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Characterization of the Major Brain Form of the Ryanodine Receptor/Ca^{2+} Release Channel*

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At least three distinct ryanodine receptor genes appear to be expressed in mammalian brain. We have used biochemical and immunological methods to characterize the major form of ryanodine binding protein purified from brain. [^{3}H]Ryanodine binding to the purified brain receptor is stimulated by Ca^{2+}, ATP, KCl, and phosphorylation and is inhibited by calmodulin, Mg^{2+}, and ruthenium red. Immunoblot and immunoprecipitation analysis using a panel of monoclonal and polyclonal antibodies against skeletal and cardiac muscle ryanodine receptors, and two novel polyclonal antibodies against the brain ryanodine receptor, reveals that the major form of ryanodine receptor expressed in brain is immunologically similar to the cardiac ryanodine receptor, but is distinct from the skeletal muscle receptor. Digestion of cardiac and brain ryanodine receptors with trypsin or α-chymotrypsin generates similar proteolytic patterns as detected by immunoblot analysis or by autoradiography after labeling with a hydrophobic probe, suggesting that the two proteins are similar in both their large cytoplasmic and hydrophobic transmembrane domains. Taken together, these data indicate that the cardiac ryanodine receptor/Ca^{2+} release channel is the major form of ryanodine receptor expressed in brain, and that it likely functions in releasing Ca^{2+} from caffeine-sensitive intracellular Ca^{2+} stores in neurons by a mechanism of regulated Ca^{2+}-induced Ca^{2+} release.

The concentration of free cytosolic Ca^{2+} is a crucial signal for a variety of neuronal processes including neurotransmitter release, control of membrane excitability, and alterations in the cytoskeleton (1). One way in which neurons regulate their free cytosolic Ca^{2+} concentration is through storage and release of Ca^{2+} from intracellular pools (2, 3). Two major intracellular Ca^{2+} pools appear to be present in neurons (4–6), one which is sensitive to inositol 1,4,5-triphosphate (IP_3)[1] and is gated by the IP_3 receptor (7), and a second which is sensitive to caffeine and ryanodine and which is gated by the ryanodine receptor/Ca^{2+} release channel (8).

Ryanodine receptors were initially identified in heavy sarcoplasmic reticulum fractions (9) from which they could be solubilized using the detergent CHAPS in the presence of high ionic strength (10). The receptors were subsequently purified from skeletal (11–13) and cardiac (14, 15) muscle and demonstrated to be involved in the release of Ca^{2+} from the sarcoplasmic reticulum (16). The regulation of ryanodine receptor functioning in skeletal *versus* cardiac muscle appears to be different. In skeletal muscle, activation of sarcoplasmic reticulum Ca^{2+} release is independent of Ca^{2+} influx (17, 18) and is possibly due to a direct interaction of the ryanodine receptor with the dihydropyridine receptor (19). In contrast, activation of the cardiac ryanodine receptor is dependent on elevated cytosolic Ca^{2+} concentrations from Ca^{2+} influx (18, 20), and it appears that the cardiac ryanodine receptor is a Ca^{2+}-induced Ca^{2+} release channel. The recent localization of the cardiac ryanodine receptor to the corbular sarcoplasmic reticulum of heart myocytes, well removed from the sarcolemma, provides a morphological basis for Ca^{2+}-induced Ca^{2+} release in the heart (21).

In order to understand the nature of Ca^{2+} release from caffeine-sensitive Ca^{2+} pools in neurons, several studies have been performed aimed at characterization of the brain ryanodine receptor. The receptor was initially identified in brain membranes utilizing [^{3}H]ryanodine binding (22–26) and was purified from brain and shown to function as a caffeine-sensitive Ca^{2+} release channel (8). The ryanodine receptor has been localized to membranes of the endoplasmic reticulum in chicken cerebellum Purkinje cells (27), and its distribution throughout the nervous system has been studied (28, 29). Recently, it has been demonstrated that several forms of the ryanodine receptor are expressed in brain. In mouse brain, a protein which is immunoreactive with an antibody raised against a peptide sequence specific to the skeletal muscle ryanodine receptor (30) is present in cerebellar Purkinje cells, whereas an antibody against a peptide sequence specific to the cardiac ryanodine receptor (31) detects a protein throughout the central nervous system (32). Further, a protein with approximately 70% homology with both the cardiac and skeletal muscle ryanodine receptors has recently been cloned from brain (33). Thus, it appears that the brain expresses several forms of ryanodine receptor. The relative significance of each of these forms to neuronal Ca^{2+} handling remains unclear.

