The Calcium Release Channel of Sarcoplasmic Reticulum Is Modulated by FK-506-binding Protein

DISSOCIATION AND RECONSTITUTION OF FKBP-12 TO THE CALCIUM RELEASE CHANNEL OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM

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The ryanodine receptor/calcium release channel (CRC) of rabbit skeletal muscle terminal cisternae (TC) of sarcoplasmic reticulum (SR) has been found to be tightly associated with FK-506 binding protein (FKBP-12), the cytosolic receptor (immunophilin) for the immunosuppressant drug FK-506 (Jayaraman, T., Brillantes, A. M., Timmerman, A. P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and Marks, A. (1992) J. Biol. Chem. 267, 9474-9477). In this study, a procedure is described to dissociate FKBP from TC and reconstitute human recombinant FKBP-12 back to the ryanodine receptor so that the role of the immunophilin on CRC activity can be assessed. Titration of TC vesicles with FK-506 dissociates FKBP from the ryanodine receptor. Sedimentation of FK-506-treated vesicles effectively separates the TC from the soluble FKBP-FK506 complex which remains in the supernatant. The FKBP-deficient TC vesicles have altered functional characteristics: 1) the ATP-stimulated calcium uptake rate of TC vesicles is reduced 2-fold; and 2) the threshold concentration of caffeine required to induce calcium release from TC vesicles is decreased. These changes appear to reflect modification of the calcium release channel since: 1) severalfold higher concentrations of FK-506 do not alter the calcium uptake rate of either longitudinal tubules of SR, or TC vesicles in the presence of ruthenium red; 2) human recombinant FKBP reassociates with FKBP-deficient TC but not with control TC or longitudinal tubules of SR; and 3) the reduced Ca\(^{2+}\) uptake rate in FKBP-deficient TC is restored to control values in the FKBP-reconstituted TC. These studies demonstrate that FKBP-12 modulates the CRC of rabbit skeletal muscle sarcoplasmic reticulum.

Skeletal muscle contraction is triggered by calcium release

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¶ The abbreviations used are: SR, sarcoplasmic reticulum; BSA, bovine serum albumin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; CRC, calcium release channel; CsA, cyclosporin A; DTT, dithiothreitol; FKBP-12 (or FKBP), FK-506 binding protein; IHM, imidazole homogenization medium; LT, longitudinal tubules; RR, ruthenium red; TBST, Tris-buffered saline-Tween; 7C, terminal cisternae; PAGE, polyacrylamide gel electrophoresis.

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muscle ryanodine receptor has been found to be tightly associated with the calcium release channel of rabbit skeletal muscle sarcoplasmic reticulum provides a system to study the role of this immunophilin in skeletal muscle excitation-contraction coupling. In this study, we describe a procedure to dissociate FKBP-12 from TC and then rebind recombinant FKBP-12 to FKBP-deficient TC. As shown in this report, this procedure is useful to assess the role of FKBP-12 on ryanodine receptor function.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods**

**Materials**—[3H]Ryanodine was purchased from New England Nuclear. Human recombinant FKBP-12, [3H]FK-816 (a dihydropyridine derivative of FK-506), FK-506, rapamycin, and CsA were provided from Merck Research Laboratories (Rahway, NJ). Sephadex LH-20 and Sepharose 6B were obtained from Pharmacia LKB Biotechnologies Inc. (Piscataway, NJ). Goat anti-rabbit alkaline phosphatase conjugate and NBT/BCIP color development reagents for Western analysis were purchased from Promega Corporation (Madison, WI). Immunoblot-P membrane was purchased from Millipore (Bedford, MA). CHAPS, antipyrilazo III, caffeine, and ruthenium red were obtained from Sigma. Sodium dodecyl sulfate, acrylamide, methylene bis-acrylamide, and SDS-PAGE molecular weight standards were obtained from Bio-Rad.

**General Methods**—The protein concentration of SR and human recombinant FKBP-12 preparations was estimated by the folin reaction using bovine serum albumin as protein standard. SDS-PAGE was performed with a mini-slab gel apparatus (Hoeffer Scientific) using the buffer system described by Laemmli.

**Isolation of Longitudinal and Terminal Cisternae Vesicles from Rabbit Skeletal Muscle**

Membrane fractions referable to the terminal cisternae and longitudinal tubules of sarcoplasmic reticulum were isolated from New Zealand White rabbit skeletal muscle as described previously.

