing properties of Ca2+-induced Ca2+ release (CICR) (2, 3).

Knowledge about RyR3, in contrast, is much less than that about RyR1 or RyR2 because of its minuscule amount. An excess amount of other coexisting isoforms of similar biochemical characteristics has prevented us from isolating RyR3 by conventional purification procedures. To overcome this difficulty, we utilized an antibody specific to RyR3 (anti-RyR3) as a tool for isolation of the protein. Frog skeletal muscle expresses two isoforms of RyRs (α- and β-RyR) in almost equal amounts (10). Cloning and sequencing of their cDNAs revealed that α- and β-RyRs are homologues of RyR1 and RyR3, respectively (11). Taking advantage of the similarity between RyR3 and β-RyR, we have successfully raised and purified anti-RyR3 against a peptide of amino acid sequence 4375–4387 of rabbit RyR3 (12). Selective immunoprecipitation with the antibody revealed properties of RyR3 in rabbit brain (12) and diaphragm (13). RyR3 forms a homotetramer of ~550-kDa subunit, which is slightly smaller than those of RyR1 and RyR2, as is the case for β-RyR. It shows high affinity [3H]ryanodine binding which is activated by micromolar Ca2+, adenine nucleotides, and caffeine, and inhibited by millimolar concentrations of Ca2+ and Mg2+, procaine, and ruthenium red, indicating that it may function as a CICR channel. The contents of RyR3 in rabbit diaphragm and brain were estimated to be less than 1 and 0.06%, respectively, of RyR1 in skeletal muscle (12, 13).

In this paper, we describe the structural and functional characterization of RyR3 that was successfully purified from rabbit diaphragm by immunoaffinity chromatography. [3H]Ry-
andonic binding and single channel recordings of the molecule revealed several unique properties of RyR3. We also defined the location of the differential segment in the assembly of the RyR1 molecule, which could be one of the origins for the differences in function between the two isoforms. During the course of publication of our results, Jeyakumar et al. (14) independently reported the purification of RyR3.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-RyR3 antibody against a peptide corresponding to the amino acid sequence 4575–4587 of rabbit RyR3 (RyR3-peptide) was produced in rabbits by affinity chromatography with a porcine-β-RyR-bound polyvinylidene difluoride (PVDF) membranes (12, 13). Monoclonal antibody 3F4 against a 12-kDa FK506-binding protein (FKBP12) and human recombinant FKBP12 (hFKBP12) were produced as described previously (15, 16). Monoclonal antibody XA7, which reacts with RyR1 (17) and S4C, which recognizes all the three mammalian RyR isoforms (18) were purchased from Upstate Biotechnology, Inc., and Affinity Bioreagents, Inc., respectively. [3H]Ryanodine (60–90 Ci/mmol) was purchased from NEN Life Science Products. Goat anti-rabbit IgG-agarose and glutathione-agarose were obtained from Sigma. Egg lecithin (egg total phosphate extract) was from Avanti Polar-Lipids. All other reagents were of analytical grade.

**Isolation of Sarcoplasmic Reticulum (SR) Vesicles**—Heavy fraction of SR vesicles was prepared from rabbit diaphragm according to Murray et al. (10). The isolation was quickly frozen in liquid N2 and stored at −80 °C until used.

**Purification of RyR3—**RyR3 was purified by a combination of sucrose gradient ultracentrifugation and immunoprecipitation. Anti-RyR3 beads were prepared by mixing anti-rabbit IgG-agarose beads with the purified anti-RyR3 antibody, followed by washing with a buffer containing 0.15 M NaCl, 20 mM sodium phosphate, pH 7.2. These anti-RyR3 beads can be stored at 4 °C for 3 months without a significant decrease in the RyR3-binding activity. Rabbit diaphragm SR vesicles (10 mg) were solubilized in 4% CHAPS and 2% egg lecithin in 2.5 ml of 0.5 M NaCl, 0.1% Triton-HCl, pH 7.4, and 2 mM dithiothreitol (DTT) including the above protease inhibitors (buffer A) to isolate tetrameric RyRs by ultracentrifugation on 5–20% linear gradients of sucrose (10). The fractions around ~15% sucrose containing tetrameric RyRs were incubated overnight at 4 °C with 100 μl of the anti-RyR3 beads. The beads were washed five times with buffer A containing 1% CHAPS and 0.5% egg lecithin, and then incubated overnight with 30 μl of RyR3-peptide in the same buffer to dissociate RyR3 from the antibody. Finally, the supernatant containing RyR3 was applied to a small gel filtration column (Centri-Sep, Applied Biosystems Inc.) to remove a large amount of the RyR3-peptide.

**Protein Assay**—Protein concentrations of the purified RyR1 were determined by the method of Kaplan and Pedersen (19) with Amido Black staining SDS-polyacrylamide gel with a MasterScan densitometer. The separated proteins were electrophoretically transferred onto PVDF membranes at 40 V in the presence of 0.02% SDS (10, 13). Immunodetection was carried out with an ECL system (Amersham Pharmacia Biotech) using peroxidase-conjugated secondary antibodies. Primary antibodies were diluted as follows: 1:1,000 for anti-RyR3, 1:5,000 for XA7, 1:5,000 for S4C, and 1:1,000 for 3F4. When probed by these antibodies, the same transferred PVDF membranes were run together with the purified anti-RyR3 antibody, followed by washing twice with buffer B, the beads were incubated for 1 h at 37 °C with 1 μg of the purified RyR1 or RyR3 in buffer B. The beads were washed three times with buffer B and proteins bound to the beads were subjected to SDS-PAGE.

**[3H]Ryanodine Binding Assay**—To determine with high sensitivity the [3H]ryanodine binding activity with a small amount of RyR3 protein, we separated protein-bound ryanodine from the free ligand using a small scale gel filtration column (Centri-Sep) in a centrifuge. The purified RyR3 was incubated with 8.5 nM [3H]ryanodine for 5 h at 25 °C in 30–50 μl of a binding buffer containing 0.17 M NaCl, 10 mM MOPS/OH, pH 6.8, 2 mM DTT, 1% CHAPS, 0.5% egg lecithin, 2 mM AMP, and various concentrations of Ca2+ (buffer 10 with 10 mM EGTA. Free Ca2+ concentrations were calculated using the value of 8.79 × 105 s−1 M−1 at 4 °C with 10 mM EGTA. Binding assays were performed independently under identical conditions using a 1:1 ratio of the sample (10–20 μl) was applied to the Centri-Sep column that had been equilibrated with 1.1 mM NaCl, 10 mM MOPS/OH, pH 6.8, 1% CHAPS, 0.5% egg lecithin, 2 mM DTT, and 0.1 mM CaCl2. The radioactivity of the eluate was determined by a liquid scintillation counter. NonSpecific radioactivity was determined in the presence of 50 μM nonradioactive ryanodine. By using this assay system, we were able to determine the ryanodine binding activity with only 20 ng of the purified RyR3 protein, which is one-tenth the minimum amount for the conventional filtration assay. [3H]Ryanodine binding to the purified RyR3 was determined under the identical condition to the filtration assay with polyethyleneimine-treated glass fibers as described (10).