Here we have characterized the structural, immunological, and ryanodine binding properties of the major form of ryanodine receptor purified from brain. The purified brain receptor is regulated by Ca^{2+}, ATP, KCl, Mg^{2+}, ruthenium red, calmodulin, and protein kinase A phosphorylation. Immunoblot and immunoprecipitation analysis indicates that the purified brain ryanodine receptor is similar to the cardiac ryanodine receptor, but distinct from the skeletal muscle receptor. Proteolytic digestion of ryanodine receptor purified from heart and brain generates very similar patterns. Taken together, these data demonstrate that the cardiac ryanodine receptor/Ca^{2+} release channel is the major form of ryanodine receptor

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[1] The abbreviations used are: IP_3, inositol 1,4,5-triphosphate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazerine; NS, nonspecific.

expressed in mammalian brain, and that it likely functions in releasing Ca^{2+} from caffeine-sensitive intracellular Ca^{2+} stores in neurons by a mechanism of regulated Ca^{2+}-induced Ca^{2+} release.

EXPERIMENTAL PROCEDURES

Purification of Ryanodine Receptor—Ryanodine receptor was purified from whole rabbit brain as described (8). Ryanodine receptor was purified from rabbit skeletal and cardiac muscle as described for brain except that the DEAE-agarose step was omitted.

Ryanodine Binding Assay—Purified brain ryanodine receptor (75 μl, approximately 0.05 μg) was incubated in 10 mM sodium HEPES, pH 7.4, containing 5 nM [^{3}H]ryanodine, 250–1000 mM KCl, 1 mM EGTA, 10 mM ATP, and varying $CaCl_2$ concentrations to equal 100 μM free Ca^{2+}, with or without 1 μM ryanodine for 1 h at 37 °C. After incubation, 100 μl of carrier protein (0.5% bovine serum albumin, 0.5% γ-globulin, 50 mM Tris-Cl, pH 7.4) and 3 ml of 10% polyethylene glycol were added. The samples were incubated on ice for 15 min, and the precipitated proteins were collected on Whatman GF-B filters on a Brandel Cell Harvester. For specific experiments, the concentrations of ATP, Ca^{2+}, Mg^{2+}, KCl, calmodulin, and ruthenium red were varied individually while keeping all other variables constant. The concentration of free divalent cations was determined using the computer program of Fabiato (34).

Production of Antibodies against Ryanodine Receptor—Monoclonal antibody XA7 and polyclonal antibody GP-23 against the skeletal muscle ryanodine receptor were prepared as described (35, 36). Monoclonal antibodies RyR-1 and RyR-3 against the cardiac ryanodine receptor were generous gifts of Drs. Toshiaki Imagawa and Manekazu Shigekawa. Anti-peptide antibody rabbit-46 was prepared as previously described (8). Antibody GP-561 was prepared by injection of purified brain ryanodine receptor (~6 μg) in complete Freund's adjuvant into a guinea pig. The animal was boosted three times at 2-week intervals with ~6 μg of receptor in incomplete adjuvant. Goat-43 was prepared by injection of purified brain ryanodine receptor (~50 μg in complete Freund's adjuvant) into a goat at multiple epidermal sites. The animal was given intravenous boosts three times at 2-month intervals using ~50 μg of brain ryanodine receptor. Serum from GP-561 and goat-43 were tested for antibody production by immunoblot analysis against purified receptor.

SDS-PAGE and Immunoblot Analysis—Purified ryanodine receptors (~1 μg) were analyzed by SDS-PAGE (3–12% gradient gels) using the buffer system of Laemmli (37). Samples were transferred to nitrocellulose membranes and analyzed with antibodies as described (23). For staining of proteolyzed proteins, the Visiblot system (U. S. Biochemical Corp.) was used for increased sensitivity.

Immunoprecipitation Analysis—Cardiac microsomes (375 μg) and brain crude membranes (10 mg) were incubated in 10 mM sodium HEPES, pH 7.4, containing 2.5 nM [^{3}H]ryanodine, 1 M KCl, 0.8 mM $CaCl_2$ (100 μM free Ca^{2+}), and 10 mM ATP for 1 h at 37 °C. Binding was performed in the absence (total) or presence (NS) of 10 μM unlabeled ryanodine. The membranes were pelleted at 135,000 × g for 15 min, resuspended in buffer A (10 mM sodium HEPES, pH 7.4, 0.8 mM $CaCl_2$, 0.83 nM benzamidine, and 0.23 mM phenylmethylsulfonyl fluoride) containing 2.5% CHAPS, 1.0% phosphatidylcholine, and 1 M NaCl, and mixed for 1 h at 4 °C, and insoluble material was removed by centrifugation at 135,000 × g for 15 min. The specific [^{3}H]ryanodine binding in the supernatant was determined by polyethylene glycol precipitation of aliquots of the total and NS tubes as described above. Total and NS supernatants were diluted 4-fold in buffer A, and aliquots of the diluted material were added to increasing amounts of ryanodine receptor antibodies conjugated to protein A-Sepharose (GP-561) or protein G-Sepharose (goat-43). The total amount of protein A-Sepharose or protein G-Sepharose was kept identical between tubes by adding unconjugated Sepharose beads. Antibodies were conjugated to Sepharose beads by incubation of the serum overnight with the beads, followed by several washes in buffer B (buffer A containing 0.625% CHAPS, 0.25% phosphatidylcholine, and 0.25 M NaCl) with microcentrifugation. The [^{3}H]ryanodine-labeled aliquots (total and NS in separate tubes) with added Sepharose beads were incubated overnight at 4 °C, and the beads were washed with 3 × 500 μl of buffer B by microcentrifugation. The final pellet was resuspended in 10 ml of scintillation mixture for determination of radioactivity.