**Drug Binding Assays**

**[3H]Ryanodine Binding**—The concentration of the high affinity ryanodine-binding site in SR membranes was measured by Scatchard analysis of ryanodine-binding isotherms from 2 to 60 nM [3H]ryanodine. Ryanodine binding was measured essentially as described previously, briefly, SR vesicles (25 μg of TC or 100 μg of LT protein) were incubated for 1 h at room temperature in 0.125 ml of buffer containing 10 μM K-Hepes, pH 7.4, 100 mM NaCl, 5 μM CaCl2, and 2-60 nM [3H]Ryanodine (8500 counts/min/subunit). Nonspecific binding was estimated in the presence of 8 μM cold ryanodine. Free ligand was separated from bound by sedimenting the TC vesicles in a Beckman TJ-6 tabletop centrifuge collecting the eluate (1.7 ml) in a 16 by 100-mm test tube. For comparison of [3H]ryanodine binding by the spin column method versus control (TL 100.1 centrifugation method), TC vesicles (41 pg/mL) were incubated for 1 h at room temperature as described above in the presence of 60 nM [3H]ryanodine. Specific activity (counts/min/pmol) free from bound ligand, samples (20 μg of TC protein) were either directly centrifuged in the TL-100.1 rotor or solubilized in 50 μl of FK-816 binding buffer containing 1 μM NaCl and 5 mg/mL BSA and then loaded onto the Sepharose 6B/LH-20 spin column. For comparison of [3H]FK-816 binding by the spin column method versus control (2 ml LH-20 column method), TC vesicles (1.25 μg of protein) were incubated with 10 nM [3H]FK-816 in 0.125 ml of binding buffer for 30 min at 37 °C. After incubation, samples (0.5 μg of protein) were applied to either a 2-ml LH-20 column or a 3-ml Sepharose 6B/LH-20 spin column.

**Purification of Skeletal Muscle Ryanodine Receptor**

The ryanodine receptor was purified from solubilized TC vesicles utilizing a modification of the sucrose gradient method described by Lai et al. (6). Briefly, TC vesicles were solubilized as described by Inui et al. (5) at 3 mg of protein/ml in 20 mM Tris-Cl, pH 7.2, 1 mM NaCl, 2 mM DTT, 2 μg/mL leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 4% CHAPS, and 2% soy bean lipid. The solubilized vesicles were incubated at room temperature for 1 h in the presence or absence of 10 μM FK-506. Solubilized vesicles (5 ml) were loaded on top of a step sucrose gradient containing 8 ml each 10, 15, 17.5, and 20% sucrose in solubilization buffer (lipid was omitted from the 15, 17.5, and 20% sucrose steps) and centrifuged in a Beckman Ti-50 rotor at 45,000 revolutions/min. The tubes were fractionated into 12 ml of elution buffer allowing the columns to be reused several times.

**Dissoociation of FKBP from TC Vesicles**

TC vesicles (2 mg protein/ml) were preincubated in imidazole homogenization medium (IHM) (5 mM imidazole-Cl, pH 7.4, and 0.3 M sucrose) containing 0.5 μM FK-506 at room temperature for 1 h. Samples of 50 μl were centrifuged at 95,000 revolutions/min in a Beckman TL-100.1 rotor for 10 min at 2 °C to yield a pellet and supernatant fraction. The supernatant contains the soluble FKBP-12 protein. The supernatant was further purified by refractionation (Centriprep 30) to about 0.2 μg/mL as determined by two-dimensional densitometry of the high molecular weight band (ryanodine receptor protein) versus bovine serum albumin.
at a constant power of 2 watts/gel. One gel was visualized by silver staining while the other was transferred to an Immobilon-P membrane (Millipore, Bedford MA) for 30 min at 150 mA constant current in transfer buffer composed of 200 mM glycine, 25 mM Tris-Cl, pH 8.5, and 0.01% SDS. The blotted membrane was blocked in TBST solution (0.05% Tween 20 in PBS, pH 8.0, 0.05% NaCl) for 30 min containing 5% (w/v) dry milk protein for 1 h then probed for 90 min with anti-FKBP antiserum (19) at a dilution of 1:2000 in blocking buffer. The membrane was washed repetitively with TBST then probed for 1 h with secondary antibody (goat anti-rabbit IgG) conjugated to alkaline phosphatase at a 1:5000 dilution in blocking buffer. The membrane was again washed with TBST and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates.

