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Solubilization and Biochemical Characterization of the High Affinity [^{3}H]Ryanodine Receptor from Rabbit Brain Membranes*

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A high affinity [^{3}H]ryanodine receptor has been solubilized from rabbit brain membranes and biochemically characterized. [^{3}H]Ryanodine binding to rabbit brain membranes is specific and saturable, with a K_d of 1.3 nM. [^{3}H]Ryanodine binding is enriched in membranes from the hippocampus but is significantly lower in membranes from the brain stem and spinal cord. Approximately 60% of [^{3}H]ryanodine-labeled receptor is solubilized from brain membranes using 2.5% CHAPS and 10 mg/ml phosphatidylcholine containing 1 M NaCl. The solubilized brain [^{3}H]ryanodine receptor sediments through sucrose gradients like the skeletal receptor as a large (~30 S) complex. Solubilized receptor is specifically immunoprecipitated by sheep polyclonal antibodies against purified skeletal muscle ryanodine receptor coupled to protein A-Sepharose. [^{3}H]Ryanodine-labeled receptor binds to heparin-agarose, and a protein of ~400,000 Da, which is cross-reactive with two polyclonal antibodies raised against the skeletal muscle ryanodine receptor, elutes from the column and is enriched in peak [^{3}H]ryanodine binding fractions. These results suggest that the ~400,000-Da protein is the brain form of the high affinity ryanodine receptor and that it shares several properties with the skeletal ryanodine receptor including a large oligomeric structure composed of ~400,000-Da subunits.

Skeletal muscle contraction is initiated by Ca^{2+} release from the sarcoplasmic reticulum upon depolarization of the transverse tubular membrane. The skeletal muscle receptor for the plant alkaloid ryanodine (1) has been shown to be identical with the Ca^{2+} release channel of the sarcoplasmic reticulum (2, 3) and the "foot" structures at the junction between the sarcoplasmic reticulum and transverse tubular membranes (3-5). Interaction between the foot structures and the dihydropyridine receptor of the transverse tubule has been proposed to be responsible for excitation-contraction coupling in skeletal muscle (5, 6).

The concentration of free cytosolic Ca^{2+} is an important regulator of function in noncontractile cells as well as in muscle. In neurons, the concentration of free cytosolic Ca^{2+} is a crucial signal for a variety of processes including neurotransmitter release (7) and alterations in the cytoskeleton (8). Inositol 1,4,5-triphosphate (IP_3),[1] produced after receptor activation, is an important second messenger involved in the release of stored Ca^{2+} from intracellular compartments (9). The IP_3 receptor from rat cerebellum has been purified (10), cloned (11, 12), and localized to the endoplasmic reticulum of cerebellar Purkinje cells (12).

Although IP_3 appears to have a major role in the regulation of free cytosolic Ca^{2+} concentration within neurons, pharmacological evidence has indicated the presence of intracellular Ca^{2+} stores within neurons which are distinct from IP_3-gated pools (13-16). In sensory and sympathetic neurons, ryanodine has been shown to block caffeine-induced Ca^{2+} release from intracellular stores which are distinct from IP_3-gated Ca^{2+} pools (14, 15). In adrenal chromaffin cells, ryanodine affects spontaneous Ca^{2+} fluctuations which have been shown to be independent of IP_3 (16). Further, dantrolene sodium, a drug known to interfere with Ca^{2+} release in skeletal muscle (17), can inhibit release of stored Ca^{2+} from hippocampal cells (18).

Recently, Ashley (19) has demonstrated that [^{3}H]ryanodine binds to rat brain microsomes with a K_d of 3 nM, and that brain microsomes contain a Ca^{2+} conductance modifiable by ryanodine. The brain Ca^{2+} current has a slope conductance of 100 pS (19), which is similar to the 75- and 100-pS Ca^{2+} conductances seen in native sarcoplasmic reticulum from heart (20) and skeletal muscle (2), respectively. These values are significantly different from the 10-pS conductance for Ca^{2+} channel activity reconstituted from arterial smooth muscle (21). Thus, pharmacological and biophysical studies have indicated the presence of non-IP_3-gated Ca^{2+} pools in neurons which may be related to ryanodine receptor-gated pools in skeletal muscle.

Here, we report the solubilization of the [^{3}H]ryanodine-labeled receptor from rabbit brain membranes and identify the receptor as a protein of ~400,000 Da which cross-reacts with antibodies against sarcoplasmic reticulum ryanodine receptor. The ~400,000-Da protein migrates as a large, oligomeric complex of ~30 S on sucrose density gradients.

EXPERIMENTAL PROCEDURES

Isolation of Rabbit Brain and Skeletal Muscle Membranes—Either adult rabbit brain minus the brain stem, dissected brain regions, or fast twitch muscle from rabbit back and hind legs was homogenized in 50 mM Tris-Cl, pH 7.4, in the presence of protease inhibitors: aprotinin (76.8 nm), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and phenylmethylsulfonyl fluoride (0.23 mM). Homogenates were centrifuged at 35,000 × *g* for 15 min, and pellets were resuspended in 0.303 M sucrose in 20 mM

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[1] The abbreviations used are: IP_3, inositol 1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

Tris maleate, pH 7.0, containing protease inhibitors and stored at −135 °C. Protein was measured by the method of Lowry *et al.* (22) as modified by Peterson (23), with bovine serum albumin as a standard.