cAMP-dependent Protein Kinase Phosphorylation—Brain ryanodine receptor (~1 μg in 160 μl of 10 mM sodium HEPES, pH 7.4, 0.36% CHAPS, 0.14% phosphatidylcholine, 0.8 mM $CaCl_2$, 10 mM $MgCl_2$, 40 μM ATP) was incubated with or without 1 μg of cAMP-dependent protein kinase catalytic subunit (generous gift of Dr. Richard Maurer, University of Iowa) for 10 min at room temperature. After incubation, samples were processed for [^{3}H]ryanodine binding as described.

Hydrophobic Labeling—Purified brain and cardiac ryanodine receptor (~1 μg in 160 μl of 10 mM sodium HEPES, pH 7.4, containing 0.8 mM $CaCl_2$, 0.36% CHAPS, 0.14% phosphatidylcholine, and 0.5 M NaCl) were incubated for 30 min on ice with 5 μCi of the hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazerine ([^{125}I]TID), followed by a 30-min exposure to uv light (365 nm, ~2 cm). Proteins were then separated on SDS-PAGE, and gels were stained with Coomassie Brilliant Blue, dried, and exposed to x-ray film at −80 °C with an intensifier screen.

Proteolytic Digestion of Ryanodine Receptor—Cardiac and brain ryanodine receptor (~6 μg) or receptor labeled with [^{125}I]TID (~1 μg) in 160 μl of 10 mM sodium HEPES, pH 7.4, containing 0.8 mM $CaCl_2$, 0.36% CHAPS, 0.14% phosphatidylcholine, and 0.5 M NaCl were incubated with trypsin or α-chymotrypsin (1:50, enzyme:receptor, w/w) for 75 min on ice. Reactions were terminated by the addition of protease inhibitors and Laemmli sample buffer, followed by boiling for 2 min.

Materials—Horseradish peroxidase-conjugated secondary antibodies were from Boehringer Mannheim. Prestained molecular weight standards were from Bethesda Research Laboratories. [^{3}H]Ryanodine and $^{45}Ca^{2+}$ were from Du Pont-New England Nuclear, and [^{125}I]TID was from Amersham. Visiblot immunodetection system was from United States Biochemical Corp. Phosphatidylcholine, molecular weight standards, protein A-Sepharose, and heparin-agarose were from Sigma. All other chemicals were of reagent grade.

RESULTS

Ryanodine Binding Analysis on Purified Brain Ryanodine Receptor—A large number of compounds have been demonstrated to stimulate or inhibit the release of Ca^{2+} from skeletal and cardiac muscle sarcoplasmic reticulum (16), and several of these agents appear to affect ryanodine binding to brain membranes in a similar manner (38). We have previously purified the major ryanodine binding protein in brain using a combination of heparin-agarose chromatography and sucrose density gradient centrifugation (8). To characterize the brain receptor, we performed [^{3}H]ryanodine binding analysis on the purified protein in the presence of several of these agents to assess their interaction with the ryanodine receptor. The brain ryanodine receptor is sensitive to ATP, which can maximally increase ryanodine binding at 1 mM (from 4630 specific dpm/μg in the absence of ATP to 8564 specific dpm/μg at 1 mM ATP) (Fig. 1). This value is in good agreement with ATP effects on ryanodine binding to brain membranes (38). [^{3}H]Ryanodine binding to the purified brain receptor is also sensitive to changes in ionic strength as KCl can stimulate binding to 447% of control levels (from 2095 specific dpm/μg in the absence of KCl to 9364 dpm/μg in optimal KCl) (Fig. 1). The cardiac ryanodine receptor can be phosphorylated by cAMP-dependent protein kinase (39, 40), and Yoshida *et al.* (41) recently demonstrated that the brain ryanodine receptor can also be phosphorylated by this enzyme. We have determined that phosphorylation of the brain ryanodine receptor with protein kinase A leads to an increase in [^{3}H]ryanodine binding to the purified brain receptor to 174% of the levels for the nonphosphorylated form of the receptor (data not shown).

