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AFFINITY PURIFICATION OF THE RYANODINE RECEPTOR/CALCIUM RELEASE CHANNEL FROM FAST TWITCH SKELETAL MUSCLE BASED ON ITS TIGHT ASSOCIATION WITH FKBP12

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The ryanodine receptor (RyR)/calcium release channel isolated from skeletal muscle terminal cisternae (TC) of sarcoplasmic reticulum (SR) is tightly associated with FK506 binding protein of 12.0 kDa (FKBP12) (Jayaraman et al., (1992) J.Biol.Chem. **267**, 9474-9477). In this study, we describe a new method of affinity chromatography for purifying the RyR from skeletal muscle SR based on: 1) its tight association with FKBP12; and 2) the finding that bound FKBP on the RyR can be exchanged with soluble FKBP12 (Timerman et al., (1995) J.Biol.Chem. **270**, 2451-2459). Soluble glutathione S- transferase/FKBP12 (GST/FKBP12) fusion protein was first exchanged with bound FKBP12 on the RyR of TC. The TC were then solubilized with CHAPS and the complex of RyR•GST/FKBP12 was specifically adsorbed by glutathione Sepharose 4B and then eluted with glutathione. The RyR, purified by this method, has similar characteristics by SDS-PAGE, radioligand binding and immuno-reactivity as the RyR purified by multiple sequential column chromatography.

Skeletal muscle contraction is triggered by a rise in intracellular Ca^{2+} mobilized via the calcium release channel/ryanodine receptor of the sarcoplasmic reticulum (SR) (1). The ryanodine receptor (RyR) has been purified from skeletal muscle SR and identified as the calcium release channel (CRC) involved in skeletal muscle excitation-contraction coupling (2-5). The RyR has been identified morphologically as the foot structure of the triad junction, involved in the association of the transverse tubule with the terminal cisternae (TC) of SR (3,6,7). FKBP12, a cytosolic receptor

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Abbreviations Used: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CRC, calcium release channel; DTT, dithiothreitol; FKBP12, a 12 kDa FK506 binding protein; GST/FKBP12, a fusion protein of glutathione S-transferase and FKBP12; IHM, imidazole homogenization medium; PBS, phosphate buffered saline; PMSF, phenylmethanesulfonyl fluoride; RyR, ryanodine receptor; SBL soybean lecithin; SR, sarcoplasmic reticulum; TC, terminal cisternae of sarcoplasmic reticulum.

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for the immunosuppressive drug FK506, was found to be tightly associated with the RyR/CRC isolated from skeletal muscle TC of SR (8) with a stoichiometry of four FKBP12 per RyR (9). That is, the structural formula of the ryanodine receptor is (RyR protomer)$_4$(FKBP12)$_4$. FKBP12 is not only tightly associated with RyR/CRC from skeletal muscle SR, but also modulates its Ca^{2+} release function (9-11). Recently, we found that soluble FKBP can exchange with bound FKBP12 on the RyR of isolated skeletal muscle TC of SR (12). In the present study, we describe a new method for purifying the RyR/CRC from skeletal muscle SR using glutathione Sepharose affinity chromatography for the fusion protein following exchange of the fusion protein with FKBP12 bound to the TC of SR. A preliminary communication has appeared (13).

MATERIALS AND METHODS

Materials and General Methods [^{3}H]Ryanodine, Western Blot chemiluminescence reagents and horseradish peroxidase labelled second antibody were purchased from New England Nuclear (DuPont). Human FKBP12 and GST/FKBP12 expression constructs, [^{35}S]labeled FKBP12, [^{3}H]FK816 (L-682, 816) and FK590 (L-683, 590, FK520) (closely related structural analogues of FK506), were provided by Merck Research Laboratories (Rahway, NJ). Sephadex LH-20, Glutathione Sepharose 4B was obtained from Pharmacia LKB Biotechnologies Inc. (Piscataway, NJ). Immobilon-P membranes were purchased from Millipore (Bedford, MA). Sodium dodecyl sulfate, polyacrylamide, methylene bis-acrylamide and SDS-PAGE molecular weight standards were obtained from Bio-Rad. CHAPS, SBL and other reagents were obtained from Sigma. The protein concentration of SR membrane fractions and GST/FKBP12 preparations was estimated by the Lowry procedure using bovine serum albumin as a standard (14). The protein concentration of purified RyR preparations was determined by scanning densitometry of Coomassie Blue stained SDS-PAGE gels with an automated gel analysis and image processing system (Technology Resources Inc., Nashville, TN), with bovine serum albumin as standard (9). SDS-PAGE was performed with a mini-slab gel apparatus (Hoeffer Scientific) using the buffer system described by Laemmli (15). Western blot analysis was detected by chemiluminescence and was carried out as described by the manufacturer (Dupont).