**Electron Microscopy**—The structure of RyR molecules was examined by electron microscopy after negative staining. A 1:100 dilution of the purified antibody was used to completely eliminate phospholipid and to reduce detergent from the preparation, since their presence hampered the visualization of structural details of the proteinaceous receptors. To meet such requirements, the materials for the structural study were treated as follows. The RyR3 immunoprecipitated by anti-RyR3 beads was washed with a buffer containing 0.1% CHAPS, instead of 1% CHAPS and 0.5% egg lecithin, to finally pinpoint the precise site. The antibody was visualized with a probe large enough to be easily found, and later with the hard to identify by itself, in the thick stain layer embedding the total molecule (10). The ryanodine binding activity with RyR3 was restored by negative staining but with use of a specific anti-D2-region polyclonal antibody as a probe. Since the size of the IgG molecule is quite small and thin (~150 kDa) as compared with the giant RyR molecule (~2,200 kDa) standing upright on the carbon-support, it was anticipated that the IgG probe bound to the receptor molecule might be hard to identify by itself, in the thick stain layer embedding the total receptor assembly. Thus, we used a two-step procedure (24, 25) to search for the epitope site of the antibody, tentatively with IgG conjugated with a probe large enough to be easily found, and later with the antibody by itself, to finally pinpoint the precise site. The purified RyR3 was produced in rabbits and purified by affinity chromatography with frog FKBP12 (20). The plasmid construction of GST-FKBP was essentially the same as previously reported (21). A plasmid pBFKFP333 for hFKBP12 (15) was subjected to PCR to place S9F4. When probed by these antibodies, the same transferred PVDF membranes were run on a 2–3 μl of the sample was applied to fresh thin carbon film over a 200-mesh copper grid. After 3 min, the residual solution was thoroughly rinsed off with the above buffer without CHAPS, and the sample was stained negatively with 1% uranyl acetate containing catarin (23). Specimens were set in a side entry goniometer for the JEOL 2000EX electron microscope with the carbon side facing upward. Stereo-paired micrographs were taken by tilting ±10° at 80 kV acceleration voltage (23).

**Expression** of purified Type 3 Ryanodine Receptor

The location of the D2 segment in the RyR1 architecture was determined similarly by negative staining but with use of a specific anti-D2-region polyclonal antibody as a probe. Since the dimension of the IgG molecule is quite small and thin (~150 kDa) as compared with the gigantic RyR molecule (~2,200 kDa) standing upright on the carbon-support, it was anticipated that the IgG probe bound to the receptor molecule might be hard to identify by itself, in the thick stain layer embedding the total receptor assembly. Thus, we used a two-step procedure (24, 25) to search for the epitope site of the antibody, tentatively with IgG conjugated with a probe large enough to be easily found, and later with the antibody by itself, to finally pinpoint the precise site. The purified RyR3 was produced in rabbits and purified with rabbits using GST-fusion protein as an antigen. A cDNA fragment (residues 4073–4239) was obtained from a cDNA library RYR1cds (26) and cloned into the pGEX-3X vector (Amersham Pharmacia Biotech). Expression and purification of the GST-fusion protein was carried out according to the manufacturer's instructions. The IgG was affinity-purified by the RyR1-bound PVDF membranes (10). This anti-
body stained a single band of RyR1 on Western blot analysis of SR vesicles which, in turn, were specifically immunoprecipitated by the same antibody (data not shown). In order to produce a probe with a large marker, the antibody was biotinylated and conjugated with oligomeric avidin, a linear rod-like assembly. Biotinylation of the antibody was carried out by incubation of the antisem with sulfos-NHS-LC-biotin (Pierce). An oligomeric avidin was prepared by addition of a divalent biotin (generous gift from Dr. Kazuo Sutoh to E. K.) to neutravidin (Pierce) at a molar ratio of 2:1 (divalent biotin/neutravidin) (27) and was mixed with the biotinylated antibody before use. For negative staining, purified RyR1 receptor was first applied onto a carbon film and left standing for 3 min. The grid was briefly washed and the solution containing the antibody with or without avidin conjugate was added. After 3–5 min incubation period, it was exhaustively washed with the buffer and stained negatively with uranyl acetate, as described above.

**Planar Lipid Bilayer Experiments and Single Channel Data Acquisition**—Single channel recordings were carried out as described previously (28). Briefly, lipid bilayer consisting of a mixture of L-a-phosphatidyl-diyethanolamine, L-a-phosphatidyl-L-serine, and L-a-phosphatidylcholine (5:3:2 by weight) in decane (40 mg/ml) was formed across a hole (~200 μm in diameter) in a polystyrene partition separating two chambers referred to as cis (volume 1 ml) and trans (volume 1.5 ml). The trans chamber was held at virtual ground potential, and the cis chamber was voltage-clamped at −40 mV relative to the ground, unless noted otherwise. Incorporation of the purified RyR channel was performed in asymmetrical KCl solutions containing 500:50 μM (cis/ trans) KCl, 20 μM HEPES/Tris, pH 7.4, and 0.1 mM CaCl2. The protein was added to the cis chamber. After confirming the channel incorporation by the occurrence of flickering currents, further incorporation of the protein was prevented by supplement of an aliquot of 3 mM KCl dissolved in 20 mM HEPES/Tris to the trans chamber. Single channel currents were recorded in symmetrical solutions containing 500 mM KCl, 20 mM HEPES/Tris, pH 7.4, and various concentrations of free Ca2+ buffered with 1 mM EGTA. Free Ca2+ was calculated using the apparent binding constant of EGTA for Ca2+ by Harafuji and Ogawa (22) and was confirmed by potentiometry with a handmade ETH1001-based a-cd electrode. Only bilayers containing a single channel were used in this study. Experiments were carried out at room temperature (18–22 °C). We found that the purified RyRs could be incorporated into the bilayers in either orientation. The sidedness of the single channel was determined by response to Ca2+, because the RyR channel sensitively responded to cytoplasmic Ca2+. About 90% of the RyR channels was blocked by lowering the cis free Ca2+ to 10–100 nM with EGTA, indicating that the cytoplasmic side of most channels faced the cis chamber. Single channel currents amplified by an Axopatch 1D patch clamp amplifier (Axon Instrument, CA) were displayed on an oscilloscope, filtered at 1 kHz using an eight-pole low-pass Bessel filter, and digitized at 5 kHz for analysis. Data were saved on the hard disk of an IBM personal computer. Pp and the lifetime of open and closed events from records of duration >2 min were calculated by 50% threshold analysis using pClamp (version 6.0.4) software. The results were presented as means ± S.E.