Sarcoplasmic reticulum Ca^{2+} release can be inhibited by several factors including Mg^{2+} and ruthenium red (16). These agents are also inhibitory to ryanodine binding to the purified brain ryanodine receptor (Fig. 1). With binding performed in the presence of 250 mM KCl, Mg^{2+} inhibits ryanodine binding with an IC_{50} of approximately 10 mM. With binding performed in the presence of 100 mM KCl, ruthenium red inhibits ryanodine binding with an IC_{50} of approximately 3 μM (Fig.

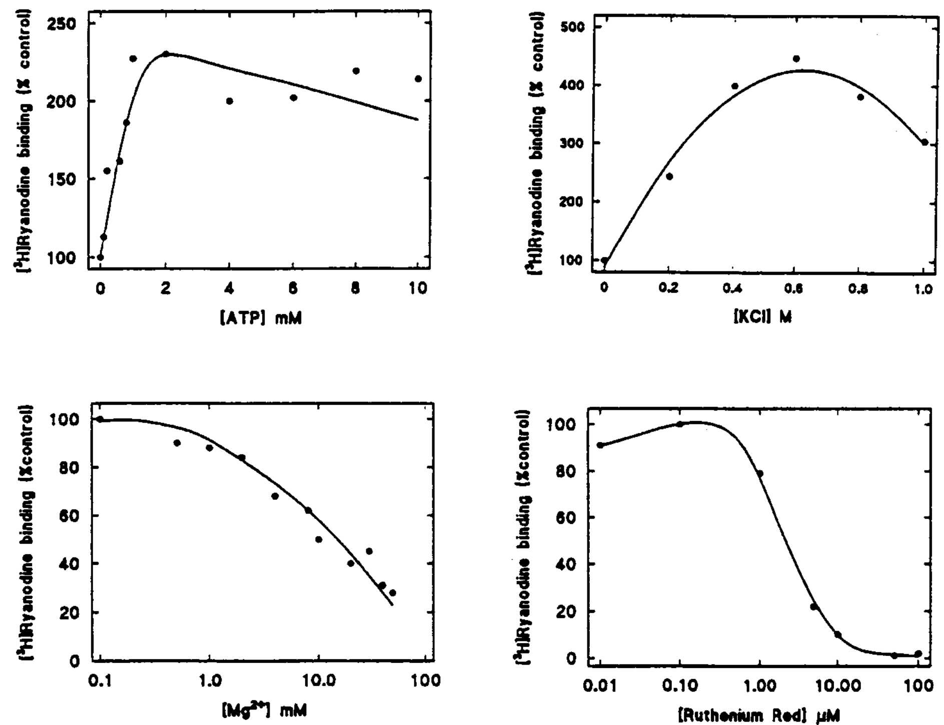

FIG. 1. **Analysis of ATP, KCl, Mg^{2+}, and ruthenium red on [^{3}H]ryanodine binding to purified brain ryanodine receptor.** [^{3}H]Ryanodine binding was performed as described under "Experimental Procedures." In all cases, the free Ca^{2+} concentration was maintained at 100 μM. The Mg^{2+} and ATP curves were performed in the presence of 250 mM KCl. The ruthenium red curve was performed in the presence of 100 mM KCl. The values for specific binding at 100% for each curve are as follows: ATP, 4,630 dpm/μg; KCl, 2,095 dpm/μg; Mg^{2+}, 12,801 dpm/μg; ruthenium red, 11,042 dpm/μg. The *curves* represent the average of three or more experiments.

1). When binding was performed in the presence of 1 M KCl, the ruthenium red dose-response curve was shifted approximately 1 full order of magnitude to the right (data not shown).

Ca^{2+} is an important regulator of sarcoplasmic reticulum Ca^{2+} release, and it is believed to be the "trigger" for Ca^{2+}-induced Ca^{2+} release in cardiac excitation-contraction coupling. Therefore, we examined the interaction of Ca^{2+} with the major form of ryanodine receptor purified from brain. Ca^{2+} increases ryanodine binding to the purified brain ryanodine receptor at concentrations between 50 nM and 100 μM, with half-maximal stimulation occurring at approximately 2 μM (Fig. 2). The resting free Ca^{2+} concentration in neurons is approximately 50 nM and rises to several hundred nanomolar during stimulation (3). Over this range, ryanodine binding rises from undetectable levels to near 40% maximal activation (Fig. 2), similar to what is seen for Ca^{2+} activation of sarcoplasmic reticulum Ca^{2+} release. These values are also in good agreement with Ca^{2+} activation of ryanodine-sensitive currents from brain membranes incorporated in planar lipid bilayers (42). However, the physiological significance of our data must be interpreted cautiously as the binding assays were performed in the presence of high ionic strength (1 M KCl). Fig. 2 also demonstrates that calmodulin inhibits [^{3}H]ryanodine binding to the pure brain receptor at concentrations between 100 nM and 5 μM.

