Characterization of Type 3 Ryanodine Receptor (RyR3) of Sarcoplasmic Reticulum from Rabbit Skeletal Muscles*

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Takashi Murayama and Yasuo Ogawa‡

From the Department of Pharmacology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

We investigated type 3 isoform (RyR3) of ryanodine receptor in rabbit skeletal muscles using an antibody specific for RyR3. By Western blot analysis and by immunoprecipitation, a single polypeptide for RyR3 was detected in sarcoplasmic reticulum vesicles from rabbit diaphragm but not in those from back muscle. The molecular mass was slightly smaller than that of RyR1, the major isoform in skeletal muscles. Each of RyR1 and RyR3 formed a homotetramer in rabbit diaphragm. RyR3 showed about a 7-fold lower Ca\textsuperscript{2+} sensitivity than RyR1. Interestingly, RyR3 showed a 7-fold lower Ca\textsuperscript{2+} sensitivity for caffeine, as is true of RyR1, and was stimulated further by adenine nucleotide, caffeine, or high salt concentration. Procaine and ruthenium red inhibited the binding. RyR3 was more resistant to Mg\textsuperscript{2+} inhibition than RyR1. Interestingly, RyR3 showed a single class of \([3\text{H}]\)ryanodine binding sites of high affinity \((K_D = 1.6 \text{ nm})\). From the \(B_{\text{max}}\) of the binding, the content of RyR3 was estimated to be only 0.6% of RyR1 in rabbit diaphragm. \([3\text{H}]\)Ryanodine binding to RyR3 was biphasically dependent on Ca\textsuperscript{2+}, as is true of RyR1, and was stimulated further by adenine nucleotide, caffeine, or high salt concentration. Procaine and ruthenium red inhibited the binding. RyR3 was more resistant to Mg\textsuperscript{2+} inhibition than RyR1. Interestingly, RyR3 showed a 7-fold lower Ca\textsuperscript{2+} sensitivity than RyR1. Comparison with the counter parts in bullfrog skeletal muscles indicates that the Ca\textsuperscript{2+} sensitivities of RyR3 homologs are similar to each other, whereas those of RyR1 homologs are species-specific.

Ryanodine receptor (RyR)\textsuperscript{1} is one of the Ca\textsuperscript{2+} release channels of intracellular Ca\textsuperscript{2+} stores and may play important roles not only in muscles but also in various other cells (1–5). Molecular cloning of cDNAs encoding mammalian RyRs has shown that there are three distinct isoforms of RyR (RyR1–3) encoded by different genes (6–11). Although recent studies by analysis of its mRNA showed that there were several tissue-specific alternative splicing variants of RyR3, especially between brain and peripheral tissues (11, 21). These alternatively spliced variants might generate potential heterogeneity in the function of RyR3 among tissues. mRNA for RyR3 was also found in mammalian skeletal muscles that express primarily RyR1 (14, 15, 17). Conti et al. (22) recently demonstrated minor amounts of RyR3 protein in mammalian skeletal muscles by Western blot analysis. Interestingly, the content of RyR3 varied among different muscles in rat: higher levels in diaphragm and soleus, lower levels in abdominal muscles and tibialis anterior, and no detectable amounts in the extensor digitorum longus. A particularly high content of RyR3 in the diaphragm was observed in several mammals examined (rat, mouse, rabbit, and cow). To learn whether there are functional differences in RyR3 between brain and skeletal muscles, we identified and characterized here RyR3 expressed in rabbit skeletal muscles using the anti-RyR3 antibody (20). The results of this study show that a homotetramer of RyR3 is expressed in rabbit diaphragm but is undetectable in back muscle. It may function as a CICR channel that is similar to RyR3 in rabbit brain. Further unique properties of RyR3 are also revealed.