**RESULTS**

**Purification of RyR3 from Rabbit Diaphragm SR**—RyR3 was purified from rabbit diaphragm using immunoprecipitation with an antibody specific to RyR3 (anti-RyR3) (12, 13) which was raised against a synthetic peptide corresponding to residues 4375–4387 of rabbit RyR3 (RyR3-peptide) (see “Experimental Procedures”). The diaphragm was found to express about 10-fold more abundant RyR3 than brain in rabbits (13). As shown in Fig. 1A, SR protein prepared from rabbit diaphragm contained a large amount of RyR1 that was easily seen on Coomassie Brilliant Blue-stained gel and positively detectable on Western blot with an antibody to RyR1 monoclonal antibody XA7 and a low level of RyR3 that was identified on anti-RyR3 blot. After solubilization with CHAPS, SR proteins were incubated with anti-RyR3-agarose beads to precipitate RyR3 (12, 13). The band for RyR3 was clearly detected in the precipitate on both protein staining and anti-RyR3 blotting of rabbit diaphragm SR vesicles. 30 μg of the SR vesicles was subjected to SDS-PAGE on a 2–12% linear gradient gel and stained with Coomassie Brilliant Blue (CBB). Similar gel was transferred onto a PVDF membrane and probed with anti-RyR1 monoclonal antibody XA7 (XA7) or with anti-RyR3 (Anti-RyR3). B, selective immunoprecipitation of RyR3. Five mg of the solubilized rabbit diaphragm SR was incubated with anti-RyR3 beads in the absence (Control) and presence (RyR3-pep.) of 30 μM RyR3-peptide. The resultant beads were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (panel CBB) or probed with the anti-RyR3 antibody (panel Anti-RyR3). C, recovery of RyR3 by the addition of the RyR3-peptide. RyR3 immunoprecipitated with anti-RyR3 beads was incubated with (panel RyR3-pep.) or without (panel Control) 30 μM RyR3-peptide. Proteins in the supernatant (Sup.) and the precipitated beads (Ppt.) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Arrowheads indicate the position of RyR3. The RyR3-peptide is detected as a heavily stained band near dye front (asterisk).

**Fig. 1. Selective immunoprecipitation by the anti-RyR3 antibody and recovery of RyR3 by the RyR3-peptide.** A, Western blotting of rabbit diaphragm SR vesicles. 30 μg of the SR vesicles was subjected to SDS-PAGE on a 2–12% linear gradient gel and stained with Coomassie Brilliant Blue (CBB). Similar gel was transferred onto a PVDF membrane and probed with anti-RyR1 monoclonal antibody XA7 (XA7) or with anti-RyR3 (Anti-RyR3). B, selective immunoprecipitation of RyR3. Five mg of the solubilized rabbit diaphragm SR was incubated with anti-RyR3 beads in the absence (Control) and presence (RyR3-pep.) of 30 μM RyR3-peptide. The resultant beads were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (panel CBB) or probed with the anti-RyR3 antibody (panel Anti-RyR3). C, recovery of RyR3 by the addition of the RyR3-peptide. RyR3 immunoprecipitated with anti-RyR3 beads was incubated with (panel RyR3-pep.) or without (panel Control) 30 μM RyR3-peptide. Proteins in the supernatant (Sup.) and the precipitated beads (Ppt.) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Arrowheads indicate the position of RyR3. The RyR3-peptide is detected as a heavily stained band near dye front (asterisk).
RyR3 preparation, indicating no contaminating RyR1 in the purified RyR3. Anti-RyR3 reacted with RyR3 but not with the purified RyR1. These findings confirm our previous results of homotetrameric formation of each isoform (13).

FKBP12 Binding of RyR3—It has been demonstrated that FKBP12 is tightly associated with RyR1 with a stoichiometry of 1 mol/mol of the monomer and modulates channel activity (24, 29, 30). To know whether RyR3 associated with FKBP12 as well, we carried out Western blotting using an antibody against FKBP12. As shown in Fig. 3A, rabbit diaphragm SR vesicles contained a band recognized with anti-FKBP12 monoclonal antibody 3F4 whose mobility was almost identical with human recombinant FKBP12 (hFKBP12) (Fig. 3B). This corresponds to previous results that RyR1 associates with FKBP12 in skeletal muscles (29, 31). In contrast, no band was detectable in either RyR1 or RyR3 preparation. Because the charged amount of the RyR in these experiments was comparable to that in the SR vesicles on the basis of the density of RyR bands (upper panel), negative immunoreactivity is not due to a small amount of charged proteins. These results indicate that FKBP12 may be dissociated from RyR1 or RyR3 during purification procedures. This is consistent with the results of Wagenknecht et al. (24) who reported a substantial loss of FKBP12 from the RyR1 preparation on addition of CHAPS.

To address whether RyR3 possesses an ability to bind FKBP12, we carried out FKBP12 binding assays using glutathione S-transferase (GST)-hFKBP12 fusion protein (GST-FKBP) (Fig. 3B). RyR1 (left panel) and RyR3 (right panel) were incubated with glutathione-agarose beads that had been incubated with GST-FKBP or control GST in a buffer containing 0.17 M NaCl and 0.1% CHAPS. After washing the beads, proteins bound to the beads were detected by SDS-PAGE. When incubated with GST-FKBP beads, RyR1 and RyR3 were bound to the beads (lane 2). When incubated with control GST beads (lane 1), in contrast, bound RyRs were hardly detectable. In the presence of 10 μM FK506, which was known to dissociate FKBP12 from RyR1, RyRs were also severely prevented from binding the GST-FKBP beads (lane 3). These results strongly indicate that RyR3 can specifically bind FKBP12, as is true of RyR1.

Electron Microscopy of Purified RyR3—Fig. 4 exhibits negatively stained images of RyR3 particles, in comparison with those of RyR1. As shown in the general view (Fig. 4A), RyR3 preparation showed homogeneous distribution of the particles of rectangular shape similar to the reported image of RyR1. A gallery of the selected particles shows that both isoforms are indistinguishable in shape or in overall dimension (about 25 nm on each side) from each other (compare Fig. 4, B with C). They showed 4-fold symmetry reflecting their tetrameric nature, and the substructures were well defined under higher magnification. The central core with a crossed groove and outer regions with several cavities or pockets were the features commonly found in both isoforms, although closer examination revealed delicate differences in appearance from particle to particle, probably owing to uncontrollable differences in stain depth. It is also notable that both isoforms shared a pinwheel-like appearance with the same rotational handedness.