Immunological Characterization of Brain Ryanodine Receptor Isoforms—Recent data suggest that the brain expresses at least three ryanodine receptor-like proteins, including skeletal and cardiac-like forms (32) and a third form which was initially isolated from epithelial cells (43), and is approximately 70% homologous to both the cardiac and skeletal ryanodine receptors (33). In order to determine which of these forms of ryanodine receptor is the major form expressed in brain, the purified brain ryanodine receptor, corresponding to the major [^{3}H]ryanodine binding protein in brain, was compared to ryanodine receptors purified from skeletal and cardiac muscle by SDS-PAGE followed by Coomassie Blue staining and immunoblot analysis. Ryanodine receptors from cardiac muscle and brain have an apparently identical mobility on SDS-PAGE, which is slightly faster than receptor from skeletal muscle (Fig. 3). Antibody rabbit-46 against the C-terminal 15 amino acids of the skeletal muscle ryanodine receptor recognizes all three proteins. Monoclonal (XA7) and polyclonal (GP-23) antibodies against the skeletal muscle ryanodine receptor recognize the skeletal muscle receptor specifically. Monoclonal antibodies against the cardiac ryanodine receptor (RyR-1, RyR-3) recognize the cardiac and brain ryanodine receptors, but do not recognize the skeletal receptor (Fig. 3). Interestingly, two novel polyclonal antibodies raised against the purified brain ryanodine receptor (GP-561, goat-43) also recognize the cardiac and brain receptors, but do not recognize the skeletal receptor. These data indicate that the major form of ryanodine receptor expressed in brain is immunologically distinct from the skeletal muscle ryanodine receptor, but is similar to the cardiac form.

Here we report the production of two novel polyclonal antibodies (GP-561, goat-43), the first antibodies raised directly against the brain ryanodine receptor. In an effort to further characterize these antibodies, as well as to confirm the immunological similarity of ryanodine receptors from cardiac muscle and brain, we performed immunoprecipitation assays after prelabeling with [^{3}H]ryanodine. Both antibodies are able to effectively precipitate ryanodine receptor from the two tissues (Fig. 4), although immunoprecipitation with goat-43 is less efficient than that seen with GP-561.

Structural Characterization of Cardiac and Brain Ryanodine Receptors—Based on the immunological characterization described above, it was not possible to determine if there are any structural differences between ryanodine receptor from cardiac muscle and brain. Therefore, we performed proteolytic digestions of the cardiac and brain ryanodine receptors in order to compare their structures. Partial digestion of purified cardiac and brain ryanodine receptors with trypsin and α-chymotrypsin reveals similar proteolytic patterns (Fig. 5), suggesting that the two proteins are similar in their primary structure. However, several of the proteolyzed fragments of

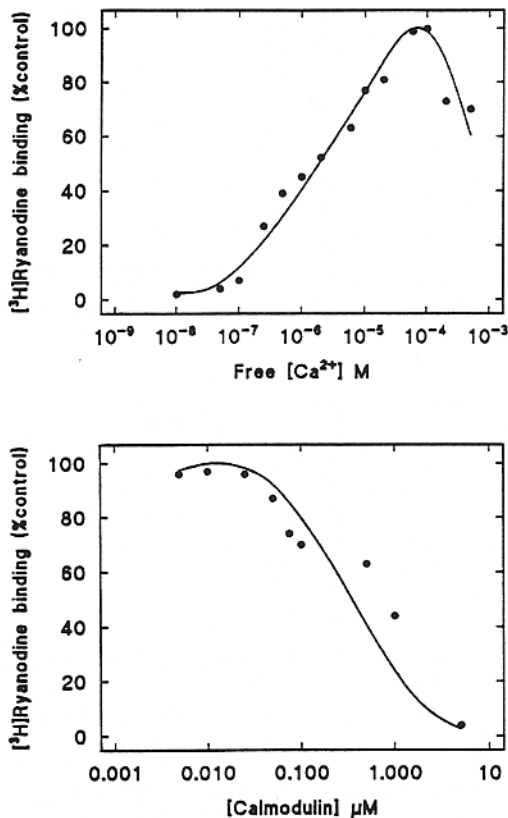

FIG. 2. **Analysis of Ca^{2+} and calmodulin effects on [^{3}H]ryanodine binding to the purified brain ryanodine receptor.** [^{3}H] Ryanodine binding was performed on purified ryanodine receptor in the presence of increasing free Ca^{2+} concentrations or increasing calmodulin concentrations. The KCl concentrations used in the Ca^{2+} curve and the calmodulin curve were 1 M and 250 mM, respectively. The values for specific binding at 100% for each curve were as follows: Ca^{2+}, 8,474 dpm/μg; calmodulin, 11,785 dpm/μg. The *curves* represent the average of three or more experiments with each experiment expressed as percentage of the maximal binding observed.