EXPERIMENTAL PROCEDURES

Materials—The peptide corresponding to the amino acid sequence 4375–4387 of the rabbit RyR3 (RyR3-peptide) was synthesized at the Central Laboratory of Medical Sciences, Division of Biochemical Analysis, Juntendo University School of Medicine (20). \([3\text{H}]\)Ryanodine (60–90 Ci/mmol) was purchased from NEN Life Science Products. Goat anti-rabbit IgG-agarose was from Sigma. Egg lecithin (egg total phosphatide 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 or back muscle according to Murayama and Ogawa (18) in the presence of a mixture of protease inhibitors (2 \(\mu\text{g}/\text{ml} \) aprotinin, 2 \(\mu\text{g}/\text{ml} \) leupeptin, 1 \(\mu\text{g}/\text{ml} \) antipain, 2 \(\mu\text{g}/\text{ml} \) pepstatin A, and 2 \(\mu\text{g}/\text{ml} \) chymostatin). The isolated
membranes were quickly frozen in liquid N2, and stored at −80 °C until use.

**Solubilization of SR Vesicles and Sucrose Gradient Ultracentrifugation**—SR vesicles (2–4 mg/ml at the final concentration) were incubated for 15 min on ice with 2% CHAPS and 1% egg lecithin in a buffer containing 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, and a mixture of protease inhibitors. The supernatant after centrifugation at 100,000 × g for 30 min was collected and used for detection and characterization of RyR3. Sucrose gradient ultracentrifugation with 5–20% linear gradients was performed as described (18).

**SDS-Polyacrylamide Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was performed with 2–12% linear gradient gels (18, 20). The molecular mass (in kDa) of standards used here was 205 (myosin heavy chain), 116 (β-galactosidase), 97.4 (phosphorylase b), 66 (bovine serum albumin), 45 (ovalbumin), and 29 (carbonic anhydrase). Gels were stained with Coomassie Brilliant Blue. For Western blotting, the separated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes at 40 V overnight in the presence of 0.02% SDS to facilitate the transfer of high molecular weight proteins (18, 20).

**Anti-RyR3 Antibody and Western Blot Analysis**—Anti-RyR3 antibody was produced in rabbits against the synthetic peptide corresponding to the amino acid sequence 4375–4387 of rabbit RyR3, and was purified with protein-bound polyvinylidene difluoride membranes of β-RyR from bullfrog skeletal muscle as described in Murayama and Ogawa (20). The antibody reacted with RyR3 of mammalian brain and β-RyR of frog or chicken skeletal muscle, but no cross-reaction was observed with mammalian RyR1, RyR2, or α-RyR of non-mammalian vertebrate skeletal muscles (20). Western blotting was carried out colorimetrically as described previously (18) using peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody and 3,3′-diaminobenzidine as the substrate.

**Immunoprecipitation**—Immunoprecipitation of RyR3 was performed using the purified anti-RyR3 antibody and goat anti-ribonuclease A-agarose beads (20). Solubilized SR vesicles (1–2 mg of protein) were incubated overnight at 4 °C with 100 µl of anti-RyR3 antibody and 30 µl of anti-ribonuclease A-agarose beads. For detection of the polypeptide band for RyR3, the beads were washed five times with a buffer containing 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween 20, 0.1% CHAPS, and 2 mM dithiothreitol and were resuspended in 40 µl of 2× Laemml sample buffer (23) containing 0.1 mM dithiothreitol. Aliquots of 15–20 µl were subjected to SDS-polyacrylamide gel electrophoresis.