[^{3}H]Ryanodine Binding Assay—Brain or skeletal muscle membranes (0.5 mg/ml) were incubated with 0.1 to 15 nM [^{3}H]ryanodine for 1 h at 37 °C in the presence or absence of 10 μM ryanodine in 10 mM sodium HEPES, pH 7.4, containing 1.5 M KCl, 10 mM ATP, and 0.8 mM $CaCl_2$ (100 μM free Ca^{2+}). The amount of [^{3}H]ryanodine bound was determined by membrane filtration as previously described (24).

Solubilization of [^{3}H]Ryanodine Receptor from Rabbit Brain Membranes—Brain membranes were labeled as described above using 10 nM [^{3}H]ryanodine in the presence or absence of 10 μM unlabeled ryanodine, centrifuged at 35,000 × *g* for 15 min, and resuspended at 5.0 mg of protein /ml in Buffer A (10 mM sodium HEPES, pH 7.4, 0.8 mM $CaCl_2$, and protease inhibitors) containing 1 M NaCl and CHAPS-phosphatidylcholine (0–5% CHAPS containing 0–20 mg/ml phosphatidylcholine). After 1.5 h of gentle stirring at 4 °C, the mixture was centrifuged for 15 min at 135,000 × *g*. Pellets were resuspended and washed by membrane filtration as described (24). Levels of [^{3}H]ryanodine-labeled receptor in the supernatant were determined by polyethylene glycol (PEG) precipitation. To aliquots of soluble supernatant (300 μl) was added 100 μl of carrier protein (0.5% bovine serum albumin, 0.5% bovine γ-globulin in 50 mM Tris-Cl, pH 7.4), and 3.5 ml of ice cold PEG buffer (10 mM $MgCl_2$, 10% PEG in 20 mM Tris-Cl, pH 7.4). Samples were vortexed, and, after 15 min at 4 °C, were washed by membrane filtration using PEG buffer.

Sucrose Density Gradient Centrifugation—A sample of 5 mg of brain membranes or 2.5 mg of skeletal muscle triad membranes (2) were labeled with 10 nM [^{3}H]ryanodine in the presence or absence of 10 μM unlabeled ryanodine, and solubilized as described above using 2.5% CHAPS and 10 mg/ml phosphatidylcholine. Approximately 1 ml of soluble receptor was layered onto a 12.5-ml linear sucrose gradient of 10–30% sucrose in Buffer A containing 0.5 M NaCl, 1.25% CHAPS, and 5 mg/ml phosphatidylcholine and centrifuged for 75 min in a Beckman VTi 65.1 rotor at 238,000 × *g* using slow acceleration and deceleration profiles. The density gradient was collected from the top in twenty 0.6-ml fractions which were added to scintillation fluid for determination of radioactivity.

Immunoprecipitation of [^{3}H]Ryanodine-labeled Receptor from Solubilized Brain Membranes—Brain membranes were labeled with 10 nM [^{3}H]ryanodine in the presence or absence of 10 μM unlabeled ryanodine and solubilized as described above. Aliquots of 250 μl of soluble supernatant were diluted to 1 ml with 10 mM sodium HEPES, 0.8 mM $CaCl_2$ to a final mixture of 10 mM sodium HEPES, 0.8 mM $CaCl_2$, 0.25 M NaCl, 0.625% CHAPS, 2.5 mg/ml phosphatidylcholine (bead equilibration buffer). Increasing concentrations of protein A-Sepharose or protein A-Sepharose which had been incubated overnight with polyclonal antibodies against the sarcoplasmic reticulum ryanodine receptor were added, after several washes in the bead equilibration buffer using microcentrifugation, to the mixture. The samples were incubated overnight with gentle mixing at 4 °C, followed by three washes in bead equilibration buffer. The final pellet was resuspended in 10 ml of scintillation fluid for determination of radioactivity.

Heparin-Agarose and Wheat Germ Agglutinin-Sepharose Bead Assays—Brain membranes were labeled with 10 nM [^{3}H]ryanodine and solubilized, and aliquots of 250 μl of soluble supernatant were diluted as described above. Heparin-agarose and wheat germ agglutinin-Sepharose beads (50 μl) were added to the samples after several washes in bead equilibration buffer. Additional samples contained 0.5 M *N*-acetylglucosamine or 0.6 M NaCl. The samples were incubated overnight with gentle mixing at 4 °C, followed by three washes in bead equilibration buffer. The final pellet was resuspended in 10 ml of scintillation fluid for determination of radioactivity.

Heparin-Agarose Chromatography of Brain [^{3}H]Ryanodine Receptor—Brain membranes (600 mg) were labeled with 10 nM [^{3}H]ryanodine and solubilized as described above. The supernatant was diluted 3.3-fold in buffer A and passed over a 25-ml heparin-agarose column equilibrated in buffer A containing 0.3 M NaCl, 0.75% CHAPS, 3 mg/ml phosphatidylcholine. The column was washed with 10 column volumes of buffer A containing 0.3 M NaCl, 1% CHAPS, and 4 mg/ml phosphatidylcholine followed by elution with buffer A containing 0.6 M NaCl, 1% CHAPS, and 4 mg/ml phosphatidylcholine. Aliquots of each 5-ml fraction were used to determine radioactivity and for analysis on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot.