We also obtained several images of RyR3 particles with less contribution of phospholipid and/or detergent. Four pairs of stereo-micrographs for RyR3 (alternate columns in each row of Fig. 4D) show the tubular network-like structure forming several stain-filling pockets, which appears very similar to the published solid models of RyR1 (32, 33). Despite careful comparison of the two isoforms, we could not find any significant difference between them which exceeds the image variance due
the amino acid sequence of RyR is close to the membrane-spanning region, it might not be an easy task to identify the epitope site in the geometry of the total RyR assembly that is attached to the carbon support through the membrane-spanning region. So, we decided to use the other antibody probe which is not only easier to visualize but also specific to some functionally important region.

The fact that RyR3 lacks a stretch of about 100 amino acid residues corresponding to 1305–1405 in RyR1 is one of the most substantial differences between them (34). The region including this stretch is generally termed D2 because of its high divergency among RyR1–3 (35). As an appropriate candidate to search for the subtle difference between RyR1 and RyR3 in the local architecture, we made an attempt to define the location of the D2 region in the organized structure of the RyR1 assembly by negative staining. We utilized an antibody specific to the D2 region of RyR1 (anti-D2 antibody) that was produced against the amino acid sequence 1358–1413 of rabbit RyR1 (see “Experimental Procedures”). Because this antibody could immunoprecipitate native RyR molecules in solution, it is clear that the antibody can attach to RyR without significantly affecting its organized architecture. In order to visualize unambiguously the bound antibody in negatively stained images, we initially used the antibody conjugated with an oligomeric avidin which might form a characteristic rod-like shape and would be easily distinguished from the other structures (27). Fig. 5A exhibits a general view of such specimen. Most of the receptor molecules in the field were accentuated with strongly stain-excluding dots and many of them with short rod-like structures (indicated by black arrows), both close to the corners of the square-shaped assembly. This indicates that the anti-D2 antibody should be associated with the RyR1 assembly at the sites close to the corner of the square. This probe, however, was too large to pin-point the precise epitope site on the assembly. With the knowledge that the D2-specific antibody might bind close to the corners of the RyR1, we then carried out similar experiments with the unconjugated antibody. Fig. 5B shows the images obtained in such way. After incubation with antibody solution and exhaustive washing, many receptor molecules appeared as square-shaped particles with small but strongly stain-excluding spots close to the corners. A substantial fraction of them also had the protrusions from the same position close to the corner of the square assembly. These spots often consisted of a triangular or tripodal structure that would represent the anti-D2 IgG molecule (free IgG molecules are shown in the insets as reference). They were reproducibly found at several nanometers apart counterclockwise from the exact corners of the rectangular receptor molecules. More convincing evidence for the location of the epitope site was the presence of cross-linked particles through fine Y- or V-shaped pieces, the dimension of which is similar to that of free IgG, connected to the sites equivalent to those suggested above as the epitope site. It is notable that the disposition of the two adjacent receptors seen here was opposite to the model motif II for self-association of the purified RyR which was suggested by Wagenknecht et al. (25). Hence, it seems unlikely that these connected receptors were due to self-association without the antibody. These lines of evidence strongly suggest that the epitope site of anti-D2 antibody, i.e. the D2 region, exists at the position several nanometers apart counterclockwise from the corner of the tetrameric receptor particles. It has been shown that RyR adheres almost exclusively with its transmembrane side toward the carbon film (36). Because the images in this paper are printed so that the receptor molecules are observed from their cytoplasmic side, the location of the D2 region may be safely interpreted to be at or close to “domain 9” in the three-dimensional archi-
properties of purified RyR3 were determined by gel filtration method (see “Experimental Procedures”). The preliminary results indicate that properties of [3H]ryanodine binding to the purified RyR3 are consistent with those already reported with the immunoprecipitated receptors (12, 13); it was activated by micromolar Ca^{2+}, adenine nucleotides, and caffeine, inhibited by millimolar concentrations of Ca^{2+} and Mg^{2+}, procaine, and ruthenium red, and affected by salt concentration of the medium (data not shown). In this study, we focused on some important characteristics that so far remained to be determined or were controversial.

Fig. 6 shows dose-dependent [3H]ryanodine binding to the purified RyR3. The amounts of the bound [3H]ryanodine in the medium containing 1 M NaCl, 1 mM AMP-PCP, and 0.1 mM Ca^{2+} increased with increases in [3H]ryanodine concentrations and approached the asymptotic value. The Scatchard plot (inset) gave a straight line within the range of 0.5–21 nM [3H]ryanodine, indicating a single class of high affinity ryanodine-binding sites with an apparent KD of 1.5 nM, which is similar to that of the immunoprecipitated RyR3 (1.6 nM) (13). In the previous study (13), we were unable to estimate the stoichiometry of ryanodine bound to RyR3, because we could not determine the amount of the receptor. The B_{max} of the purified RyR3 (522 pmol/mg protein) shown here revealed that it binds [3H]ryanodine with a stoichiometry of 1 mol of ryanodine per 1 mol of the homotetramer, as is true of the other isoforms (3). Based on the binding sites of [3H]ryanodine binding to RyR3 in rabbit diaphragm SR (B_{max} = 0.065 pmol/mg) (13), the amount of RyR3 in 10 mg of the SR is calculated to be 1.2 μg (10 × 0.065 ÷ 522 × 1,000). We obtained 0.5–0.7 μg of the purified RyR3 from 10 mg of the SR vesicles. These results show that
Ca²⁺ that both receptors were effectively inactivated by millimolar protein, respectively.

inhibited by Mg²⁺ recordings (Fig. 9).

tion columns as described under “Experimental Procedures.” The Linear Scatchard plot (inset) indicates that RyR3 had a single class of binding sites. The $K_D$ and $B_{max}$ values were 1.5 nM and 522 pmol/mg protein, respectively.

the yield of RyR3 was 42–58% and that the extent of purification was more than 8,000-fold.