**Calcium Loading and Release Assays**

TC vesicles (2 mg/ml) were preincubated at room temperature for 1 h in IIM buffer containing 0–10 μM FK-506. The vesicles were then stored on ice until the calcium loading and release assays were performed. The vesicles were often stored on ice for up to 7 h followed by quick freezing in liquid nitrogen and overnight storage at −80 °C. The loading rates of control vesicles was not compromised by such storage. The loading rates of FK-506-treated TC vesicles, however, was occasionally further reduced after prolonged storage. Calcium loading and caffeine-induced release assays in the presence of 100 mM phosphate were monitored spectrophotometrically with the calcium indicator antipyrylazo III essentially as described previously (4, 27). Briefly, TC vesicles (25 μg of protein) were added to 1 ml (final volume) of loading medium (100 mM KPO4, pH 7.0, 5 mM antipyrilazo III, 4 mM MgCl2, and 1 mM ATP), then aliquots of CaCl2 (7.5 or 15 mM) were repetitively spiked into the cuvette, and the calcium uptake rate was monitored by dual wavelength spectrophotometry (816 at 37 °C for 30 min as described under “Experimental Procedures.”) Scatchard analysis of [3H]FK-816 binding to TC vesicles, B, Scatchard analysis of [3H]FK-816 binding to recombinant FKBP-12. The data are representative of five different TC preparations. The average Kd (± standard deviation) obtained from five preparations was 6.8 ± 1.4 nM with an average Bmax of 124.5 ± 25.0 pmol/mg protein. The average correlation coefficient (r ± S.D.) for the five preparations was -0.966 ± 0.024.

**RESULTS**

**Characteristics of [3H]FK-816 Binding in Terminal Cisternae Preparations—Binding of [3H]FK-816, a dihydropriopyl derivative of FK-506, to TC was measured in the presence of 0.5% CHAPS. The binding isotherm follows a simple hyperbolic response. That is, Scatchard analysis of the binding data (Fig. 1A) gives a straight line, which is consistent with a single class of [3H]FK-816-binding sites. The binding parameters, obtained with five different TC preparations, gives a dissociation constant (Kd) of 6.8 ± 1.4 nM and Bmax of 124.5 ± 25.0 pmol/mg TC. The binding parameters obtained from three different preparations of recombinant FKBP-12 (Fig. 1B) gave a Kd of 5.9 ± 1.7 nM with a Bmax of 106 ± 31 nmol/mg protein.3

3The Bmax value that we report for three different recombinant FKBP-12 (106 ± 31 nmol/mg) preparations is based upon the Lowry et al. (21) protein assay as described under “Experimental Procedures.” Siekierka et al. (27) reported a Bmax for FK-506 binding to FKBP-12 isolated from Jurkat cells of 92.5 nmol/mg utilizing the Bradford dye binding assay. As first reported by Siekierka et al. (27), we also find that the Bradford dye binding method overestimates the protein concentration by a factor of 2.5–3-fold in comparison to...
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The difference in total binding at 10 nM [3H]FK-816 versus that in the presence of 1 μM FK-506 defines specific binding in this assay. The data represent the average ± S.D. of five TC preparations.

Competition binding studies (Fig. 2) indicate that [3H]FK-816 binding (at 10 nM) to TC preparations is competed for with 1 μM of FK-506 or rapamycin, whereas 1 μM of CsA does not compete for [3H]FK-816 binding. In comparison to previous reports by Siekierka et al. (26), the affinity of FKBP-12 appeared to be lower in our binding assay due to the presence of 0.5% CHAPS. However, when measured under similar conditions in the presence of 0.5% CHAPS, the dissociation constant and specificity of [3H]FK-816 binding to solubilized rabbit skeletal muscle TC preparations is similar to that of human recombinant FKBP-12 (26, 28).

Stoichiometry of FKBP/Ryanodine Receptor—The ryanodine receptor contains a single high affinity ryanodine binding site/homotetramer (5, 24); therefore, the ratio of binding (Bmax) for [3H]FK-816 to high affinity [3H]ryanodine in TC provides an estimate of the molar ratio of FKBP/calcium release channel. Table I shows that the Bmax for FK-816 binding to solubilized TC vesicles (124.5 pmol/mg) is roughly four times greater than the high affinity ryanodine-binding site density (28 pmol/mg). Thus, TC vesicles have approximately 4 mol of FKBP/homotetramer, i.e. one FKBP for each protomer of the calcium release channel. Binding of [3H]FK-816 to preparations of longitudinal tubules is a small fraction of that found in TC vesicles (Table I). The small amount of ryanodine binding detected in the longitudinal tubules preparations reflects contamination by TC vesicles (about 10%) which accounts for the [3H]FK-816 binding in this fraction.