Polyclonal Antibodies against Skeletal Muscle Ryanodine Receptor (Ca^{2+} Release Channel)—Polyclonal antibodies against the rabbit skeletal muscle Ca^{2+} release channel were prepared by injection of guinea pigs using gel slices from 10 μg of purified skeletal muscle ryanodine receptor (2) as previously described for the subunits of the dihydropyridine receptor (25). Antibodies were screened by testing reactivity on immunoblot with purified skeletal muscle ryanodine receptor. Sheep polyclonal antibodies (26) and monoclonal antibody XA7 (27) against the skeletal muscle ryanodine receptor were prepared as previously described.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis—Heparin-agarose column fractions were analyzed by SDS-PAGE (3–12% gradient gels) using the buffer system of Laemmli (28) and stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes according to Towbin *et al.* (29). The nitrocellulose transfers were blocked for 1 h with phosphate-buffered saline (154 mM NaCl, 50 mM NaH_2PO_4, pH 7.5 with NaOH) containing 5% nonfat dry milk and subsequently incubated overnight with primary antibody. Immunoblots were then washed three times in phosphate-buffered saline-milk, incubated for 4 h with secondary anti-IgG antibodies covalently linked to horseradish peroxidase, washed with phosphate-buffered saline-milk, and finally developed in Tris-buffered saline (200 mM NaCl, 20 mM Tris-HCl, pH 7.5) using 4-chloro-1-naphthol as the substrate.

Materials—Skeletal muscle ryanodine receptor was purified as previously described (2, 30). Horseradish peroxidase-conjugated secondary antibodies were obtained from Boehringer Mannheim. Prestained molecular weight standards were from Bethesda Research Laboratories. [^{3}H]Ryanodine was from Du Pont-New England Nuclear. Phosphatidylcholine, molecular weight standards, and heparin-agarose (Type 1) were from Sigma. CHAPS was obtained from Pierce. All other chemicals were of reagent grade.

RESULTS

[^{3}H]Ryanodine Binding Analysis on Rabbit Brain and Skeletal Muscle Membranes—In order to characterize the ryanodine receptor in mammalian brain, binding experiments were performed on rabbit brain membranes utilizing [^{3}H]ryanodine. Initial binding data using 100 μg of membrane protein per assay tube yielded 300–500 total dpm with specific binding accounting for 50% of the total. An increase in membrane protein to 500 μg led to approximately 3200 dpm total binding, of which 75% was specific. Fig. 1*A* shows saturation analysis and a corresponding Scatchard plot from a typical experiment for [^{3}H]ryanodine binding using 500 μg of membrane protein per tube. Values (± S.E.) for K_d (1.3 ± 0.2 nM) and B_{max} (56.3 ± 2.9 fmol/mg) were determined from three separate experiments. The K_d values are similar to those for [^{3}H]ryanodine binding to skeletal muscle membranes prepared in a manner identical with that from brain. Fig. 1*B* shows saturation analysis and a corresponding Scatchard plot from a typical experiment for [^{3}H]ryanodine binding to skeletal muscle membranes. Values for K_d (1.7 ± 0.3 nM) and B_{max} (556 ± 139 fmol/mg) were determined from three separate experiments. Skeletal muscle yields approximately 50% more membrane protein than brain per tissue wet weight (data not shown). Therefore, the B_{max} values indicate that [^{3}H]ryanodine receptor is only about 15-fold more abundant in skeletal muscle than in brain per tissue wet weight.

Fig. 2 shows the distribution of [^{3}H]ryanodine (2.5 nM) binding within the central nervous system. Spinal cord, brain stem, frontal quadrants of the cerebral hemispheres, and cerebellum had 21.1 ± 9.3, 35.9 ± 4.6, 93.0 ± 13.2, and 92.0 ± 11.8% binding of the whole brain. The highest binding was observed in the hippocampus (138.0 ± 5.4%). Binding was dependent on Ca^{2+} as the addition of 1 mM EGTA led to 5.1 ± 1.0% of control binding (data not shown). Removal of KCl caused binding to drop to 19.5 ± 1.2% of control (data not shown).

Solubilization and Sucrose Density Gradient Centrifugation of [^{3}H]Ryanodine-labeled Receptor from Rabbit Brain Membranes—Brain membranes were labeled with 10 nM