We previously reported that RyR3 in rabbit diaphragm showed unique characteristics with regard to sensitivity to Ca²⁺ and Mg²⁺; it was less sensitive to activating Ca²⁺ than RyR1 and resistant to inhibition by Mg²⁺ (13). Since these conclusions were obtained from the results of [³H]ryanodine binding with the immunoprecipitated receptors, one might assume that binding of anti-RyR3 antibody may alter the sensitivity of RyR3. We therefore determined Ca²⁺- and Mg²⁺-sensitivities of RyR3 with the purified receptors. Fig. 7A shows the Ca²⁺-dependence of the [³H]ryanodine binding to the purified RyR1 and RyR3 in an isotonic medium containing 0.17 M NaCl. The Ca²⁺ sensitivity of RyR3 for activation (open circles) ($EC_{50} = 14 \mu M$) was 4-fold lower than that of RyR1 (filled circles) ($EC_{50} = 3.5 \mu M$). On the other hand, it was apparent that both receptors were effectively inactivated by millimolar Ca²⁺; IC₅₀ values were calculated to be 1.9 and 2.9 mM for RyR1 and RyR3, respectively. These results confirm the previous conclusion (13) that RyR3 shows a lower sensitivity to activating Ca²⁺ than RyR1, whereas they are similar with regard to the Ca²⁺-sensitivity in inactivation.

The effect of Mg²⁺ on [³H]ryanodine binding of the purified RyR3 is shown in Fig. 7B. The amount of binding to RyR3 that had been activated by 100 mM Ca²⁺ was dose-dependently reduced by Mg²⁺ with IC₅₀ of 2.1 mM, which was similar to that of RyR1 (2.5 mM). The IC₅₀ value for Mg²⁺ was consistent with that for inactivating Ca²⁺ (see Fig. 7A). Because dose-dependent inhibition by Mg²⁺ was also observed on single channel recordings (Fig. 9C), it is concluded that RyR3 is as effectively inhibited by Mg²⁺ as RyR1. This is in contrast to our previous results with the immunoprecipitated receptors (13). It is unlikely that binding of anti-RyR3 antibody may alter the sensitivity to Mg²⁺ of RyR3, because the inhibition by Mg²⁺ of the purified RyR3 was unchanged, irrespective of the presence or absence of the antibody (data not shown). The reason for the discrepancy remains to be solved.

Single Channel Recordings of the Purified RyR3 Channel—Single channel currents through the purified RyR3 were recorded in a solution containing symmetrical 500 mM KCl, 20 mM HEPES/Tris, pH 7.4. As shown in the right column in Fig. 8A, the RyR3 channel was activated by exposure to cis-pCa 6.7 ($P_o = 0.236$). The $P_o$ remained unchanged at voltages between 

$-60$ and $+60$ mV, indicating no voltage dependence of the open probability. The counterparts for RyR1 with a similar $P_o$ (0.259) in the presence of 10 μM cis Ca²⁺ were drawn in the left column of Fig. 8A. It should be noted that the subconduction opening was not as common as claimed to be characteristic of FKBP12-free RyR, although the frequency of subconduction opening was only slightly higher with the purified RyR1 than that with the receptor in SR vesicles. The current-voltage plot for RyR3 (open circles in Fig. 8B) gave a linear relationship between $-60$ and $+60$ mV, as is true of RyR1 (filled circles in Fig. 8B). This indicates the non-rectifying property of either channel. The conductance of the RyR3 channel ranged from 719 to 794 pS with an average of $743 \pm 11$ pS (mean ± S.E., n = 6). This value was significantly larger than $630 \pm 9$ pS (n = 8) for the RyR1 channel (ranging from 582 to 669 pS), when K⁺ was used as current carrier.

The gating kinetics for RyR1 and RyR3 channels were determined by open and closed time histograms. Typical results are shown in Fig. 8C where RyR1 and RyR3 showed similar $P_o$ values of 0.2–0.25. Mean open time for the RyR3 channel (4.85 ± 1.16 ms, n = 7) was significantly ($p < 0.05$) longer than that for the RyR1 channel (2.58 ± 0.35 ms, n = 10). The open lifetime distributions were best fit by two exponentials for both channels (dashed lines). The fast ($\tau_o$) and the slow ($\tau_{0}$) open time constants and their relative areas (in parentheses) which were averaged from independent experiments were $0.50 \pm 0.04$ ms (80.2 ± 2.3%) and $2.59 \pm 0.25$ ms (19.8 ± 2.3%) for the RyR1 channel (n = 10), and $0.53 \pm 0.05$ ms (48.0 ± 12.8%) and 5.61 ±
Fig. 8. Conductance and gating kinetics of purified RyR1 and RyR3 channels. The purified RyR1 and RyR3 were incorporated into lipid bilayers as described under "Experimental Procedures." A, single channel currents of the RyR1 channel activated by 10 μM Ca²⁺ (left column) and the RyR3 channel activated by 0.2 μM Ca²⁺ (right column) were recorded in a symmetrical recording solution containing 500 mM KC1 and 20 mM HEPES/Tris, pH 7.4, at indicated holding potentials. The levels of the base lines are indicated by a short line to the right of each current trace. B, current-voltage relationships of the purified RyR1 (filled circles) and the purified RyR3 (open circles) channels. Data were mean ± S.E. of eight and six independent experiments for RyR1 and RyR3, respectively. C, histograms of the dwell time for the RyR1 (left) and RyR3 (right) channels. The histograms were constructed from channels with similar open probabilities of RyR1 (P₀ = 0.259) and RyR3 (P₀ = 0.236). Open time constants and their relative areas (in parentheses) were calculated as follows: τCA = 0.58 ms (76%), τC₂ = 1.97 ms (24%) for RyR1 channel; τC₁ = 0.59 ms (57%), τC₂ = 2.51 ms (43%) for RyR3 channel. Calculated closed time constants and their relative areas (in parentheses) are as follows: τC₁ = 0.57 ms (36%), τC₂ = 2.53 ms (46%), τC₃ = 8.85 ms (18%) for RyR1 channel; τC₁ = 0.31 ms (27%), τC₂ = 4.48 ms (40%), τC₃ = 17.04 ms (33%) for RyR3 channel.

1.75 ms (52.0 ± 12.8%) for the RyR3 channel (n = 7), respectively. Thus, the longer mean open time for RyR3 is due both to a larger fraction of the τC₂ component and to its longer dwell time. In contrast, mean closed time for the RyR3 channel (8.93 ± 1.39 ms) was similar to that for the RyR1 channel (8.68 ± 2.36 ms). Closed time constants in both channels were best fit by three similar exponentials (dashed lines). The fast (τC₁), medium (τC₂), and slow (τC₃) components of closed time constants and their relative areas (in parentheses) were 0.52 ± 0.06 ms (34.0 ± 3.2%), 2.91 ± 0.34 ms (43.3 ± 2.9%), and 11.94 ± 1.46 ms (22.7 ± 3.3%) for the RyR1 channel and 0.55 ± 0.10 ms (32.4 ± 9.7%), 3.01 ± 0.59 ms (35.6 ± 5.1%), and 12.31 ± 1.26 ms (32.0 ± 7.9%) for the RyR3 channel, respectively.