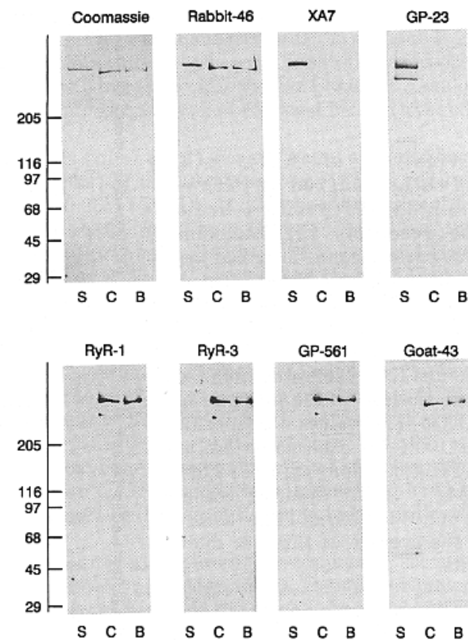

FIG. 3. **Western blot analysis of ryanodine receptor from skeletal and cardiac muscle and brain.** Ryanodine receptor was purified from skeletal muscle (*S*), cardiac muscle (*C*), and brain (*B*), and ~1 μg of each sample was run on SDS-PAGE. The samples were stained with Coomassie Brilliant Blue (Coomassie) or stained with antibodies against the skeletal ryanodine receptor (*Rabbit 46, XA7, GP-23*), the cardiac ryanodine receptor (*RyR-1, RyR-3*), or the brain ryanodine receptor (*GP-561, Goat-43*). The *band* at approximately 350 kDa in several samples is a known proteolytic fragment of the ryanodine receptor. The migratory position of molecular weight markers is indicated on the *left*.

the brain ryanodine receptor have slightly greater mobilities than the corresponding fragments of the cardiac receptor, indicating a subtle structural difference between the two proteins (Fig. 5). This difference in mobility was highly reproducible in six separate digestions. Analysis of proteolytic fragments of the skeletal muscle ryanodine receptor by antibodies has been used to successfully detect the single amino acid change between receptor from normal and malignant hyperthermic pigs (44, 45), indicating the power of this technique to detect subtle changes in protein structure. The greater mobility of the fragments of the brain ryanodine receptor may be due to a small amino acid deletion compared to the cardiac receptor, or it may be due to differences in post-translational modifications between the two proteins.

In an effort to characterize the membrane domains of the brain ryanodine receptor, we performed studies with the hydrophobic probe [^{125}I]TID. This probe readily partitions into hydrophobic domains of proteins, where it can be covalently attached by exposure to uv light (46). Ryanodine receptor from both cardiac muscle and brain are readily labeled with this probe to an identical level as determined by densitometric scanning of Coomassie-stained gels and autoradiographs (data not shown), indicating that the two proteins have a similar hydrophobic nature. Digestion of the labeled proteins with trypsin and α-chymotrypsin suggests that the brain ryanodine receptor has transmembrane domains similar to the cardiac ryanodine receptor, as the proteolytic patterns generated are similar (Fig. 6). However, the increased electrophoretic mobility of several of the fragments from the brain ryanodine receptor described above is again observed (Fig. 6). Taken together, these data suggest that the major form of ryanodine receptor expressed in brain is the cardiac form, although it may be subject to different translational or post-translational modifications.

DISCUSSION

This paper describes the characterization of the major form of ryanodine receptor purified from brain as a protein which is immunologically and structurally similar to the ryanodine receptor of cardiac muscle. Thus, although the brain appears to express several forms of ryanodine receptor (32, 33), it is likely that the cardiac form is the predominant regulator of Ca^{2+} release from caffeine-sensitive intracellular Ca^{2+} pools in neurons and that it functions by a process of highly regulated Ca^{2+}-induced Ca^{2+} release. Although the cardiac ryanodine receptor appears to be the major form of ryanodine receptor expressed in neurons, there are subtle differences in the structure of the protein expressed in heart and brain (Figs. 5 and 6). It has been suggested that the cardiac ryanodine receptor may be subject to alternative splicing (47), and it is interesting to speculate that the cardiac form of ryanodine receptor expressed in brain may be an alternatively spliced form of the protein expressed in heart.