[3H]Ryanodine Binding Assay—Assays were carried out as described previously (20). SR vesicles (0.2 mg) were incubated with [3H]ryanodine (2–21 nM) for 4 h at 25 °C in 0.5 ml of a binding buffer containing 0.3 or 1 mM NaCl, 10 mM MOPS/NaOH, pH 6.8, 2 mM dithiothreitol, 1% CHAPS, 0.5% egg lecithin, the mixture of protease inhibitors, and a specified concentration of free Ca2+ buffered with 10 mM EGTA. Then RyR3 was immunoprecipitated with 100 µl of anti-RyR3 and 30 µl of anti-ribonuclease A-agarose beads. The radioactivity of the beads after five washings with a buffer (1 mM NaCl, 10 mM MOPS/NaOH, pH 6.8, 1% CHAPS, 0.5% egg lecithin, 2 mM dithiothreitol, and 0.1 mM CaCl2) was determined by scintillation counting as the activity for RyR3. The nonspecific radioactivity was determined in the absence of anti-RyR3 in each experiment. The value was similar to that determined with the addition of 30 µM RyR3-peptide (see Fig. 5). The nonspecific activity was decreased as the salt concentration of the medium was increased and approached a value similar to the result on the addition of excess unlabeled ryanodine (10–50 µM) at 0.3 M NaCl. These findings indicate that it may be caused probably by direct binding of [3H]ryanodine and RyR1 to the agarose beads (see Fig. 2) rather than weak binding to anti-RyR3. For compensating for the nonspecific radioactivity thus determined, [3H]ryanodine binding specific to RyR3 can be obtained. In this study, the [3H]ryanodine binding to RyR3 could not be determined accurately in an isotonic medium containing 0.17 M NaCl because of a high nonspecific radioactivity. Instead, assays were carried out in a medium containing 0.5 M NaCl where the properties are assumed to be more physiological than those in 1 M NaCl (see Figs. 8 and 9, Tables I and II). The binding for RyR1 was measured by filtering an aliquot of the remaining supernatants after immunoprecipitation through polyethyleneimine-treated Whatman GF/B glass filters (18). Free Ca2+ was calculated using values of 8.79 × 10−6 M−1 and 1.92 × 105 M−1 as the apparent binding constants for Ca2+ of EGTA (24) and of AMP-PCP (25), respectively.

**RESULTS**

**Identification of RyR3 in Rabbit Skeletal Muscles**—To identify RyR3 in skeletal muscles, we prepared terminal cisternae-rich fractions of SR vesicles from rabbit skeletal muscles where RyR3 is reportedly to be localized (22). Because the content of RyR3 varied among different muscles in rat (22), we used diaphragm (which was reported to express the highest content of RyR3 among skeletal muscles examined) and back muscle (material commonly used for SR vesicles) as materials. Fig. 1A shows a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel of SR vesicles from diaphragm and back muscle. The two specimens showed very similar patterns of protein composition. Mammalian skeletal muscles express primarily RyR1, in contrast to skeletal muscles of non-mammalian vertebrates which have nearly equal amounts of two RyR isoforms (α- and β-RyR). Consistently, single bands for RyR1 of nearly equal density were clearly detected at the low mobility range of the gel (arrowhead) in both SR preparations, and the band for RyR3 could not be identified in diaphragm or back muscle SR on the Coomassie-stained gel.

When the transferred membrane was probed with anti-RyR3, the antibody raised against a synthetic peptide (RyR3-peptide) corresponding to 4375–4387 of rabbit RyR3 which reacts highly specifically with mammalian RyR3 among the three isofoms (20), a single band for RyR3 was faintly but significantly reacted just below the RyR1 band in diaphragm SR, but not in back muscle SR (Fig. 1B). Thus, a minute amount of RyR3 in addition to dominant RyR1 was expressed in rabbit diaphragm, whereas no RyR3 was detected in back muscle SR.

The expression of RyR3 was also examined by immunoprecipitation using antibody-conjugated agarse beads (see “Experimental Procedures”). Fig. 2 shows Coomassie Brilliant Blue-stained gels of the proteins immunoprecipitated with the anti-RyR3 from CHAPS/egg lecithin-solubilized SR. In diaphragm SR, a single high molecular weight band (arrowhead) was observed, which specifically disappeared by the addition of 30 µM RyR3-peptide during immunoprecipitation (panel A). This band was reacted with anti-RyR3 on Western blot analysis (data not shown). These results indicate that RyR3 is definitely expressed in rabbit diaphragm. A band seen above RyR3 band was a minute contamination of RyR1 because of its positive activity for RyR3; see “Experimental Procedures”). Fig. 2 shows Coomassie Brilliant Blue-stained gels of the proteins immunoprecipitated with the anti-RyR3 from CHAPS/egg lecithin-solubilized SR. In diaphragm SR, a single high molecular weight band (arrowhead) was observed, which specifically disappeared by the addition of 30 µM RyR3-peptide during immunoprecipitation (panel A). This band was reacted with anti-RyR3 on Western blot analysis (data not shown). These results indicate that RyR3 is definitely expressed in rabbit diaphragm. A band seen above RyR3 band was a minute contamination of RyR1 because of its positive
reaction with anti-RyR1 antibody (data not shown). Because the band did not disappear by the RyR3-peptide, the precipitation of RyR1 may be the result of nonspecific binding to the agarose beads rather than weak binding to anti-RyR3. No specific bands, in contrast, were immunoprecipitated from back muscle SR (panel B), suggesting that there are no detectable amounts of RyR3 in back muscle, the same conclusion as shown in Fig. 1B. The varied contents of RyR3 in contrast to similar amounts of RyR1 between diaphragm and back muscle corresponded well to the results with rat skeletal muscles (22). The following experiments to characterize RyR3 in skeletal muscles were therefore carried out with diaphragm muscle SR (arrowhead).