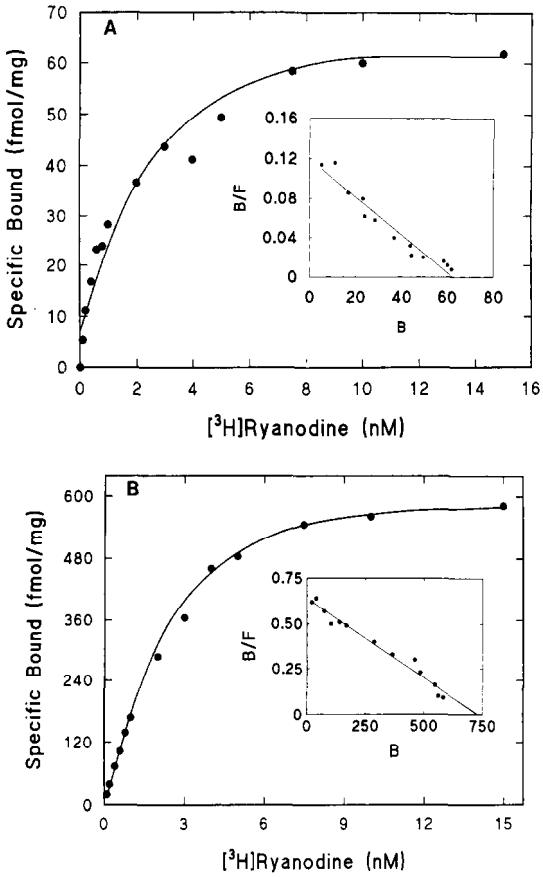

FIG. 1. **[³H]Ryanodine binding to isolated rabbit brain and skeletal muscle membranes.** Whole rabbit brain (*A*) or skeletal muscle (*B*) membranes were incubated with various [³H]ryanodine concentrations (0.1–15 nM) for 1 h at 37 °C, as described under "Experiental Procedures." Specific binding was derived by subtracting nonspecific binding determined in the presence of 10 μM unlabeled ryanodine. All experiments were performed in triplicate, and a representative example of each experiment is presented. Scatchard analysis of brain membranes (*A*) yielded apparent B_{max} and K_d values (± S.E.) of 56.3 ± 2.9 fmol/mg and 1.3 ± 0.2 nM, respectively. Scatchard analysis of skeletal muscle membranes (*B*) yielded apparent B_{max} and K_d values of 556 ± 139 fmol/mg and 1.7 ± 0.3 nM, respectively.

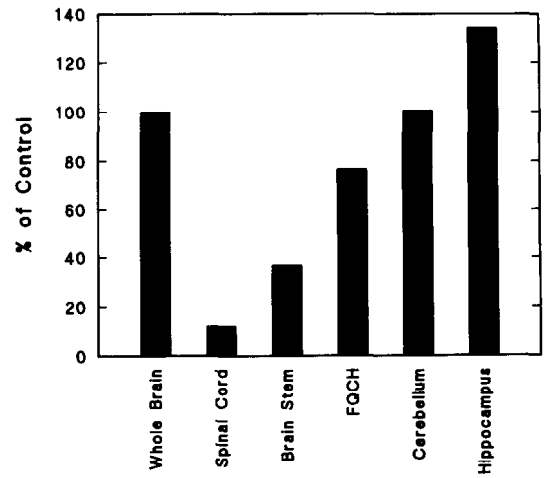

FIG. 2. **[³H]Ryanodine binding to membranes from isolated central nervous system regions.** [³H]Ryanodine binding to membranes isolated from various central nervous system regions was measured in the presence of 2.5 nM [³H]ryanodine, as described under "Experimental Procedures," and expressed as percentage of control of binding for whole brain membranes (*FQCH* = frontal quadrants of the cerebral hemispheres).

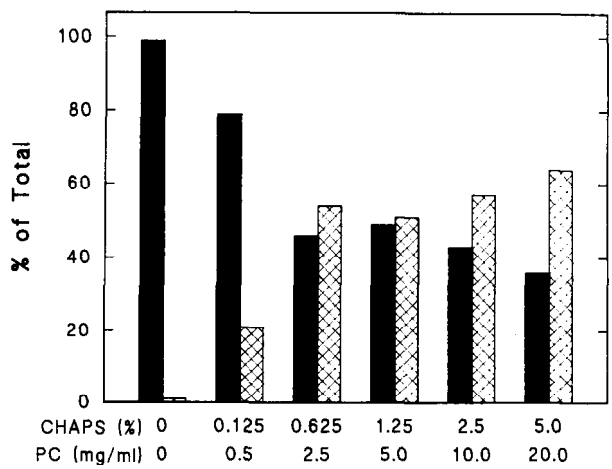

FIG. 3. **Solubilization of [³H]ryanodine-labeled receptor from rabbit brain membranes.** Brain membranes were labeled with 10 nM [³H]ryanodine in the presence or absence of 10 μM unlabeled ryanodine as described under "Experimental Procedures." Labeled membranes were incubated for 1.5 h at 4 °C in 0.8 mM $CaCl_2$, 1 M NaCl, and the indicated concentrations of CHAPS and phosphatidylcholine. After centrifugation at 135,000 × *g*, the amount of specifically labeled [³H]ryanodine receptor in the supernatant (■) and pellet (▨) was determined and expressed as percent of total.

[³H]ryanodine, treated with increasing concentrations of CHAPS-phosphatidylcholine, and centrifuged at 135,000 × *g* for 15 min. An increase in the amount of specifically labeled [³H]ryanodine receptor remaining in the supernatant was seen with increasing CHAPS-phosphatidylcholine concentrations (Fig. 3). Solubilization was near-maximal (60%) at 2.5% CHAPS, 10 mg/ml phosphatidylcholine, and these concentrations were therefore used routinely. Sucrose density gradient centrifugation was then performed on solubilized, [³H]ryanodine-labeled membranes from skeletal muscle (Fig. 4*A*) and brain (Fig. 4*B*) as described under "Experimental Procedures." In both tissues, [³H]ryanodine-labeled receptor showed a peak sedimentation to fraction 14 of the 10–30% sucrose gradient, thus indicating a similar size for both brain and skeletal muscle [³H]ryanodine receptor. [³H]Ryanodine receptor from skeletal muscle (3) and heart (20) are known to sediment on sucrose gradients as characteristically large complexes of approximately 30 S.