The effects of Ca^{2+}, ATP, Mg^{2+}, and ruthenium red on [^{3}H] ryanodine binding to the purified brain ryanodine receptor reported here are similar to those observed for [^{3}H]ryanodine binding to brain membranes (38). It is important to study interactions of these agents with the pure receptor to confirm that the effects observed are due to allosteric regulation of

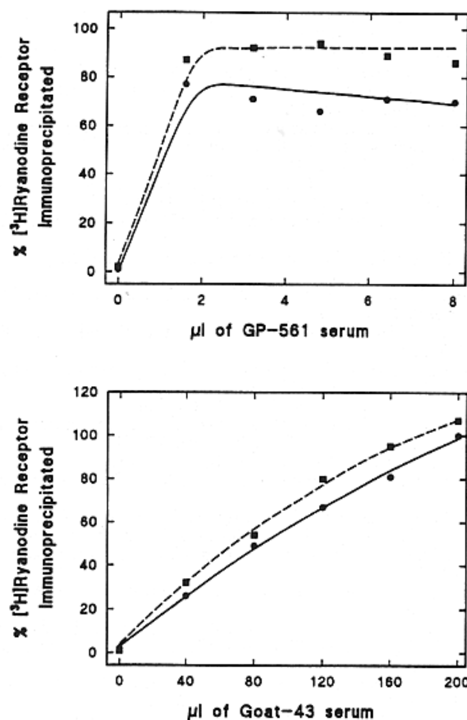

FIG. 4. **Immunoprecipitation of ryanodine receptor from cardiac muscle and brain.** Brain membranes (*circle, solid line*) and cardiac microsomes (*square, dashed line*) were labeled with [^{3}H] ryanodine in the absence (total) or presence (NS) of 10 μM unlabeled ryanodine, solubilized, and the solubilized extracts were incubated with protein A-Sepharose coupled to GP-561 or protein G-Sepharose coupled to goat-43. After incubation, the Sepharose beads were pelleted and washed, and the radioactivity on the beads for total and NS was determined. The amount of specific [^{3}H]ryanodine binding activity (disintegrations/min in beads incubated with total samples minus disintegrations/min in beads incubated with NS samples) precipitated by the beads is expressed as a percentage of the total specific [^{3}H]ryanodine binding activity added to the beads (determined by polyethylene glycol precipitation of total and NS aliquots). For GP-561, 5,992 specific dpm (cardiac) and 6,790 specific dpm (brain) were added per tube. For goat-43, 2,108 specific dpm (cardiac) and 1738 specific dpm (brain) were added per tube. The incubation tubes had increasing amounts of antibody-coupled Sepharose, but a constant amount of Sepharose.

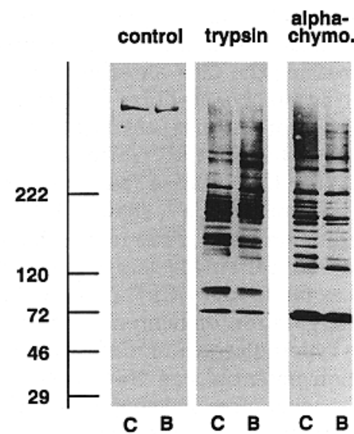

FIG. 5. **Proteolytic digestion of purified ryanodine receptor.** Purified ryanodine receptor from cardiac muscle (*C*) or brain (*B*) was fractionated on SDS-PAGE and stained with GP-561 before (*control*) or after proteolytic digestion with trypsin (*trypsin*) or α-chymotrypsin (*alpha-chymo*). Approximately 1 μg of protein was loaded per lane for control, whereas ~6 μg of protein was loaded per lane in the digested samples. The migratory position of molecular weight markers is indicated on the *left*.

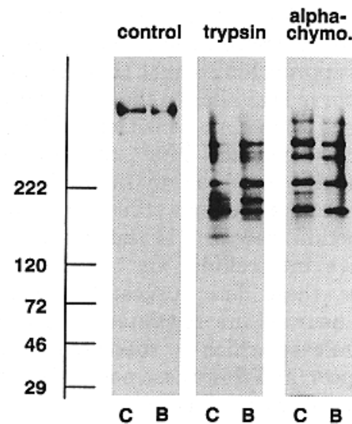

FIG. 6. **Proteolytic digestion of purified ryanodine receptor after hydrophobic labeling.** Purified ryanodine receptor from cardiac muscle (*C*) or brain (*B*) (approximately 1 μg of protein per lane) was labeled with the hydrophobic probe [^{125}I]TID, and the labeled samples were fractionated on SDS-PAGE before (*control*) or after proteolytic digestion with trypsin (*trypsin*) or α-chymotrypsin (*alpha-chymo*). Gels were dried and exposed to x-ray film. The migratory position of molecular weight markers is indicated on the *left*.

the protein and not due to secondary effects. For example, Ca^{2+} was initially shown to be inhibitory to IP_3 binding in brain membranes (48), but, upon purification of the protein, the Ca^{2+} inhibition was lost (49). It has recently been shown that Ca^{2+} inhibition of IP_3 binding in membranes is due to Ca^{2+} activation of phospholipase C and the generation of IP_3 (50). The binding studies performed here demonstrate that the brain ryanodine receptor has binding sites for Ca^{2+}, ATP, Mg^{2+}, and calmodulin as has been predicted for the cardiac ryanodine receptor from cDNA cloning (31, 47).