Dissociation of FKBP from Terminal Cisternae—The kinetics of [3H]FK-816 binding to TC was found to be slower than [3H]FK-816 binding to human recombinant FKBP-12. Saturation binding data, the coefficient of variation (S.D./mean) of 18-20% for [3H]FK-816 binding to human recombinant FKBP-12 (26, 28).

Dissociation of FKBP from Terminal Cisternae—The ratio of FKBP/calcium release channel (CRC) in five different TC and two different LT preparations was evaluated from the ratio of [3H]FK-816 and high affinity [3H]ryanodine binding-site densities (Bmax) obtained by Scatchard analysis of [3H]FK-816 binding isotherms from 1 to 30 nM (23, 24) (see Fig. 1A) and [3H]ryanodine binding isotherms from 2 to 60 nM [3H]ryanodine as described under "Experiment Procedures." Scatchard analysis of the ryanodine binding data gave only a straight line (r = -0.976 ± 0.016) compatible with a single high affinity ryanodine-binding site as described previously (24). The Kd for the high affinity ryanodine-binding site in the TC vesicles used in this study was 8.5 ± 1.9 nM (n = 5). The difference in total binding at 10 nM [3H]FK-816 binding in this fraction binding data are expressed as the mean ± S.D. for five preparations. The LT fraction binding data are expressed as the mean ± the range for two preparations. With regard to the TC-binding data, the coefficient of variation (S.D./mean) of 18-20% for FK-816 binding was complete in less than 20 min at room temperature, while [3H]FK-816 binding to TC required over 1 h to reach saturation (data not shown). [3H]FK-816 binding to TC reached saturation at 37 °C in less than 30 min. For this reason, FK-816 binding assays were performed at 37 °C for 30 min.

The difference in the binding kinetics, albeit yielding similar Kd values for FK-816 binding to TC preparations versus recombinant FKBP, suggested that [3H]FK-816 binding in TC preparations is influenced by the association of FKBP with the ryanodine receptor. A binding assay utilizing a spin column composed of Sepharose 6B and Sephadex LH-20 (upper layer) was devised to determine whether [3H]FK-816 bound to free FKBP-12 or to the FKBP-ryanodine receptor complex (see "Experimental Procedures"). In this assay using TC, the percentage of specific [3H]FK-816 binding recovered in the void volume of a Sephrose 6B spin column was compared with the amount of specific [3H]ryanodine binding recovered on identical columns. If the FK-816-binding site was associated with the ryanodine receptor, similar percentages of ryanodine and FK-816 binding should be recovered in the void volume of the Sephrose 6B. The results of this experiment are summarized in Fig. 3. Note, [3H]FK-816 binding was measured at 10 nM FK-816 which approximates the Kd for [3H]FK-816 binding, whereas [3H]ryanodine binding was measured at saturating levels of ryanodine (60 nM). Control values for [3H]FK-816 binding and [3H]ryanodine binding were 53 and 28 pmol/mg protein, respectively. Only 23% of control [3H]FK-816 binding (12.4 pmol/mg protein) was recovered in the void volume of the Sephrose 6B column versus 82% of control ryanodine binding (23.2 pmol/mg protein). These results indicated the FK-856 binding is complicated by dissociation of FKBP from the ryanodine receptor and that the FKBP-FK-506 complex (M, of 12,000) remains in the included volume of the Sephrose 6B rather than excluded together with the larger ryanodine receptor (mass of 2.3 million) in the void volume of the column.

Dissociation of FKBP from TC vesicles by FK-506 was confirmed by Western blot analysis using antisera raised against the NH2-terminal sequence of FKBP-12 (19). The

Table I

| Molar ratio (FKBP/CRC) | pmol/mg |
|-----------------------|---------|
| Control                | 28.1 ± 5.0 | 4.4 ± 0.26 (n = 5) |
| FK-506                | 2.8 ± 1.4 | 4.5 ± 0.5 (n = 2) |
| CsA                   | 12.6 ± 1.6 |                     |
| Rapamycin             | 12.45 ± 2.1 |                     |

**FIG. 2. Competition of [3H]FK-816 binding by FK-506, CsA, and rapamycin.** Competition of [3H]FK-816 binding (10 nM [3H]FK-816 to TC vesicles by 1 μM FK-506, CsA, and rapamycin. The different in total binding at 10 nM [3H]FK-816 versus that in the presence of 1 μM FK-506 defines specific binding in this assay. The data represent the average ± S.D. of five TC preparations.