Subunit Molecular Mass and Tetramer Formation of RyR3—

The molecular mass of rabbit RyR3 protein is estimated to be 552 kDa from its predicted amino acid sequence (10), which is slightly smaller than that of rabbit RyR1 (565 kDa) (6, 7). As shown in Fig. 3, the mobility of immunoprecipitated RyR3 was slightly but significantly larger than that of RyR1 on the Coomassie Brilliant Blue-stained SDS-polyacrylamide gel. The mobility of RyR3 was significantly larger than that of rabbit RyR1 (565 kDa) (6, 7). As shown in Fig. 4, the mobility of immunoprecipitated RyR3 was slightly but significantly larger than that of RyR1 on the Coomassie Brilliant Blue-stained SDS-polyacrylamide gel. The mobilities of RyR1 and RyR3 were similar to those of bullfrog α- and β-RyR, the homologs of RyR1 and RyR3, respectively, in non-mammalian vertebrates (18, 19).

Formation of tetramer is one of the typical characteristics of RyR3. As described above, rabbit diaphragm expresses considerable amounts of RyR1 and minor levels of RyR3 (see Fig. 1). It is therefore important to determine whether RyR3 forms a homotetramer of its subunit or heterotetramer with RyR1. The tetramer formation of RyR is detected easily by the sedimentation pattern of sucrose density gradients: a tetrameric RyR is detected easily by the sedimentation pattern of sucrose density gradients. The mobility of RyR3 was significantly larger than that of rabbit RyR1 (565 kDa) (6, 7). As shown in Fig. 4B, the bands for RyR3 were detected in the immunoprecipitated products with anti-RyR3 from 4 ml of the fraction (panel B) was resolved on a 2–12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The mobility of RyR3 was significantly larger than that of RyR1. Note that the mobilities of rabbit RyR1 and RyR3 correspond to bullfrog α- and β-RyR, the homologs of RyR1 and RyR3, respectively.

Gradient. This pattern corresponds well with our previous results with α- and β-RyR of bullfrog skeletal muscle (18) and RyR2 and RyR3 of rabbit brain microsomes (20) under identical conditions, as is true of RyR of rabbit skeletal muscle SR (26, 27). The sedimentation pattern of RyR3 through the sucrose gradient was similarly examined on the Coomassie Brilliant Blue-stained gel after immunoprecipitation of a large volume of the gradient fractions (4 ml of each fraction) with anti-RyR3. As shown in Fig. 4B, the bands for RyR3 were detected in the immunoprecipitated products on each lane of the gel. If RyR3 forms a heterotetramer with RyR1, the two should be coprecipitated, resulting in the detection of dual bands on the Coomassie Brilliant Blue-stained gel because of their different mobilities (see Fig. 3). The absence of an RyR1 band clearly excludes the possibility of heterotetramer formation of RyR3 with RyR1. These results indicate that each of RyR1 and RyR3 coexpressed in rabbit diaphragm exclusively forms a homotetramer. Heterotetramer formation of RyR3 with RyR1 and suggests that each of RyR1 and RyR3 forms a homotetramer.
formation of RyR3 is also observed in rabbit brain, which expresses a high content of RyR2 (20).