Fig. 9. Ligand gating properties of the purified RyR3 channel. Mean open probability (P₀) is indicated at the right of each trace. A, effects of Ca²⁺ and caffeine. The channel was first inhibited by 1.63 mM EGTA (0.1 μM free Ca²⁺) and then activated partially by 0.2 μM Ca²⁺. The concentrations of ligands shown in the figure stand for their final concentrations. Subsequent application of 1 mM caffeine increased the P₀ of the channel. B, effect of ATP on the purified RyR3 channel. 1 mM ATP was subsequently added to the cis chamber in the presence of 0.25 μM cis-[3H]ryanodine. C, effects of Mg²⁺, ryanodine, and ruthenium red. MgCl₂ (1 and 10 mM) dose-dependently reduced the P₀ of the RyR3 channel that had been activated by 10 μM Ca²⁺. Subsequent addition of 10 μM ryanodine shifted the channel to a long lasting open state with about half of normal conductance. The channel was completely blocked by the addition of 5 μM ruthenium red.
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Fig. 10. Ca^{2+} dependence of the purified RyR3 channel. A, typical current traces of the purified RyR3 channel. The RyR3 channel was activated by 0.2 μM or more Ca^{2+} and inhibited by 3 mM Ca^{2+}. Note that the RyR3 channel was nearly fully activated (P_o ≈ 1) over the whole range of pCa 6–3. B, typical current traces of the RyR1 channel with low open probability (low P_o, left column) and high open probability (high P_o, right column). C, pCa-P_o relationship of each type of channel. Data were mean ± S.E. of 4–7, 9–12, and 4–7 separate experiments for RyR3 (open circles), low P_o RyR1 (open triangles), and high P_o RyR1 (filled circles) channels, respectively. There was no spontaneous transition between low and high P_o channels.

Fig. 10 depicts Ca^{2+} dependence of single channel activities of purified RyR1 and RyR3 channels. The channels were activated by Ca^{2+} alone without any addition of other ligands such as ATP or caffeine. The RyR3 channel displayed no open event at pCa 7, and partial open events (P_o = 0.172) were seen at pCa 6.7 (Fig. 10A). The channel was further activated to a great extent (P_o = 0.817) at pCa 6.3 and showed almost full opening (P_o = 1.0) over the wide range of pCa 6–3. Around 3 mM Ca^{2+}, on the contrary, the channel activity markedly declined (P_o = 0.191). P_o values that were obtained from 4 to 7 separate experiments are plotted against varied cis Ca^{2+} concentrations in Fig. 10C. At 0.1 μM Ca^{2+}, five of six determinations showed no open events, whereas one experiment showed P_o = 0.181. Thus, the threshold of Ca^{2+} concentration for the RyR3 channel opening seemed to be around 0.1 μM, and the channel was already fully activated at 1 μM or more Ca^{2+}. The pCa-P_o relationship was very steep and appeared to be in almost all-or-none fashion. This Ca^{2+} dependence is similar to the results reported with RyR2 (1, 2) as well as those with RyR3 which were determined by lipid bilayer experiments (14, 37). The RyR3 channel was inactivated by Ca^{2+} concentrations above 1 mM. The dose dependence of inactivation by high Ca^{2+} appeared similar to that of inhibition by Mg^{2+} (see Fig. 9).

We determined Ca^{2+} dependence of the RyR1 channel activity to compare with that of the RyR3 channel (Fig. 10B). RyR1 channels demonstrated the following two distinct populations of single channel current fluctuations: high and low open probability (high and low P_o, channel, respectively), just as recently revealed with terminal cisterna of rabbit skeletal muscle SR (38, 39). The high P_o channel (7 of 19 determinations) was activated by cis Ca^{2+} concentrations up to 10 μM and inhibited at higher than 100 μM Ca^{2+} (right column in Fig. 10B). In contrast, the low P_o channel (12 of 19 determinations) showed only a few open events even at the optimum Ca^{2+}, although it showed biphasic Ca^{2+} dependences (left column in Fig. 10B). P_o values against pCa values for high P_o (n = 4–7) and low P_o (n = 9–12) channel are also plotted in Fig. 10C. Their Ca^{2+} dependences appeared to be similar, although the relationship for low P_o channel was less clear. Whereas the RyR3 channel showed homogeneous channel activity with nearly full opening (P_o ≈ 1), RyR1 displayed heterogeneous populations of the channel activity with high P_o (–0.3) and low P_o (<0.05), respectively, at the optimum Ca^{2+}. Ca^{2+} concentrations that would give half the maximum activation were estimated to be ~1 and ~0.3 μM for RyR1 and RyR3 channels, respectively. In the presence of 0.1 μM Ca^{2+}, interestingly, the high P_o RyR1 showed a larger P_o than the RyR3 channel.

It has been demonstrated that the RyR channel is modulated by the redox state of RyR molecules that could be attained by sulphydryl-oxidizing and -reducing reagents (39–43). We therefore examined effects of sulphydryl reagents on single channel activity of RyR1 and RyR3 channels. Typical data are depicted in Fig. 11. Addition of 50 μM DTT to the cis side of the high P_o RyR1 channel led to a remarkable reduction in P_o (from P_o = 0.217 ± 0.061 in controls to P_o = 0.071 ± 0.046, n = 7; see Fig. 11A for a typical result). Increase in DTT to 500 μM induced a further decrease in P_o (P_o = 0.035 ± 0.002, n = 7). All DTT-treated channels were modified by 10 μM ryanodine and blocked by 5 μM ruthenium red (data not shown). The channel activity inhibited by application of 100–500 μM DTT (from P_o = 0.104 ± 0.051 in controls to P_o = 0.002 ± 0.001, n = 3) was overshoot to P_o = 0.313 ± 0.006 by subsequent cis addition of 100–500 μM p-chloromercuri phenyl sulfonic acid (pCMPS), a specific organic sulphydryl reagent (bottom traces in Fig. 11A). On the other hand, the low P_o RyR1 channel was activated by the addition of 10–50 μM cis pCMPS (from P_o = 0.004 ± 0.001 in controls to P_o = 0.116 ± 0.034, n = 9), which was similar to the high P_o state (Fig. 11B). Subsequent exposure of such pCMPS-activated channel to 25–50 μM DTT reversed the P_o to a low level (0.019 ± 0.008, n = 4). In 3 of 14 experiments, cis-pCMPS elicited irreversible closure after transient increase in P_o, which could not be recovered by any ligands including a large amount of DTT (data not shown), suggesting the existence of at least two different sulphydryls associated with activation and inactivation of the channel in the RyR1 molecule. Similar results on sulphydryl reagents were observed with RyR channels in frog skeletal muscle SR (43). The conversion between low P_o and high P_o channels occurred immediately after addition of these reagents. During channel activation or inactivation by these sulphydryl-modifying reagents, the channel conductance remained unchanged. These findings suggest that the two populations of the RyR1 channel showing different P_o against pCa values might reflect the extent of their redox state.
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RyR3 channel showing low $P_o$ (0.054 ± 0.026, $n = 4$) at 0.13–0.3 μM Ca$^{2+}$, on the other hand, was markedly activated by the addition of 10 μM pCMPS to the cis side to high $P_o$ (0.599 ± 0.192) (Fig. 11D). Thus, the effect of sulfhydryl reagents on the RyR3 channel was similar to that on the RyR1 channel; a sulfhydryl-oxidizing reagent activates the channel activity, whereas a sulfhydryl-reducing reagent inhibits it. The channel activity of RyR3, however, appears to depend steeply on the redox state showing almost all-or-none fashion, whereas the dependence is more gradual with RyR1. This may be reflected in the observation that the RyR3 channel showed no opening in the closed state after addition of DTT, whereas some opening was still detected with RyR1 even at $P_o < 0.001$.