Immunoprecipitation of [³H]Ryanodine-labeled Receptor—Having identified a high affinity [³H]ryanodine receptor in brain membranes, with a sedimentation rate similar to that in striated muscle, immunoprecipitation of the brain [³H] ryanodine receptor was performed using sheep polyclonal antibodies raised against the skeletal muscle ryanodine receptor (Fig. 5*A*). Increasing concentrations of protein A-Sepharose beads, preincubated with sheep polyclonal antibodies, led to an increase in the precipitation of solubilized, [³H]ryanodine-labeled receptor. Protein A-Sepharose beads alone did not cause appreciable precipitation. The specificity of the immunoprecipitation assay was further characterized (Fig. 5*B*). Sheep antibodies, coupled to protein A-Sepharose beads, did not cause significant precipitation of [³H]ryanodine label when [³H]ryanodine binding was performed in the presence of excess (10 μM) unlabeled ryanodine. Further, the addition of 10 μg of purified skeletal muscle ryanodine receptor (2, 30) to the immunoprecipitation mixture significantly inhibited immunoprecipitation of the brain [³H]ryanodine receptor by the sheep antibody. In contrast to the sheep polyclonal antibody, monoclonal antibody XA7 against the skeletal muscle ryanodine receptor (27), linked to goat anti-mouse beads, was

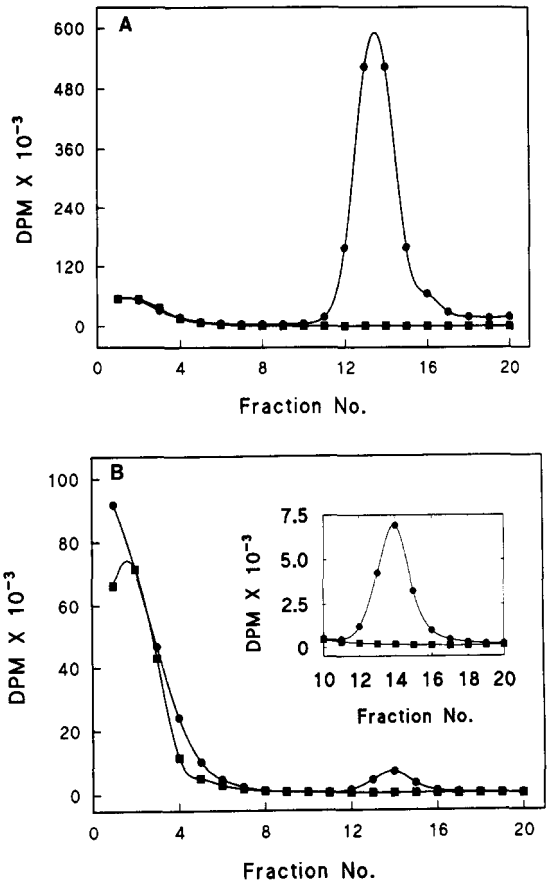

FIG. 4. **Sucrose density gradient centrifugation of solubilized, [^{3}H]ryanodine-labeled membranes from brain and skeletal muscle.** Triad membranes from skeletal muscle (2.5 mg) (*A*) or whole brain membranes (5 mg) (*B*) were labeled with 10 nM [^{3}H] ryanodine in the presence or absence of excess unlabeled ryanodine (10 μM) and solubilized as described under "Experimental Procedures." The solubilized membranes (1 ml) were placed on 12.5-ml 10–30% sucrose gradients and centrifuged in a VTi65.1 Beckman rotor for 75 min at 238,000 × *g* as described under "Experimental Procedures." Total (●) and nonspecific (■) binding are shown. The *inset* (*B*) shows fractions 10–20 on an expanded scale.

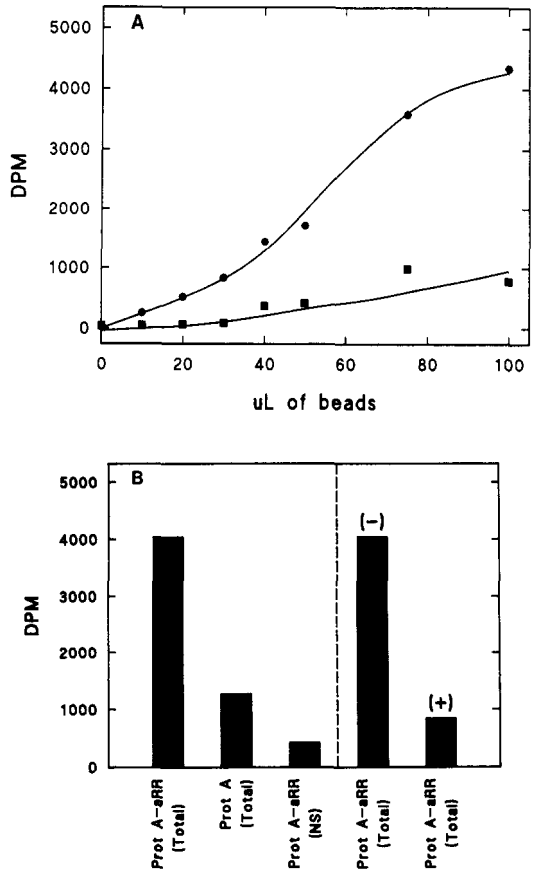

FIG. 5. **Immunoprecipitation of solubilized, [^{3}H]ryanodine-labeled brain membranes.** Whole brain membranes were labeled with 10 nM [^{3}H]ryanodine and solubilized as described under "Experimental Procedures." *A*, samples were incubated with protein A-Sepharose coupled to sheep polyclonal antibodies raised against the skeletal muscle ryanodine receptor (●) or to protein A-Sepharose beads alone (■). *B*, immunoprecipitations were performed using 100 μl of protein A-Sepharose beads alone (*Prot A*) or beads coupled to sheep polyclonal antibodies (*Prot A-αRR*). Immunoprecipitations using protein A coupled to ryanodine receptor antibodies were also performed on membranes labeled in the presence of excess unlabeled ryanodine (*NS*) and in the presence (+) or absence (−) of 10 μg of pure skeletal muscle ryanodine receptor prepared as described (2, 30).

unable to immunoprecipitate [^{3}H]ryanodine-labeled receptor (data not shown). Thus, the brain ryanodine receptor appears to be immunologically similar, but not identical with the skeletal muscle ryanodine receptor.