Isolation of Junctional Terminal Cisternae of SR from Skeletal Muscle and Purification of Ryanodine Receptor by Sequential Column Chromatography Membrane fraction referable to junctional terminal cisternae of SR were prepared from rabbit fast twitch skeletal muscle as described previously (3,16). The terminal cisternae were solubilized using CHAPS and SBL and the purification of RyR was performed by sequential column chromatography on heparin-agarose and hydroxylapatite in the presence of CHAPS as described previously (3).

Expression and Purification of FKBP12 and GST/FKBP12 Fusion Protein FKBP12 was expressed in E. coli and purified by HPLC using a TSK G3000SW column as described previously (17). The expression and purification of GST/FKBP12 fusion protein are briefly described as follows: the JM 101/pGST/FKBP12 were grown in 1 liter of LB medium containing ampicillin (final concentration: 50 μg/ml) at 37^{0}C. At an OD_{600} of 0.6-0.8, the expression of the fusion protein was induced by incubation with 1 mM IPTG for 6 hours. The cells were then harvested, resuspended in 20 ml of PBS (pH 7.4), and lysed using a sonicator (50% pulse, 30 sec intervals for 3 min, Branson Sonic Power Co.) on ice bath. The nucleic acids in the crude lysate were precipitated by protamine sulfate (final concentration: 0.04%) followed by centrifugation (15,000 x g, 30 min). The GST/FKBP12 fusion protein was purified from the supernatant by affinity chromatography. Briefly, the supernatant was incubated with glutathione Sepharose 4B resin (5 ml of bed volume) for 2 hrs

at 4°C with gentle agitation. The resin was recovered by centrifugation at 500 x g for 5 min in this and subsequent steps. The resin was washed three times with 10 bed volumes of PBS, and then the bound fusion protein was eluted from the matrix by incubation for 10 min at room temperature with 1 bed volume of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione). The eluates containing the fusion protein (monitored by SDS-PAGE assay) were collected and frozen in liquid nitrogen and stored at -80°C.

[^{3}H]Ryanodine Binding and [^{3}H]FK816 Binding Assay [^{3}H]Ryanodine binding to TC vesicles (500 μg/ml) or to purified ryanodine receptor (10-20 μg/ml) was measured essentially as described previously (3,18). [^{3}H]FK816 binding to FKBP12 (0.2 μg/ml) and GST/FKBP12 (0.6 μg/ml) were measured in the presence of detergent (CHAPS) using a hydrophobic resin (Sephadex LH-20) to separate free from bound ligand essentially as described previously (9).

Prelabelling of Terminal Cisternae with [^{35}S]FKBP12 and Exchange of GST/FKBP12 with Prelabelled TC Prelabelling and exchange experiments were carried out as described previously (12). Prelabeled [^{35}S]FKBP12 TC vesicles were incubated with varying concentrations of GST/FKBP12 or FKBP12 at 37°C for 30 min, or by incubating 10 μM of GST/FKBP12 or FKBP12 at 37°C at varying incubation times.