**FIG. 3.** Dissociation of FKBP from TC vesicles by FK-506. The ratio of FKBP/calcium release channel (CRC) in five different TC and two different LT preparations was evaluated from the ratio of [3H]FK-816 and high affinity [3H]ryanodine binding-site densities (Bmax) obtained by Scatchard analysis of [3H]FK-816 binding isotherms from 1 to 30 nM (23, 24) (see Fig. 1A) and [3H]ryanodine binding isotherms from 2 to 60 nM [3H]ryanodine as described under "Experiment Procedures." Scatchard analysis of the ryanodine binding data gave only a straight line (r = -0.976 ± 0.016) compatible with a single high affinity ryanodine-binding site as described previously (24). The Kd for the high affinity ryanodine-binding site in the TC vesicles used in this study was 8.5 ± 1.9 nM (n = 5). The difference in total binding at 10 nM [3H]FK-816 binding in this fraction binding data are expressed as the mean ± S.D. for five preparations. The LT fraction binding data are expressed as the mean ± the range for two preparations. With regard to the TC-binding data, the coefficient of variation (S.D./mean) of 18-20% for FK-816 binding was complete in less than 20 min at room temperature, while [3H]FK-816 binding to TC required over 1 h to reach saturation (data not shown). [3H]FK-816 binding to TC reached saturation at 37 °C in less than 30 min. For this reason, FK-816 binding assays were performed at 37 °C for 30 min.

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The percentage of specific [3H]FK-816 was compared with specific binding obtained by control assays (standard binding methods) as described under "Experimental Procedures." Only 23% of control FK-816 binding is recovered from the Sepharose 6B spin column versus 82% of control ryanodine binding. The data represent the average ± S.D. for three different TC preparations.

Western blot analysis shown in Fig. 4B clearly indicates that all of the FKBP in untreated samples sediments with TC vesicles (Fig. 4B, sample 1 pellet (P)). Treatment with FK-506 (0.12-5.0 μM) results in a dose-dependent shift of about 80-90% of the FKBP into the supernatant fraction (Fig. 4B, samples 2-5). The EC50 for dissociation of FKBP from TC vesicles is in the concentration range between 0.12 and 0.5 μM FK-506.

The Ryanodine Receptor Isolated from FK-506-treated Terminal Cisternae Lacks FKBP—Ryanodine receptor preparations were purified from solubilized TC vesicles by sucrose gradient centrifugation after incubation of the solubilized vesicles (in the presence or absence of 10 μM FK-506). The ryanodine receptor prepared from control TC contains the 14 kDa band visualized by silver staining (lane 2 of Fig. 5A) which reacts positively to FKBP-12 antiserum (lane 2 of Fig. 5B). This band is greatly reduced or nearly absent from ryanodine receptor preparations isolated from solubilized TC vesicles which were preincubated for 1 h with 10 μM FK-506 (lane 3 of Fig. 5, A and B).

The Calcium Loading Rate of TC Is Reduced by FK-506 Treatment—To evaluate the effect of FKBP-12 on the function of the calcium release channel, we compared the ATP-stimulated calcium loading rate of control TC and TC treated with FK-506 (0-10 μM). The basis of this assay is that the calcium loading rate of TC vesicles is reduced by calcium leak from the vesicles via the calcium release channel (4). Therefore, drugs which close the calcium release channel (such as ruthenium red) enhance the calcium loading rate of TC vesicles. Ruthenium red has no effect on the loading rate of longitudinal tubules, which are devoid of ryanodine receptor. In contrast, drugs which activate the calcium release channel (such as ryanodine) reduce the calcium uptake rate of TC vesicles (4).