A notable finding of this study was the dramatic inhibition of ryanodine binding to the purified brain receptor by calmodulin. Previously, calmodulin had been demonstrated to inhibit Ca^{2+} release from sarcoplasmic reticulum of skeletal and cardiac muscle (51, 52). Here we demonstrate that calmodulin, at nanomolar concentrations, inhibits [^{3}H]ryanodine binding to purified receptor from brain (Fig. 2). Calmodulin is present in neurons at concentrations of greater than 1 μM (3). It is possible that calmodulin provides a negative feedback system to regulate intracellular Ca^{2+} concentrations. When Ca^{2+} is released from caffeine-sensitive internal stores, it could interact with calmodulin, leading to inhibition of the ryanodine receptor with a subsequent decrease in Ca^{2+} release. Unfortunately, it was not possible to test whether or not calmodulin was dependent on Ca^{2+} to inhibit the ryanodine receptor as Ca^{2+} must be present at all times for [^{3}H]ryanodine binding activity. However, such a Ca^{2+}/calmodulin feedback mechanism may possibly play a role in the phenomenon of Ca^{2+} oscillations.

Activation of the skeletal muscle ryanodine receptor/Ca^{2+} release channel is independent of extracellular Ca^{2+} (17, 18) and is possibly due to a direct interaction with the dihydropyridine receptor (19). In contrast, the signal for activation of the cardiac ryanodine receptor/Ca^{2+} release channel is Ca^{2+} itself, which functions in a process termed Ca^{2+}-induced Ca^{2+} release (18, 20, 21). Therefore, the ryanodine receptor/Ca^{2+} release channel is activated by membrane depolarization in skeletal muscle and by intracellular Ca^{2+} in cardiac muscle. In neurons, there is little evidence for intracellular Ca^{2+} release coupled to plasma membrane depolarization, but there is growing evidence of Ca^{2+}-induced Ca^{2+} release from intracellular membranes. In spinal neurons, Ca^{2+} influx through

voltage-gated channels causes a greater increase in intracellular Ca^{2+} concentrations than can be accounted for by influx alone, and decreasing the Ca^{2+} entering through these channels by decreasing the extracellular Ca^{2+} concentration does not affect the size of the intracellular Ca^{2+} response (53). These data suggest that the Ca^{2+} which enters the cell acts as a trigger for further Ca^{2+} release from internal stores which generates the bulk of the Ca^{2+} signal (53). In sympathetic neurons, ryanodine treatments can reduce, but do not abolish, Ca^{2+} transients from caffeine-sensitive stores elicited by Ca^{2+} influx (54). An example where Ca^{2+}-induced Ca^{2+} release may have a role in regulation of neuronal activity is the action potential after-hyperpolarization. Ca^{2+} which enters the cell during the action potential can lead to activation of Ca^{2+}-activated K^+ conductances causing hyperpolarization (55). This hyperpolarization can be potentiated by caffeine and attenuated by ryanodine, indicating that the source of Ca^{2+} to activate the K^+ channels is 2-fold, Ca^{2+} which enters from the outside and subsequent Ca^{2+}-activated Ca^{2+} release from a caffeine- and ryanodine-sensitive intracellular store (55).

In this study we have demonstrated that the cardiac form of the ryanodine receptor is the major form of ryanodine receptor expressed in the brain (Figs. 3–6). We have also demonstrated that Ca^{2+} can interact directly with purified ryanodine receptor from brain, activating ryanodine binding at nanomolar concentrations, but showing less activation at concentrations approaching 1 mM (Fig. 2). This bell-shaped Ca^{2+} response curve is similar to that seen for activation of a large conductance, ryanodine-sensitive Ca^{2+} channel found in cerebellum microsomes (42). $^{45}Ca^{2+}$ can also bind directly to the brain ryanodine receptor on Immobilon-P membranes (data not shown). In a previous study, we demonstrated that the brain ryanodine receptor is the gating mechanism for caffeine-sensitive intracellular Ca^{2+} stores in neurons (8). Taken together, these data suggest that caffeine-sensitive Ca^{2+} stores in neurons are regulated by a process of Ca^{2+}-induced Ca^{2+} release which is mediated by a cardiac-like ryanodine receptor, and that these pools play important roles in the regulation of neuronal activity.

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