Affinity Purification Procedure of the Ryanodine Receptor TC membrane vesicles isolated from rabbit skeletal muscle were resuspended at 4 mg protein/ml in IHM buffer (0.3 M sucrose. 5 mM imidazole, pH 7.4) and incubated with GST/FKBP12 (10 μM) at 37°C for 40 min. The mixture was centrifuged at 50,000 rpm for 10 min (Beckman TL 100.2 rotor) to separate the free GST/FKBP12 in the supernatant from the TC membrane in the pellet. The TC were resuspended with solubilization buffer containing 20 mM Tris-HCl, pH 7.4, 1 M NaCl, 0.2 mM PMSF, 2 mM DTT, 2 μg/ml leupeptin. The solubilization of the membrane was initiated by adding the CHAPS-SBL mixture (3) to make final concentrations of 2% and 1% respectively, in which the concentration of TC protein was also 4 mg/ml. The sample was incubated at 4°C for 10 min and centrifuged at 50,000 rpm for 15 min in a Beckman TL 100.2 rotor. The supernatant was diluted 5-fold with PBS to reduce the concentration of salt (0.3 M) and CHAPS/SBL (0.4%/0.2%) and incubated with Glutathione Sepharose 4B for 2 hrs in the cold room with gentle agitation. The suspension was centrifuged at 500 x g for 5 min. The Glutathione Sepharose 4B pellet was washed three times with 10 bed volumes of washing buffer containing 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 0.5 M KCl, 0.5% CHAPS, 2 μg/ml leupeptin, 2 mM DTT. Then the RyR•GST/FKBP12 complex bound to the matrix was eluted with one bed volume of elution buffer (50 mM Tris-HCl, pH 8.0, 0.5 M KCl, 0.5% CHAPS, 2 μg/ml leupeptin, 2 mM DTT, 10 mM glutathione) after 10 min incubation and recovered by centrifugation (500 x g, 5 min). The elution was repeated four times. The fractions containing RyR (usually 3-4 fractions) were monitored on 7.5% SDS-PAGE gel by Coomassie Blue staining and combined. The samples were concentrated using a Centricon-30, and then frozen in liquid nitrogen and stored at -70°C. The purification of the RyR from TC by this method takes about 5 hours.

All of the results presented in the figures and table are typical results from at least two experiments.

RESULTS AND DISCUSSION

The affinity purification of RyR involves three steps: 1) exchange of bound FKBP12 of TC of SR with GST/FKBP12; 2) solubilizing TC with CHAPS; and 3) affinity purification using glutathione Sepharose 4B.

Characterization of GST/FKBP12 Fusion Protein Scatchard analysis of [^{3}H]FK816 binding to GST/FKBP12 in CHAPS (9) showed a similar dissociation constant (K_d) as FKBP12 (Figure 1). Their K_ds are essentially the same (about 30 nM). The maximal binding (Bmax) of