The calcium loading rate of TC vesicles is reduced after pretreatment with FK-506 in a concentration-dependent manner to about 35% of the control rate (Fig. 6). The IC50 for FK-506 on the loading rate is approximately 0.2 μM. Therefore, the concentration of FK-506 required to alter TC function (Fig. 6) is in the same range as that required to dissociate FKBP-12 from the calcium release channel of TC vesicles (Fig. 4B). The loading rate of longitudinal tubules (which are devoid of ryanodine receptor) and TC vesicles in the presence of ruthenium red (which closes the calcium release channel) is not affected by 10 μM FK-506 (Fig. 6). Dissociation of FKBP from the ryanodine receptor increases the Ca2+ flux out of the TC likely due to enhanced activation of the calcium release channel as evidenced by the reduced TC calcium loading rate. We also find that rapamycin (10 μM) reduces the calcium loading rate of TC vesicles and dissociates FKBP from TC vesicles; however, CsA (10 μM) does not alter either the loading rate or association of FKBP with TC vesicles.

Treatment of TC with FK-506 Reduces the Threshold for Caffeine-induced Calcium Release—Pretreatment of TC with

FIG. 3. Separation of [3H]FK-816 from [3H]ryanodine binding in solubilized TC on a Sepharose 6B spin column. The percentage of specific [3H]FK-816 versus specific [3H]ryanodine binding to TC recovered in the void volume of a Sepharose 6B spin column was compared with specific binding obtained by control assays (standard binding methods) as described under "Experimental Procedures." The data represent the average ± S.D. for three different TC preparations.
FK-506 (0.5 μM) reduces the threshold concentration of caffeine required to induce calcium release from TC vesicles (Fig. 7). Control (untreated) vesicles (Fig. 7, A-C) more rapidly take up four to five aliquots of CaCl₂ (7.5 or 15 nmol each) until they accumulate nearly 2 μmol of calcium/mg protein. The threshold of caffeine required to induce calcium release from untreated vesicles is 2.5 mM (Fig. 7C). This is to be contrasted with TC vesicles pretreated with 0.5 μM FK-506 (Fig. 7, D and E). As expected from the results above (Fig. 6), the calcium loading rate is distinctly lower (e.g. compare A and D) in treated vesicles. Additionally, the threshold of caffeine required for release is reduced to 1 mM caffeine (Fig. 7D). These results further indicate that dissociation of FKBP-

12 from the ryanodine receptor modulates the calcium release channel of rabbit skeletal muscle SR.

Rebinding of FKBP to FKBP-deficient TC Vesicles and Reconstitution of Function—TC vesicles were preincubated for 90 min at room temperature in the presence or absence of FK-506. Duplicate pairs of samples (0.5 ml each) were washed by centrifugation through identical sucrose gradients in which the 15% step of one gradient was supplemented with recombinant FKBP-12 (see “Experimental Procedures”). The vesicles were isolated from the gradient and analyzed for FKBP by Western blot analysis using antiserum against FKBP-12 (19). The results are summarized in Fig. 8A. Control TC vesicles (without FK-506 treatment) isolated from both control and FK-506-supplemented gradients contain similar amounts of FKBP-12 (Fig. 8A, lanes 1 and 2) while FK-506-treated vesicles have a decreased content of FKBP (Fig. 8A, lane 3). Centrifugation of the FKBP-deficient vesicles through a sucrose gradient supplemented with recombinant FKBP in the 15% layer restores the level of FKBP-12 in the deficient vesicles to control levels (Fig. 8A, lane 4). To evaluate the nonspecific interaction of FKBP-12 with the membrane, longitudinal tubule vesicles treated with FK-506 were centrifuged through similar gradients. Lanes 5 and 6 of Fig. 8A shows that, unlike FKBP-deficient TC vesicles, FKBP does not bind to FK-506-treated longitudinal tubules of SR. These studies show that FKBP binds only to FKBP-deficient TC as compared with control TC or longitudinal tubules of SR.

Rebinding of FKBP-12 Restores TC Calcium Loading Rate—TC vesicles stripped of FKBP have only about half of the Ca²⁺ loading rate of controls (Fig. 8B; compare sample 3 versus sample 1). This is reflected in the 4.5-fold enhancement of the calcium loading rate with ruthenium red, as compared with the 2.4-fold ruthenium red enhancement in control TC (sample 1). The rebinding of FKBP to stripped vesicles (sample 4) restores Ca²⁺ loading to the control rate, and likewise the ruthenium red enhancement in the reconstituted vesicles is essentially the same as control TC. It should be emphasized that the calcium uptake rate in the presence of ruthenium red