Estimation of the Amounts of RyR3 in Rabbit Diaphragm by [3H]Ryanodine Binding—The functional properties of RyR3 in rabbit skeletal muscles were determined through the [3H]ryanodine binding. Solubilized SR was incubated with [3H]ryanodine to achieve ryanodine binding, and then RyR3 was specifically immunoprecipitated with anti-RyR3 as described in Murayama and Ogawa (20). Fig. 5 shows the radioactivity immunoprecipitated with anti-RyR3 from SR of rabbit diaphragm or back muscle. In diaphragm SR, significant radioactivity was precipitated with the antibody. Thirty μM RyR3-peptide, which completely prevented RyR3 from being immunoprecipitated (see Fig. 2), reduced the radioactivity to the background level. A similar reduction in the radioactivity was also observed in determination without anti-RyR3 (data not shown). Therefore, the radioactivity is caused by RyR3 itself, but not by minute amounts of contaminating RyR1. The addition of excess concentrations (10–50 μM) of unlabeled ryanodine caused almost total loss of the radioactivity (data not shown). These results suggest that RyR3 in diaphragm has specific [3H]ryanodine binding activity. In back muscle SR, in contrast, no significantly detectable radioactivity was observed, consistent with the absence of the immunoprecipitable RyR3 (see Fig. 2).

As shown in Fig. 6, the specifically immunoprecipitated ryanodine binding activity in 0.2 mg of the solubilized diaphragm SR increased with added amounts of anti-RyR3 and saturated around 0.044 pmol/mg of protein with 100 μl of antibody or more. The activity was no longer immunoprecipitated from the supernatant after immunoprecipitation with 100 μl of anti-RyR3 (data not shown). Therefore, the radioactivity is caused by RyR3 itself, but not by minute amounts of contaminating RyR1. The addition of excess concentrations (10–50 μl) of unlabeled ryanodine caused almost total loss of the radioactivity (data not shown). These results suggest that RyR3 in diaphragm has specific [3H]ryanodine binding activity. In back muscle SR, in contrast, no significantly detectable radioactivity was observed, consistent with the absence of the immunoprecipitable RyR3 (see Fig. 2).

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Fig. 7 shows dose-dependent [3H]ryanodine binding to RyR1 and RyR3 of rabbit diaphragm SR under optimum conditions. The amounts of the binding to RyR1 (upper panel) and RyR3 (lower panel) increased with the increase in [3H]ryanodine concentration and approached their asymptotic values. The Scatchard plots (inset) gave a straight line for both RyR1 and RyR3 within the range of 2–21 nM [3H]ryanodine, indicating a...
single class of binding sites. RyR1 showed a dissociation constant (K_D) for ryanodine of 2.3 mM and B_max of 11.4 pmol/mg protein. The K_D and B_max of RyR3 were calculated to be 1.6 mM and 0.065 pmol/mg protein, respectively. The K_D values correspond to those with RyR from rabbit skeletal muscle (26, 28) and with the purified α- and β-RyR from bullfrog skeletal muscle under similar conditions (18). The similarity in K_D for the high affinity ryanodine binding sites between RyR3 and RyR1 suggests that they are generally similar to each other and allows us to assume that the stoichiometry of the site for RyR3 may be identical to that for RyR1 (1 mol of ryanodine/tetramer) (26). This assumption was also verified with α- and β-RyRs from bullfrog skeletal muscle SR (18). Thus, the B_max value would directly express the content in the SR vesicles. From the ratio of B_max for RyR3 to that for RyR1, the amount of RyR3 was estimated to be only 0.6% of that of RyR1 in rabbit diaphragm SR.

Effect of CICR Modulators on [3H]Ryanodine Binding to RyR3—Table I summarizes the effect of well known CICR activators on [3H]ryanodine binding to RyR3. The experiments were carried out in a medium containing 0.3 mM NaCl, albeit a higher salt concentration than physiological medium, because a higher nonspecific radioactivity prevents sensitive detection of the specific binding in an NaCl concentration of less than 0.3 mM. In the absence of Ca^{2+} (10 mM EGTA), no significant ryanodine binding was detected. The Ca^{2+}-activated binding of 0.3 fmol/mg of protein was observed at 10 mM Ca^{2+}. Addition of 1 mM AMP-PCP, a nonhydrolyzable ATP analog, further increased the binding to 18.1 fmol/mg of protein. Caffeine (10 mM), a Ca^{2+} sensitizer of CICR, also enhanced the binding by about 3.7-fold. The binding was greatly enhanced (5.7-fold) by increasing the NaCl concentration from 0.3 to 1 mM. These properties are consistent with those of mammalian RyR1 and non-mammalian vertebrate α- and β-RyRs (3).