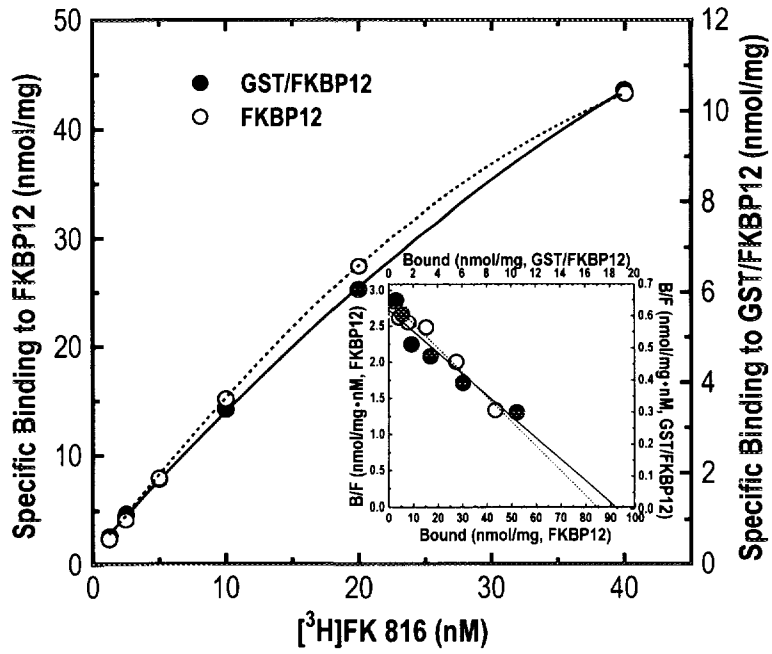

Figure 1. **[^{3}H]FK816 binding to purified GST/FKBP12.** GST/FKBP12 (•) (0.6 μg/ml) or FKBP12 (○) (0.2 μg/ml) was incubated in 40 μl at 37°C for 30 min with varying concentrations (1.25-40 nM) of [^{3}H]FK816 in binding buffer (20 mM $NaPO_4$, pH 7.2, 0.5% CHAPS, 2 mM DTT, 0.02% NaN_3 and 1.25 mg/ml BSA). [^{3}H]FK816 binding to FKBP12 and GST/FKBP12 was measured using a hydrophobic resin (Sephadex LH-20) column to separate free from bound ligand essentially as described previously (9). The K_ds for FKBP12 (29 nM) and GST/FKBP12 (30 nM) are essentially the same. The Bmax for FKBP12 (84 nmol/mg) is approximately four-fold greater than for GST/FKBP12 (19 nmol/mg),reflecting its smaller mass.

GST/FKBP12 (19 nmol/mg) is about one-fourth of that of FKBP12 (84 nmol/mg) reflecting their different molecular weights. The results show that the GST/FKBP12 fusion protein has similar binding characteristics as FKBP12.

Exchange of Soluble GST/FKBP12 with Bound FKBP12 of Terminal Cisternae of Skeletal Muscle SR The exchange experiments were first performed by incubating GST/FKBP12 with [^{35}S]FKBP12 prelabeled skeletal muscle TC vesicles. The results revealed that the fusion protein exchanges with bound FKBP12 of TC with similar dependence on concentration (Figure 2A) and time (Figure 2B), as compared with FKBP12. The exchange experiment showed that 10 μM GST/FKBP12 can effectively exchange about 80% of the bound FKBP12 on TC of SR after 30 min incubation at 37°C (Figure 2).

Purification of the Ryanodine Receptor•GST/FKBP12 Complex by Affinity Chromatography The purification of the RyR containing bound GST/FKBP12 from skeletal muscle SR involves solubilization of the TC with CHAPS•SBL mixture and affinity chromatography