DISCUSSION

In this study, we purified homotetrameric RyR3 from rabbit diaphragm by immunoprecipitation with highly specific antibody to RyR3 and by dissociation under gentle conditions using an epitope peptide. The purified RyR3 demonstrated the characteristic structure of a quatrefoil under electron microscopy. It also showed Ca$^{2+}$-dependent high affinity [$^3$H]ryanodine binding and cation channel activity. Thus, RyR3 purified by this procedure retained structural and functional integrity, which enabled us to characterize the protein.

Our recent studies with the immunoprecipitated RyR3 indicate that RyR3 forms a homotetramer of a single polypeptide with a mobility slightly larger than that of RyR1 on SDS-PAGE (12, 13). It showed Ca$^{2+}$-dependent high affinity [$^3$H]ryanodine binding which was modulated by several ligands, indicating that it constitutes a Ca$^{2+}$ release channel (12, 13). The results shown here with the purified receptor confirmed most of these previous results. In the previous results, [$^3$H]ryanodine binding was partially inhibited by Mg$^{2+}$ (13). As shown in Fig. 7B in this report, however, it was fully inhibited by Mg$^{2+}$, and its $IC_{50}$ was similar to that for Ca$^{2+}$. There is no satisfactory explanation for this discrepancy at the present time. Furthermore, several new findings were added. RyR3 was free of FKBP12, although it retained the ability to bind FKBP12. This is also the case with RyR1. Observations with negative staining electron microscopy revealed that RyR3 forms a characteristic structure with 4-fold symmetry, which is morphologically very similar to RyR1. RyR3 had a single class of [$^3$H]ryanodine-binding sites with a stoichiometry of 1 mol of [$^3$H]ryanodine per 1 mol of a homotetramer. When incorporated into planar lipid bilayers, RyR3 displayed a cation channel current with a large conductance, which is activated by micromolar Ca$^{2+}$, caffeine, and ATP, inhibited by millimolar concentrations of Ca$^{2+}$, and Mg$^{2+}$, and ruthenium red, and modified by ryanodine. Thus, RyR3 constitutes a homotetrameric Ca$^{2+}$ release channel that shares several basic properties with RyR1.

In addition to the overall similarities as mentioned above, some functional differences between RyR1 and RyR3 were also noted. First, RyR3 displays a significantly larger monovalent cation conductance (743 ± 11 pS in 500 mM KCl) than RyR1 (630 ± 9 pS) (Fig. 8). Second, the purified RyR3 channel differs in gating kinetics from the RyR1 channel. The RyR3 channel showed longer durations of channel opening than the RyR1 channel under similar $P_o$ values as reflected in a larger fraction of the component of the slow open time constant ($t_{s0}$) (Fig. 8).

Single channel recordings of RyR3 have recently been reported with recombinant rabbit uterine RyR3 that was expressed in HEK293 cells (37). The properties of the purified RyR3 channel shown here well correspond to those of the recombinant RyR3 channel in several respects as follows: (i) a large monovalent cation conductance, (ii) modulation by ligands, (iii) gating kinetics showing longer duration of open time, (iv) a high open probability, and (v) biphasic Ca$^{2+}$ de-

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**FIG. 11. Effects of sulfhydryl-oxidizing and -reducing reagents on the purified RyRs.** A, the high $P_o$ RyR1 channel ($P_o = 0.197$) at 0.1 μM Ca$^{2+}$ (control) was markedly reduced in $P_o$ to 0.049 by 50 μM DTT (+50 μM DTT). Subsequent application of 500 μM DTT further decreased $P_o$ to 0.009 (+50 μM DTT). The $P_o$ diminished by DTT rebounded remarkably ($P_o = 0.315$) by addition of 500 μM pCMPS (+500 μM pCMPS). B, the low $P_o$ RyR1 channel ($P_o = 0.001$) at 0.1 μM Ca$^{2+}$ (control) was strongly activated by addition of 10 μM pCMPS to $P_o = 0.062$ (+10 μM pCMPS). This activation disappeared ($P_o = 0.007$) by subsequent addition of 25 μM DTT (+25 μM DTT). C, the RyR3 channel was activated at 0.3 μM Ca$^{2+}$ ($P_o = 0.729$) (control). The four rows of current traces in the middle were recorded 118 s after addition of 250 μM DTT. Current fluctuations of the channel remained unchanged for the first 2 min and then suddenly ceased. The closure of the channel with no detectable open events lasted for about 10 min (+250 μM DTT). The channel activity was overshot ($P_o = 0.904$) immediately after addition of 100 μM pCMPS (+100 μM pCMPS). D, the RyR3 channel activity ($P_o = 0.020$) at 0.15 μM Ca$^{2+}$ (control) was markedly increased by 10 μM pCMPS to $P_o = 0.650$ (+10 μM pCMPS).
The importance of the D2 region in the E-C coupling of the dihydropyridine receptor of the T-tubules (45, 47, 48) has been postulated. The interaction of domain 9 or clamp-shaped domain might interact directly with the negatively stained images of the two isoforms. Although not detected in RyR1 and RyR3 preparations (Fig. 3), FKB12 was found to bind FKB12, as is true of RyR1 (Fig. 3). Because FKB12 is widely and abundantly expressed in mammalian tissues including skeletal muscles (31), this suggests that RyR3 may bind FKB12 in situ. It should also be noted that functional properties of RyR1 and RyR3 shown here were determined with receptors lacking FKB12. It has been reported that removal of FKB12 from RyR1 makes the channel unstable, resulting in increase in the number of openings to subconductance levels (30). Although we observed channels showing subconductance states in both RyR1 and RyR3 preparations, populations of such channels were minor (see Figs. 8–11). We are currently investigating the effect of FKB12 on the RyR3 channel activity.