We examined in detail the effect of Ca^{2+} on the [3H]ryanodine binding to RyR3. In a medium of a low salt concentration containing 0.3 mM NaCl, the effect of Ca^{2+} was biphasic: it increased the binding in a dose-dependent manner at lower than 0.1 mM Ca^{2+}, whereas it inhibited the binding above 0.1 mM (open circles in Fig. 8A). The Ca^{2+} concentrations that would give half the maximal binding for activation (EC_{50}) and for inactivation (IC_{50}) were 7.9 μM and 4.9 μM, respectively. The increase in NaCl concentration from 0.3 mM to 1 mM enhanced the binding at every Ca^{2+} concentration (closed circles in Fig. 8A) as described above (see Table I). Under this condition, the effect of Ca^{2+} appeared to be monophasic: only slight inactivation was observed up to 10 mM Ca^{2+}. Furthermore, the Ca^{2+} sensitivity for activation was enhanced. The binding was already at the maximum at 10 mM Ca^{2+}, which corresponds to the EC_{50} value in 0.3 mM NaCl medium, and the EC_{50} in 1 mM NaCl medium was calculated to be 2.6 μM. These characteristic modifications by high concentrations of salt were also observed with

| Ligands added | [3H]Ryanodine bound (fmol/mg protein) | Stimulation |
|---------------|--------------------------------------|------------|
| None (10 mM EGTA) | 0.3 ± 0.2 | 0.6 |
| +10 μM Ca^{2+} | 6.3 ± 0.7 | 2 |
| +10 μM Ca^{2+} + 1 mM AMP-PCP | 18.1 ± 2.1 | 3.0 |
| +10 μM Ca^{2+} + 10 mM caffeine | 22.4 ± 1.6 | 3.7 |
| +10 μM Ca^{2+}, 1 mM NaCl | 34.1 ± 1.4 | 5.7 |

Table I: Effect of CICR activators on [3H]ryanodine binding to RyR3

0.2 mg of rabbit diaphragm SR was incubated with 8.5 nM [3H]ryanodine in the medium containing 0.3 mM NaCl, 10 mM MOPS/NaOH, pH 6.8, 1% CHAPS, 0.5% egg lecithin, 2 mM diithiothreitol for 4 h at 25°C. Immunoprecipitation of RyR3 was carried out as described under “Experimental Procedures.” The data were the mean ± S.E. of four determinations.
compared with that of RyR1 (Fig. 9). Mg\(^{2+}\) dose-dependently inhibited the \(^{[3]}\)H\textit{ryanodine} binding to RyR3 to about 75% of the control up to 5 mM. However, no more significant reduction in the binding was observed even in the presence of 10 or 20 mM Mg\(^{2+}\). In contrast, the \(^{[3]}\)H\textit{ryanodine} binding to RyR3 was remarkably decreased with Mg\(^{2+}\) concentration: the IC\(_{50}\) of Mg\(^{2+}\) was around 2 mM. These results indicate that RyR3 is more resistant to inhibition by Mg\(^{2+}\) than RyR1.

**DISCUSSION**

In this study we identified homotetrameric RyR3 expressed in rabbit diaphragm. RyR3 demonstrated the \(^{[3]}\)H\textit{ryanodine} binding that was activated by micromolar to submillimolar Ca\(^{2+}\) and inactivated by millimolar or more Ca\(^{2+}\). It is stimulated further by adenine nucleotides and caffeine and inhibited by ruthenium red and procaine. These results indicate that RyR3 may function as a CICR channel in rabbit diaphragm.