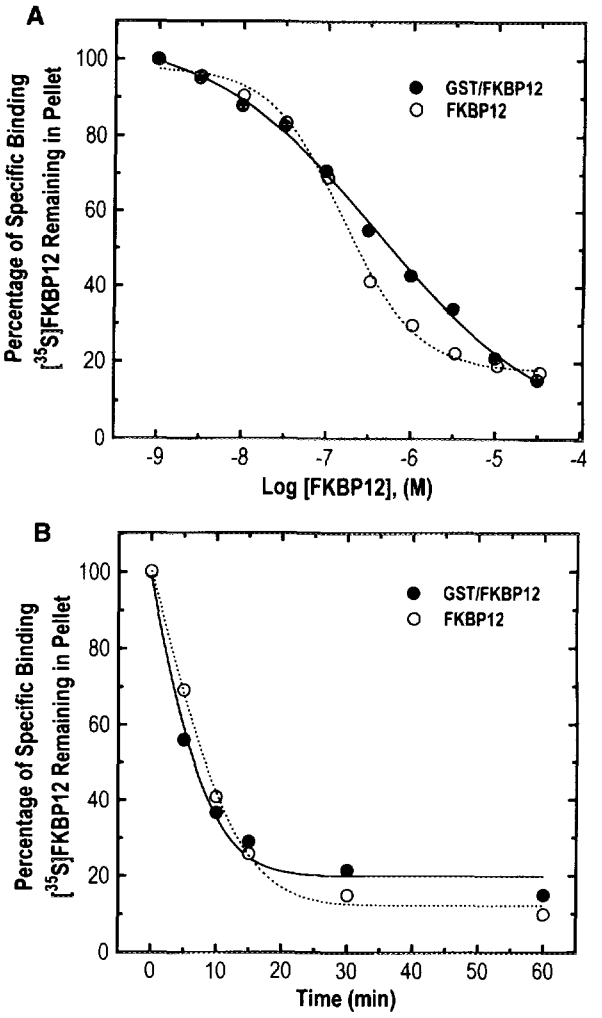

Figure 2. Concentration and Time Dependence for Exchange of GST/FKBP12 with [^{35}S]FKBP12 Prelabeled Terminal Cisternae (TC) from Skeletal Muscle Sarcoplasmic Reticulum. The exchange experiments were performed by incubating varying concentrations of GST/FKBP12 (•) and FKBP12 (○) with [^{35}S]FKBP12 prelabeled TC vesicles (2 mg/ml) at 37°C for 30 min (**Fig 2A**) or by incubating 10 μM of GST/FKBP12 (•) and FKBP12 (○) with [^{35}S]FKBP12 prelabeled TC vesicles (2 mg/ml) at 37°C for varying times (**Fig 2B**).

on glutathione Sepharose 4B. The purification is summarized in Table 1 and Figure 3. After the exchange of GST/FKBP12 with bound FKBP12 on the RyR, the TC (Figure 3A, lane 1) was solubilized with CHAPS•SBL. More than 80% of ryanodine binding activity of the membrane was solubilized under these conditions (Table 1; Figure 3A, lane 2). The CHAPS extract was diluted five fold with PBS to reduce the CHAPS and salt concentrations to 4 mg/ml and 0.3 M respectively. The sample was applied in batch to glutathione Sepharose 4B resin, the resin washed and then eluted

Table 1. **Purification of Ryanodine Receptor from Skeletal Muscle**

Fraction	Protein (mg)	[^{3}H]Ryanodine Binding* Specific activity (pmol/mg)	[^{3}H]Ryanodine Binding* Total activity (pmol)	Yield (%)	Purification (fold)
1. TC membrane	8.0[+]	24.8	198	100	1
2. Solubilized TC			168	84.8	
3. Glutathione Sepharose 4B affinity chromatography	0.19[o]	391	74	37.5	15.8

The purification procedure is described under "Materials and Methods". The values are from a typical experiment in which TC (4 mg/ml) were exchanged with GST/FKBP12 in 2.0 ml. For this amount of TC, one ml bed volume of glutathione Sepharose 4B was used.
*Ryanodine binding assay was measured with 64 nM of [^{3}H]ryanodine as described previously (18).
[+]Protein was determined according to the Lowry procedure (14).
[o]Protein was determined by densitometric scanning of Coomassie blue stained gels (9).

with glutathione. Nearly 40% of the initial binding activity in the TC is recovered as the purified receptor (Table 1; Figure 3A, lane 4).

Characterization of the Affinity Purified Ryanodine Receptor from Skeletal Muscle

The purified ryanodine receptor revealed a major band at Mr of 360,000 and a minor band

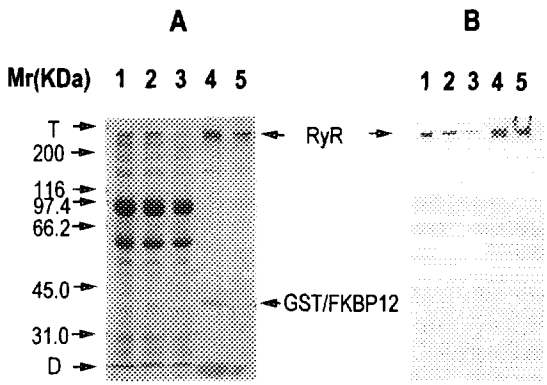

Figure 3. Affinity Purification of the ryanodine receptor from junctional terminal cisternae in skeletal muscle using GST/FKBP12. The purification procedure is described in "Materials and Methods". Electrophoresis was carried out on 7.5% SDS-PAGE gels and the RyR was detected by staining with Coomassie Blue (**Fig 3A**) or by Western Blot analysis using antibody specific for the ryanodine receptor (**Fig 3B**). Lane 1, junctional terminal cisternae vesicles (12 μg); lane 2, soluble TC membrane (12 μg) after exchange with GST/FKBP12 and solubilized with CHAPS; lane 3, the supernatant after adsorption of the RyR-GST/FKBP12 complex by Glutathione Sepharose 4B; lane 4, the RyR (1 μg) purified by affinity chromatography and concentrated by Centricon-30; lane 5, the RyR (1 μg) purified by sequential sucrose gradient centrifugation and heparin-agarose affinity chromatography. For Western Blot, the amount of sample loading was half of that for Coomassie blue staining. Positions of the molecular weight standard (BioRad) are indicated to the left and the "T" and "D" are refer to the top of the resolving gel and the bromophenol blue dye front, respectively.