Immunological Identification of Isolated [^{3}H]Ryanodine-labeled Receptor—Crude brain membranes (200 μg) were analyzed on SDS-PAGE for cross-reactivity with a variety of polyclonal and monoclonal antibodies against the skeletal muscle ryanodine receptor. No immunological staining was observed, likely owing to the low concentration (56 fmol/mg of protein) of [^{3}H]ryanodine binding sites in the crude brain membranes. Therefore, preparation of fractions enriched in [^{3}H]ryanodine receptor were necessary for immunological identification. Previously, heparin-agarose chromatography was used in purification of the skeletal and cardiac muscle ryanodine receptors (31) and the brain IP_3 receptor (10). Concanavalin A, wheat germ agglutinin lectin, and lentil lectin were used for purification of the IP_3 receptor from brain and aorta smooth muscle (10, 32, 33). Therefore, bead assays were performed on labeled brain membranes to test the affinity of [^{3}H]ryanodine receptor for heparin-agarose and various Sepharose-coupled lectins. [^{3}H]Ryanodine-labeled receptor was not appreciably precipitated by wheat grem agglutinin-Sepharose, but bound to heparin-agarose in the presence of 0.25 M NaCl (Fig. 6). Precipitation by heparin-agarose was significantly reduced when performed in the presence of 0.6 M NaCl (Fig. 6). [^{3}H]Ryanodine-labeled receptor showed no affinity for lentil lectin-Sepharose or concanavalin A-Sepharose (data not shown).

Heparin-agarose chromatography was then performed on solubilized, [^{3}H]ryanodine-labeled membranes (Fig. 7). Brain membranes (600 mg) were labeled with 10 nM [^{3}H]ryanodine and solubilized as described under "Experimental Procedures." The solubilized sample was then diluted to 0.3 M NaCl and applied to a heparin-agarose column. Approximately 87% of the bound counts were eluted using 0.6 M NaCl in CHAPS-phosphatidylcholine (Fig. 7*A*). Thus, the brain ryanodine receptor has an affinity for heparin similar to that reported for the heart and skeletal muscle ryanodine receptor (31).

Peak [^{3}H]ryanodine binding fractions from the heparin-agarose column contained a high molecular weight band of ~400,000, which showed a migration similar to the skeletal muscle ryanodine receptor (Fig. 7*B*). The ~400,000-Da protein was stained with guinea pig antisera against the skeletal muscle ryanodine receptor (Fig. 7*C*) and was also found to cross-react with the sheep polyclonal antibodies raised against rabbit skeletal muscle ryanodine receptor, either before or after affinity purification against pure muscle receptor. How-

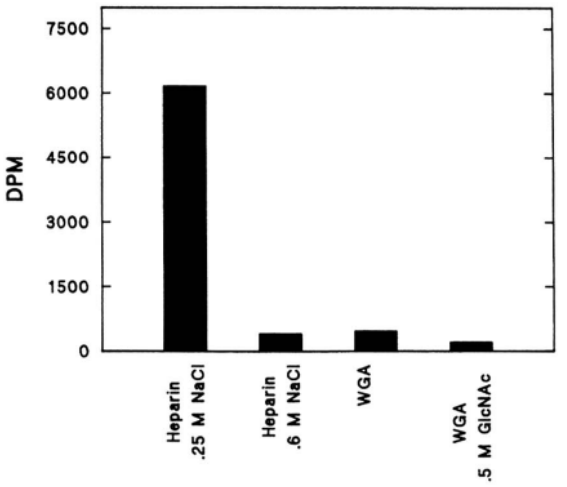

FIG. 6. **Heparin-agarose and wheat germ agglutinin-Sepharose bead assays.** Whole brain membranes were labeled with 10 nM [^{3}H]ryanodine and solubilized as described under "Experimental Procedures." Samples were incubated with 50 μl of heparin-agarose (*Heparin*) in the presence of 0.25 or 0.6 M NaCl. Identical aliquots were also incubated with 50 μl of wheat germ agglutinin-Sepharose (*WGA*) in the presence or absence of 0.5 M *N*-acetylglucosamine (*GlcNAc*). Following incubation, pellets were washed several times and radioactivity in the pellets was determined.

ever, the ~400,000-Da band did not cross-react with a second guinea pig polyclonal antibody against the skeletal muscle ryanodine receptor, nor with anti-ryanodine receptor monoclonal antibody XA7 (data not shown). Thus, it appears that the rabbit brain ryanodine receptor is similar in apparent molecular weight to the well studied skeletal and cardiac muscle forms and has maintained some, but not all, immunological cross-reactivity between tissues.