Fig. 5. Purified ryanodine receptor prepared from FK-506-treated TC is devoid of FKBP-12. The ryanodine receptor was purified by sucrose gradient centrifugation of solubilized TC vesicles on a vertical rotor following preincubation with or without (control) FK-506 as described under “Experimental Procedures.” Equivalent amounts of ryanodine receptor were separated by SDS-PAGE (15% gel) and stained with silver (A) or blotted and probed with FKBP-12 antiserum (B). Lanes 1-4 refer to: 1, recombinant FKBP-12; 2, control ryanodine receptor (1 μg); 3, ryanodine receptor (1 μg) isolated from FK-506-treated TC; 4, control ryanodine receptor (1 μg) co-loaded with recombinant FKBP-12, i.e. lanes 1 and 2 loaded on the same well. Size markers are indicated on the left, and the arrow on the right indicates the position of FKBP-12.

Fig. 6. FK-506 reduces ATP-stimulated calcium loading rate of terminal cisternae. TC vesicles (2 mg/ml) were incubated with 0–10.0 μM FK-506 for 1 h at room temperature. Active calcium loading rates of TC (25 μg/ml) were monitored following the addition of 10 nmol of CaCl₂ (10 μM) in the presence of 1 mM ATP and 4 mM MgCl₂ as described under “Experimental Procedures.” Values are expressed as the percentage of control (without addition of FK-506) loading rates and represent the average and standard deviation from three to four preparations. TC vesicles (○) had a control loading rate of 0.67 ± 0.22 μmol/min-mg (n = 4) versus 1.45 ± 0.39 (n = 4) in the presence of 3.5 μM ruthenium red (○) to give an enhancement in the loading rate by RR of 2.2. Longitudinal tubules (●) had a loading rate of 2.9 ± 0.4 μmol/min-mg (n = 3).

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is similar in control, stripped, and reconstituted vesicles. Therefore, the increased ruthenium red enhancement observed in FKBP-deficient TC reflects the lower calcium loading rate resulting from the dissociation of FKBP-12 (sample 3) from the ryanodine receptor as previously described in Fig. 6. Rebinding of recombinant FKBP decreases the Ca\(^{2+}\) leak to control values (Fig. 8B, sample 4). These data provide strong evidence that FKBP-12 modulates the gating properties of the calcium release channel of skeletal muscle sarcoplasmic reticulum.

**DISCUSSION**

We have previously reported that FKBP-12 is tightly associated with the ryanodine receptor/calcium release channel of rabbit skeletal muscle terminal cisternae (19). In this study, a procedure has been developed to dissociate FKBP-12 from the ryanodine receptor of TC and rebind FKBP to such FKBP-deficient TC. This dissociation and reconstitution procedure permits the evaluation of the effect of FKBP on the function of the ryanodine receptor. Most importantly, this study indicates that FKBP-12 specifically modulates the activity of the calcium release channel.

A key breakthrough in this study was the development of a gentle procedure to dissociate FKBP-12 from the ryanodine...
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receptor with FK-506. The clue came from studies which indicated slower binding of FK506 to TC as compared with recombinant FKBP although the binding affinities were similar (Fig. 1). An assay, using gel exclusion, designed to test FK-816 binding to the ryanodine receptor indicated that the smaller FKBP-FK506 complex is dissociated from the calcium release channel. The EC50 for FK-506 to dissociate FKBP from the ryanodine receptor was found to be in the range of 0.12 to 0.5 μM (Fig. 4B). Since the concentration of FKBP in a 2 mg/ml suspension of TC is about 0.25 μM, these results show that titration with just a small excess of FK-506 equivalents leads to dissociation of FKBP from the ryanodine receptor.

We find that FKBP specifically modulates the activity of the calcium release channel. TC vesicles treated with FK-506 have a reduced calcium uptake rate due to enhanced leak of Ca2+ from the CRC (Fig. 8A). The calcium loading rate of FKBP-deficient TC is restored by rebinding recombinant FKBP. The reassociation is specific to FKBP-deficient TC since FKBP does not bind to control TC or FK-506-treated longitudinal tubules. Our studies provide evidence that FKBP modulates the ryanodine receptor/calcium release channel of skeletal muscle SR. In developing the dissociation and rebinding of FKBP methodology, the Ca2+ loading which is explained by the increased tendency of the calcium release channel to open. Thus, FKBP appears to stabilize the closed conformation of the skeletal muscle ryanodine receptor and may thereby be important in modulating the gating kinetics of the calcium release channel during excitation-contraction coupling in skeletal muscle.

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