\(^{[3]}\)H\textit{ryanodine} binding of RyR3 was determined by immunoprecipitation of the RyR3 protein that had been incubated with \(^{[3]}\)H\textit{ryanodine}. In this case, the RyR3 showing \(^{[3]}\)H\textit{ryanodine} binding was the antibody-bound protein. It was reported that single-channel properties of RyR were modified by some anti-RyR antibodies (31, 32). Therefore, one might argue that “antibody-bound RyR3” is different in its properties from the “free RyR antibodies” (31, 32). Thus, one might argue that “an antibody-bound RyR3 showing \(^{[3]}\)H\textit{ryanodine} binding that was activated by micromolar to submillimolar Ca\(^{2+}\)” may not play a critical role in mammalian skeletal muscles on the basis of the observation of normal excitation-contraction coupling in muscles from the RyR3-null mice.

**TABLE II**

| Ligands added | \(^{[3]}\)H\textit{ryanodine} bound | Inhibition |
|---------------|----------------------------------|------------|
| None (0.1 mM Ca\(^{2+}\)) | 17.1 ± 0.4 | 100 |
| +1 \(\mu\)M ruthenium red | 4.5 ± 0.1 | 24 |
| +10 mM procaine | 3.2 ± 0.3 | 18 |
| +10 mM Mg\(^{2+}\) | 11.0 ± 0.6 | 65 |

Assays were carried out as in Table I. The data were the mean ± S.E. of four determinations. The values for 100% denote 3.22 and 0.021 pmol/mg of protein for RyR1 and RyR3, respectively.

The content of RyR3 in rabbit diaphragm is estimated to be only about 0.6% of RyR1 in rabbit diaphragm, which is reported to be the highest level of RyR3 among skeletal muscles examined (22). It is unlikely that RyR3 has extraordinary larger unit conductance or higher open probability than RyR1 because frog or chicken \(\beta\)-RyR showed single-channel kinetics similar to \(\alpha\)-RyR and mammalian RyRs (18, 35). Therefore, the contribution of RyR3 to total Ca\(^{2+}\) release channel activity in mammalian skeletal muscles cannot be much greater in magnitude than that expected from its relative content. The insignificant contribution of RyR3 to Ca\(^{2+}\) signaling in the skeletal muscles may thus be explained by its minute amount.

In this study we identified homotetrameric RyR3 expressed in rabbit diaphragm. RyR3 demonstrated the \(^{[3]}\)H\textit{ryanodine} binding that was activated by micromolar to submillimolar Ca\(^{2+}\) and inactivated by millimolar or more Ca\(^{2+}\). It is stimulated further by adenine nucleotides and caffeine and inhibited by ruthenium red and procaine. These results indicate that RyR3 may function as a CICR channel in rabbit diaphragm.

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Analysis of its mRNA revealed that there could be several tissue-specific alternative splicing variants of RyR3 especially in brain and peripheral tissues (11, 21). These alternatively spliced variants might generate potential heterogeneity in the function of RyR3 among tissues. We have recently identified and characterized RyR3 expressed in rabbit brain (20). Brain and diaphragm RyR3 proteins demonstrated similarity in several points (mobility on SDS-polyacrylamide gel, homotetramer formation, biphasically Ca\(^{2+}\)-activated \(^{[3]}\)H\textit{ryanodine} binding, and caffeine sensitivity), and no significant differences were found between them. Thus, the properties of RyR3 are similar between brain and skeletal muscle in rabbit. This is consistent with the results by Marzilli et al. (11) that the spliced variants of RyR3 might not be distinct between brain and diaphragm in mink.