(proteolytic breakdown product) at Mr of 330,000 on SDS-PAGE (Figure 3A, lane 4), and confirmed by Western Blot analysis (Figure 3B). Scatchard analysis of the high affinity [^{3}H]ryanodine binding gave a K_d of 5.4 nM with a Bmax of 406 pmol per mg of receptor protein (Figure 4). These results indicate that the ryanodine receptor purified by GST/FKBP12 has similar characteristics by SDS-PAGE, immuno-reactivity and radioligand binding as the RyR purified by heparin-agarose and additional sequential column chromatography (3).

The RyR from skeletal muscle was first purified from terminal cisternae of SR by sequential column chromatography using heparin-agarose, hydroxyapatite and gel permeation chromatography (3). The cardiac ryanodine receptor was isolated soon thereafter (19). A number of procedures have since been developed to purify the ryanodine receptor including sucrose gradient centrifugation to separate the 30S RyR (4), using an antibody affinity matrix (20), and spermine agarose chromatography (21). The affinity chromatography after exchange with GST/FKBP12 method described here is suitable to purify RyR from small amounts of TC (4-8mg). It yields a high quality receptor in purity and morphology (unpublished studies together with Dr. Terry Wagenknecht, not

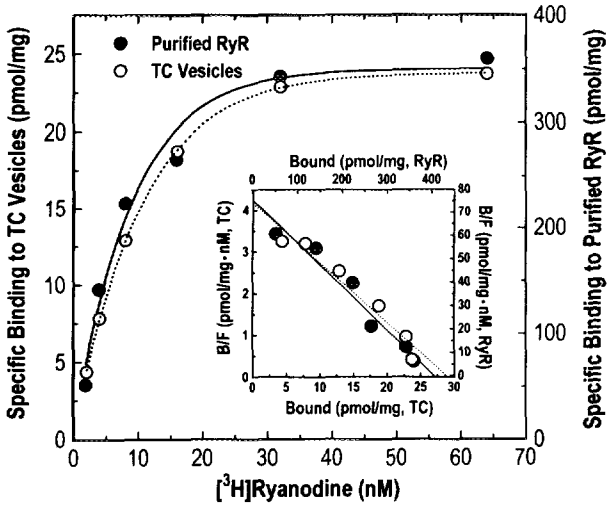

Figure 4. Comparison of Ryanodine Binding Isotherms to Affinity Purified Ryanodine Receptor (RyR) and TC. The purified RyR (•) (10-15 μg of protein/ml) or TC vesicles (○) (500 μg/ml) were incubated in 40 μl at room temperature for 1 hour with varying concentrations (2-64 nM) of [^{3}H]ryanodine in binding buffer (10 mM HEPES, pH 7.4, 25 μM $CaCl_2$, 10 mg/ml CHAPS, 5 mg/ml SBL, 2 mg/ml BSA). Nonspecific binding was determined in the presence of 10 μM cold ryanodine. The free [^{3}H]Ryanodine was separated from bound by centrifugation as described previously (9). For the purified ryanodine receptor, γ-globulin was used as carrier followed by precipitation with PEG. The K_d values for the TC (6.9 nM) and the purified ryanodine receptor (5.4 nM) are essentially the same. The Bmax values are 29 pmol/mg for TC and 406 pmol/mg,reflecting the purity of the receptor.

shown). The fact that the ryanodine receptor can be affinity purified via specific binding of FKBP12 as achieved here, provides strong evidence that the ryanodine receptor and FKBP exists as a hetero-oligomeric complex. Similar methodology can be used to purify the cardiac ryanodine receptor (22).

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