With the hope of correlating the functional differences of RyR1 and RyR3 to the specific structural features, we examined the negatively stained images of the two isoforms. Although we could not recognize any significant differences in their submolecular architecture, we found that a site-directed antibody against the D2 region of RyR1 which is absent in RyR3 binds to domain 9 or its vicinity (Fig. 5). Considering the three-dimensional architecture of the receptor molecule, the epitope site may correspond to the “clamp-shaped” domain according to the terminology of Orlova et al. (45), in which they found the occurrence of certain conformational change accompanied with opening and closing events of the channel. Several attempts were recently made to determine the interaction sites of the receptor molecules with some specific proteinaceous ligands, e.g. calmodulin (46) and FKB12 (24). Although such studies have successfully pin-pointed the binding sites of those proteins in the three-dimensional architecture of the receptor molecule, none of them has determined the position of those sites in the primary amino acid sequence. Our result thus provides the first case to link the three-dimensional architecture of RyR with its primary structure.

Since the distance between two adjacent voltage sensors of a tetrad is almost equal to the dimension of a “foot” structure, it has been postulated that the corners of a quatrefoil including domain 9 or clamp-shaped domain might interact directly with dihydropyridine receptor of the T-tubules (45, 47, 48). On the other hand, the crucial role of the D2 region in the E-C coupling of skeletal muscle was claimed based on the fact that the deletion of the D2 region causes the functional loss of Ca$^{2+}$ release activity triggered by the electrical stimulation while preserving caffeine-induced Ca$^{2+}$ release (49). Our assignment of the D2 region to domain 9 or its vicinity may reasonably link those two issues and could suggest the importance of the D2 region in the interaction of RyR1 with dihydropyridine receptor. Recently, two additional regions of RyR1 were also shown to interact with dihydropyridine receptor (50, 51). It is also of interest where these stretches are localized in the three-dimensional structure of the RyR molecule since these regions are relatively close to the D2 region in the primary structure of the receptor.

It has been demonstrated that a number of sulfhydryl-oxidizing reagents activate RyR channels (40, 41, 43). Sulfhydryl-reducing reagents such as glutathione and DTT have been shown to reduce the RyR channel activity (39, 42). On single channel recordings, we found that a sulfhydryl-oxidizing reagent pCMPS activated both RyR1 and RyR3 channels, whereas a sulfhydryl-reducing reagent DTT inhibited them (Fig. 11). The channel activity stimulated by the oxidizing reagent was reversed by the subsequent addition of the reducing reagent, and vice versa. Transition between the two states is reversible. We observed two populations of channel activity (high $P_o$ and low $P_o$ channel) in RyR1 preparation (Fig. 10). Multiple populations of RyR1 channel activity were also reported with SR vesicles prepared from skeletal muscles (38, 39). Because the channel activity of RyR is dependent on the redox state of the receptor molecule (Fig. 11), distinct populations of RyR1 channels may be attributed to the heterogeneity of the redox state of the receptor in the lipid bilayer, as recently suggested by Marengo et al. (39). In contrast, only a single population of current fluctuations with $P_o$ ~1 was detected in RyR3 preparation (Fig. 10). Consistently, no heterogeneity of RyR3 channels was so far reported (14, 37). Homogeneous channel activity was also shown in cardiac RyR (RyR2) (38). These results indicate that RyR3 may be much more steeply dependent on the redox state in the channel activity than RyR1, showing almost all-or-none fashion. This may be reflected in homogeneous channel activity of RyR3 in contrast to heterogeneous population of RyR1.

The results of the Ca$^{2+}$ sensitivity of RyR3 for activation and inactivation were so far controversial (13, 37, 52). We determined the Ca$^{2+}$ dependence of the purified RyR3 both in [3H]ryanodine binding and in single channel recordings, and surprisingly we found that they were entirely different. $E_{C_{50}}$ of RyR3 for Ca$^{2+}$ in [3H]ryanodine binding was about 14 mM which was about 4-fold larger than that of RyR1 (Fig. 7A), confirming the previous results with the immunoprecipitated RyR3 (13). This is consistent with the CICR activity of RyR1-knock-out (dyspedic) mice skeletal muscle which is probably attributed to RyR3 (52). In contrast, the RyR3 channel in single channel recordings was steeply activated to $P_o$ ~1 between 0.1 and 1 mM Ca$^{2+}$ in almost all-or-none fashion (Fig. 10C). A very similar relationship was obtained with the recombinant rabbit uterine RyR3 channel (37) and the RyR3 purified from bovine diaphragm (14). Although a higher salt concentration in the channel recordings (500 mM KCl) than in the ryanodine binding (170 mM NaCl) might be a possible cause, it is unlikely because similar results of the single channel experiments with the recombinant RyR3 were obtained in relatively low salt concentration (250 mM KCl) (37). These findings indicate that the difference in Ca$^{2+}$ dependence is simply attributed to the method adopted. RyR3 channel showed almost full opening ($P_o$ ~1) at 10 mM Ca$^{2+}$ in the absence of adenine nucleotide or caffeine (Fig. 10) (37). [3H]Ryanodine binding activity at the same Ca$^{2+}$ concentration, in contrast, was further stimulated by adenine nucleotides or caffeine (13). It was so low in the presence of Ca$^{2+}$ alone that a supplement of 4 mM AMP was required to obtain the precise results (Fig. 7A). Dithiothreitol (2 mM), on the other hand, was included in the medium for ryanodine binding but not for channel recordings. The low $P_o$ activity of RyR1 appears to correspond well to [3H]ryanodine binding in the presence of Ca$^{2+}$ alone. Marengo et al. (39) reported that the Ca$^{2+}$-dependent channel activity of RyR was sensitively dependent on the redox state. We observed that the channel activity of RyR3 was likely to be varied in almost all-or-none fashion by the redox state, whereas that of RyR1...
was gradually dependent. The steep increase in the channel activity of RyR3 between 0.1 and 1 μM Ca\(^{2+}\) (Fig. 10C) may indicate that some Ca\(^{2+}\)-dependent process(es) could also be involved in the abrupt transition of the channel activity in RyR3. This steep dependence may be a probable explanation for the difference in the Ca\(^{2+}\) dependence.

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