In mammalian skeletal muscles, RyR1 plays an important role in excitation-contraction coupling because the mice lacking RyR1 (dyspedic mice) failed normal excitation-contraction coupling (33). In addition to RyR1, we demonstrated here that RyR3 is expressed in rabbit diaphragm as a functional CICR channel. Recently, Takeshima et al. (34) concluded that RyR3 may not play a critical role in mammalian skeletal muscles on the basis of the observation of normal excitation-contraction coupling in muscles from the RyR3-null mice. We estimated the amount of RyR3 to be only about 0.6% of RyR1 in rabbit diaphragm, which is reported to be the highest level of RyR3 among skeletal muscles examined (22). It is unlikely that RyR3 has extraordinary larger unit conductance or higher open probability than RyR1 because frog or chicken \(\beta\)-RyR showed single-channel kinetics similar to \(\alpha\)-RyR and mammalian RyRs (18, 35). Therefore, the contribution of RyR3 to total Ca\(^{2+}\) release channel activity in mammalian skeletal muscles cannot be much greater in magnitude than that expected from its relative content. The insignificant contribution of RyR3 to Ca\(^{2+}\) signaling in the skeletal muscles may thus be explained by its minute amount.

The content of RyR3 in rabbit diaphragm is estimated to be only about 0.6% of RyR1. Our previous results showed that the amount of RyR3 in rabbit brain was 1.6–2% of total RyRs (20). This might lead to a misunderstanding that RyR3 is less expressed in diaphragm than in brain of rabbit. From the amounts of specific \(^{[3]}\)H\textit{ryanodine} binding sites, the total RyR in rabbit brain microsomes (primarily RyR2) is estimated to be about 3% of RyR1 in rabbit diaphragm SR. Therefore, the content of RyR3 in the brain would be at most 0.06% of RyR1 in diaphragm, which is only one-tenth of that in diaphragm. This corresponds well with the clear detection of RyR3 on Western blot analysis in rabbit diaphragm SR (Fig. 1), whereas no bands were observed in rabbit brain microsomes (20).

The results of this study demonstrate that each of RyR1 and RyR3 coexisting in rabbit diaphragm forms a homotetramer, excluding the possibility of heterotetramer formation. Rabbit diaphragm consists of several different types of muscle cells as is true of the diaphragm of other animals (36–38). If RyR1 and RyR3 were expressed separately in different types of cells, homotetramer formation of RyR1 and RyR3 should be simply the result of the lack of opportunity for heterotetramer formation. Preliminary results by immunohistochemistry with anti-RyR3 show that RyR3 is detected almost homogeneously in rat diaphragm.\(^2\) Consistently, homogeneous distribution of mRNA for RyR3 in rat diaphragm was also reported (22). These findings indicate that RyR1 and RyR3 are coexpressed in the same cell, and thereby they exclusively form a homotetramer even in the presence of different subunits. Homotetramer formation of RyR3 is also observed in rabbit brain, which expresses all three isoforms (20). Although homotetramer formation of RyR1 was believed in mammalian skeletal muscles where only the isoform was formerly acknowledged to occur, potential coexpression of RyR3 and RyR1 requires more strict evidence to prove it. In cardiac muscles where RyR2 is the primary RyR isoform,

\(^2\)T. Hijikata, personal communication.
The weak inhibition by Mg$^{2+}$ was also observed with RyR3 in rabbit brain (data not shown). This was in marked contrast to β-RyR of bullfrog which was as sensitive to the inhibitory effect of Mg$^{2+}$ as α-RyR (29). Interestingly, the amount of $[^{3}H]$ryanodine binding to RyR3 decreased with Mg$^{2+}$ concentration up to 5 mM and reached the maximum attenuation of about 25% around 5 mM Mg$^{2+}$ (Fig. 9). High concentrations of Ca$^{2+}$, on the other hand, inhibited RyR1 and RyR3 in their similar Ca$^{2+}$ dependences to very low activities (Fig. 8). It is assumed that Ca$^{2+}$ and Mg$^{2+}$ bind to the same inactivating sites of low affinity with similar affinities to inhibit the Ca$^{2+}$ release channel (30, 44). The results shown in Figs. 8 and 9, however, indicate that the cation binding sites for inactivation may be different between Ca$^{2+}$ and Mg$^{2+}$ in mammalian RyR3 and that Mg$^{2+}$ inhibits only partially, whereas Ca$^{2+}$ does so nearly completely. Another possible explanation is that the diaphragm RyR3 might be composed of two or more heterogeneous populations in its properties: one sensitive to Mg$^{2+}$ as is true of RyR1 and the other insensitive to Mg$^{2+}$. Further characterization should provide some insights into the properties of RyR3.

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