DISCUSSION

This study describes the identification of the [^{3}H]ryanodine receptor from rabbit brain membranes as a ~400,000-Da protein, with many characteristics similar to that of the skeletal and cardiac muscle forms of the receptor. [^{3}H]Ryanodine binds to rabbit brain membranes with a K_d of 1.3 nM, similar to the K_d (1.7 nM) for binding to fast twitch skeletal muscle membranes prepared in an identical manner (Fig. 1). The K_d value is also similar to that reported by Ashley (19) for [^{3}H]ryanodine binding to rat brain microsomes. [^{3}H]Ryanodine-labeled receptor from brain membranes was solubilized using CHAPS-phosphatidylcholine in the presence of 1 M NaCl (Fig. 3), conditions similar to those determined to be optimal for solubilization of the skeletal and cardiac forms of the receptor (31). Solubilized [^{3}H]ryanodine receptor from skeletal muscle and brain co-sediment on sucrose density gradients (Fig. 4). Ryanodine receptor from skeletal muscle and heart are both estimated to be 30 S complexes by this technique, presumably due to the existence of the ryanodine receptor as a stable tetramer after solubilization with CHAPS (3, 20). The brain ryanodine receptor migrates on SDS-PAGE with an apparent molecular weight similar to the skeletal muscle form (Fig. 7*C*) and is thus likely to have a similar tetrameric structure after CHAPS-phosphatidylcholine solubilization. [^{3}H]Ryanodine receptor from brain is enriched using heparin-agarose chromatography (Fig. 7) under conditions similar to those used for purification of the cardiac and skeletal muscle forms of the receptor (31). Recognition of an ~400,000-Da protein in [^{3}H]ryanodine binding fractions from the heparin-agarose column with two polyclonal antibodies against the skeletal muscle ryanodine receptor indicates that

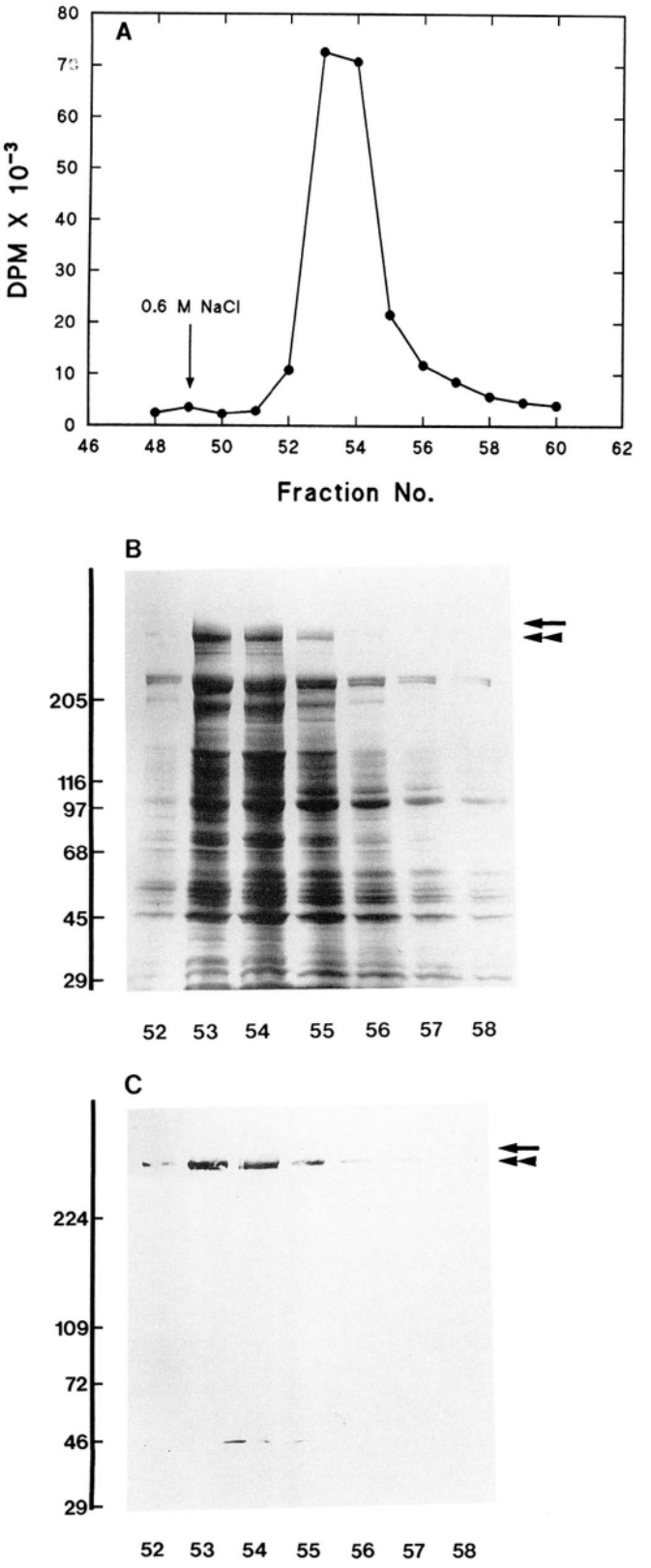

FIG. 7. **Heparin-agarose chromatography of solubilized ryanodine receptor.** Rabbit brain membranes were labeled with 10 nM [^{3}H]ryanodine, solubilized with CHAPS phosphatidylcholine using 1 M NaCl, and applied to heparin-agarose chromatography as described under "Experimental Procedures." Aliquots (500 μl) of each column fraction were used to determine radioactivity (*A*) or were analyzed on SDS-PAGE after concentration on Centricon-10 microconcentrators. Gels were stained with Coomassie Brilliant Blue (*B*) or were transferred to nitrocellulose and immunoblotted with guinea pig antiserum raised against sarcoplasmic reticulum ryanodine recep-

the brain and muscle forms have been immunologically conserved. However, failure of a third polyclonal antibody against the skeletal muscle receptor and the skeletal muscle ryanodine receptor-specific monoclonal antibody XA7 to recognize the protein indicates that the brain form of the receptor is not immunologically identical with the muscle form. Thus, the brain and muscle forms of the [^{3}H]ryanodine receptor have similar characteristics including affinity for [^{3}H]ryanodine, detergent solubilization characteristics, sedimentation on sucrose density gradients, affinity for heparin-agarose, migration on SDS-PAGE, and immunological cross-reactivity.

The brain IP_3 receptor appears to be a major protein involved in regulation of Ca^{2+} within neurons and has been found to have sequence similarities with the skeletal muscle ryanodine receptor (12). However, the brain [^{3}H]ryanodine receptor has a variety of characteristics which distinguish it from the brain IP_3 receptor. The distribution of [^{3}H]ryanodine binding within the central nervous system (Fig. 2) varies markedly from that reported for [^{3}H]IP_3 binding in rat (34) and human (35) brain. [^{3}H]Ryanodine binding is enriched in the hippocampus in relation to the whole brain, but is not enriched in the cerebellum nor the cerebral cortex. This is in contrast to the IP_3 receptor which is between 4- and 15-fold more abundant in cerebellum than hippocampus or neocortex (34, 35). The reason for the high levels of [^{3}H]ryanodine binding in hippocampus is not known. Also, [^{3}H]ryanodine-labeled receptor has no affinity for wheat germ agglutinin-Sepharose, concanavalin A-Sepharose, or lentil lectin-Sepharose (Fig. 6). This is in contrast to the IP_3 receptor which is known to have affinity for all three lectins (10, 32, 33). The brain IP_3 and [^{3}H]ryanodine receptors can also be distinguished based on their molecular weight as determined by SDS-PAGE. Brain [^{3}H]ryanodine receptor has a molecular weight of approximately 400,000 (Fig. 7), whereas the IP_3 receptor migrates with an apparent molecular weight of 260,000 (10). Such a difference in migratory ability is clearly distinguishable on the 3–12% gradient SDS-PAGE system used in this study. Further, immunoblots containing pure skeletal muscle ryanodine receptor and IP_3 receptor isolated from cerebellum show a clear distinction in migratory ability and no immuno-crossreactivity when immunoblotted with antiskeletal muscle ryanodine receptor or anti-cerebellum IP_3 receptor antibodies (data not shown).

The precise cellular location of the brain [^{3}H]ryanodine receptor remains unknown. Although there has been a preliminary report of [^{3}H]ryanodine binding in uterine smooth muscle (36), arterial smooth muscle is apparently devoid of [^{3}H]ryanodine receptor. Fleischer's laboratory (32) have purified IP_3 receptor from bovine aorta smooth muscle, but were unable to detect [^{3}H]ryanodine binding to their membrane preparation. Thus, it is unlikely that the [^{3}H]ryanodine receptor is located in brain arterial smooth muscle. Further, Ashley (19) has shown that brain microsomes contain a Ca^{2+} current, modifiable by ryanodine, with a peak slope conductance of 100 pS. This is similar to the 75- and 100-pS Ca^{2+} conductances seen in native sarcoplasmic reticulum from heart (20) and skeletal muscle (2), respectively, but is significantly different from the 10-pS conductance for Ca^{2+} channel activity recorded from arterial smooth muscle membranes (21). Also, microvessels isolated from mouse brain (37) contain approximately 0.1% of the total membrane protein content of whole brain[2] and would therefore have to contain 50 pmol/mg of protein of [^{3}H]ryanodine receptor to account for the levels of binding reported in this study. Therefore, it is likely that the [^{3}H]ryanodine receptor identified in this study is of neuronal and/or glial origin.

The physiological role of the brain ryanodine receptor remains speculative. The apparent conservation of many characteristics between the brain and muscle forms leads one to speculate that the receptor has analogous roles in the two tissues. The brain ryanodine receptor is likely to be situated in the smooth endoplasmic reticulum of neurons and/or glia, allowing for release of stored Ca^{2+} into the cytosol upon the appropriate stimulus. The brain ryanodine receptor may be activated via coupling to a voltage-activated ion channel on the cell surface, analogous to excitation-contraction coupling in skeletal muscle (5, 6), or may be activated via a Ca^{2+}-induced Ca^{2+} release mechanism as seen in heart (38). Whether or not this receptor is responsible for the non-IP_3-gated Ca^{2+} stores seen in neurons (13–16), or the ryanodine-sensitive Ca^{2+} conductance of rat brain microsomes (19), and its exact physiological role in the brain will be addressed in further studies.

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tor (*C*). The *arrow* on the *right* (*B, C*) indicates the position of the skeletal muscle ryanodine receptor (not shown), and the *double arrowheads* indicate the position of the brain ryanodine receptor shown on the immunoblot. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the *left* (*B, C*).

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