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Purified Ryanodine Receptor from Skeletal Muscle Sarcoplasmic Reticulum Is the Ca^{2+}-permeable Pore of the Calcium Release Channel*

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The ryanodine receptor of rabbit skeletal muscle sarcoplasmic reticulum was purified by immunoaffinity chromatography as a single ~450,000-Da polypeptide and it was shown to mediate single channel activity identical to that of the ryanodine-treated Ca^{2+} release channel of the sarcoplasmic reticulum. The purified receptor had a [^{3}H]ryanodine binding capacity (B_{max}) of 280 pmol/mg and a binding affinity (K_d) of 9.0 nM. [^{3}H]Ryanodine binding to the purified receptor was stimulated by ATP and Ca^{2+} with a half-maximal stimulation at 1 mM and 8–9 μM, respectively. [^{3}H]Ryanodine binding to the purified receptor was inhibited by ruthenium red and high concentrations of Ca^{2+} with an IC_{50} of 2.5 μM and >1 mM, respectively.

Reconstitution of the purified receptor in planar lipid bilayers revealed the Ca^{2+} channel activity of the purified receptor. Like the native sarcoplasmic reticulum Ca^{2+} channels treated with ryanodine, the purified receptor channels were characterized by (i) the predominance of long open states insensitive to Mg^{2+} and ruthenium red, (ii) a main slope conductance of approximately 35 pS and a less frequent 22 pS substate in 54 mM *trans*-Ca^{2+} or Ba^{2+}, and (iii) a permeability ratio P_{Ba} or P_{Ca}/P_{Tris} = 8.7. The ~450,000-Da ryanodine receptor channel thus represents the long-term open "ryanodine-altered" state of the Ca^{2+} release channel from sarcoplasmic reticulum. We propose that the ryanodine receptor constitutes the physical pore that mediates Ca^{2+} release from the sarcoplasmic reticulum of skeletal muscle.

In skeletal muscle, Ca^{2+} release from the sarcoplasmic reticulum is initiated by, and coupled to, the depolarization and repolarization of the transverse tubular membrane (1–3). Since the identification of the sarcoplasmic reticulum as the ATP-dependent relaxing factor, a wealth of information has been accumulated about the mechanism of Ca^{2+} uptake by this organelle and the pump protein responsible for this uptake (4–6). In contrast, much less is known about the proteins which function in excitation-contraction coupling and/or Ca^{2+} release. An important advancement in this area has been the identification and recording of the Ca^{2+} release channel of sarcoplasmic reticulum using planar lipid bilayer techniques (7–10). A second and crucial observation is that the plant alkaloid ryanodine activates Ca^{2+} release from sarcoplasmic reticulum and binds with high affinity to a receptor localized to the junctional sarcoplasmic reticulum membrane (11–16). These findings have provided key functional and quantitative assays for the purification of the receptor/channel protein. Recently, the ryanodine receptor of the junctional sarcoplasmic reticulum membrane has been identified as a high molecular weight protein using a monoclonal antibody which is capable of specifically immunoprecipitating the [^{3}H] ryanodine-labeled receptor (17). Here, we report the copurification of the ryanodine receptor and a ~450,000-Da protein using a monoclonal antibody affinity column and the reconstitution of ryanodine receptor channels in planar lipid bilayers. A comparison based on a single channel conductance and ionic selectivity properties revealed that purified ryanodine receptor channels and ryanodine-treated Ca^{2+} release channels from terminal cisternae sarcoplasmic reticulum (7–10) are functionally identical. Thus, our results suggest that the ~450,000-Da junctional sarcoplasmic reticulum protein is the ryanodine receptor and that the pure receptor forms the Ca^{2+}-conducting pore component of the Ca^{2+} release channel.

EXPERIMENTAL PROCEDURES

Membrane Preparations—Triads were isolated from adult rabbit skeletal muscle according to Mitchell *et al.* (18) 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 PMSF[1] (0.23 mM). Light and heavy sarcoplasmic reticulum vesicles were isolated according to Campbell *et al.* (19) in the presence of protease inhibitors. All membrane preparations were stored frozen at −135 °C in 0.25 M sucrose, 10 mM histidine (pH 7.4), 0.83 mM benzamidine, 1 mM iodoacetamide, and 58 μM PMSF. Protein was measured using the method of Lowry *et al.* (20) as modified by Peterson (21), with bovine serum albumin as a standard.

Preparation of Anti-ryanodine Receptor Monoclonal Antibody (XA7)—Anti-ryanodine receptor monoclonal antibody (XA7) was prepared as described previously (17). Hybridoma cells were dilution-cloned and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Ascites fluid was produced by injecting 5×10^6 hybridoma cells intraperitoneally into Pristane-primed BALB/c mice. The ascites fluid was delipidated with Lipoclean reagent from Behring Diagnostic.

Preparation of Immunoaffinity Adsorbent (XA7-Sepharose)—Anti-ryanodine receptor monoclonal antibody (XA7) was precipitated from ascites fluid or culture medium with 50% saturated $(NH_4)_2SO_4$, pH 7.5. The precipitate was collected by centrifugation (20,000 × *g*, 30 min) and resuspended in 4 ml of 100 mM NaCl and 50 mM sodium

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

phosphate buffer, pH 7.4. The suspension was filtered with a Millex-HA (0.45-μm, Millipore) filter. The filtrate was applied to a Sepharose CL-4B column (ϕ 2.6 × 95 cm), and the column was eluted with the above buffer. The purified monoclonal antibody (XA7) was coupled to cyanogen bromide-activated Sepharose 4B (4–5 mg of protein/ml gel) to form the immunoaffinity adsorbent (XA7-Sepharose).

Immunoaffinity Purification of Ryanodine Receptor—Isolated triads (100 mg) were solubilized with 1% digitonin and 0.5 M NaCl in buffer A (0.5 M sucrose, 0.75 mM benzamidine, 0.1 mM PMSF, and 50 mM Tris-HCl, pH 7.4) at a protein concentration of 1 mg/ml in the presence of protease inhibitors: aprotinin (3.8 μg/ml), leupeptin (7.2 μg/ml), antipain (7.2 μg/ml), chymostatin (7.2 μg/ml), and pepstatin A (0.6 μg/ml). Solubilized triads were applied to the XA7-Sepharose column (6 ml) and recycled three times. The column was washed with 5 ml of buffer A containing 1% digitonin and 0.5 M NaCl, followed by 15 ml of 0.3% digitonin and 0.5 M NaCl in buffer A. Ryanodine receptor was eluted from the XA7-Sepharose column with 0.3% digitonin and 0.5 M NaSCN in buffer A. Immediately after the elution, dithiothreitol was added to the eluted ryanodine receptor fraction to a final concentration of 5 mM. The purified ryanodine receptor was applied to a Pharmacia Biotechnology, Inc. PD-10 column pre-equilibrated with 0.3% digitonin, 5 mM dithiothreitol, and 0.5 M NaCl in buffer A to remove the NaSCN.

[^{3}H]Ryanodine Binding Assay—Isolated triads (50 μg/ml) were incubated with 10 nM [^{3}H]ryanodine (54.7 Ci/mmol, Du Pont-New England Nuclear) for 1 h at 37 °C in a solution containing 150 mM KCl, 5 μM PMSF, 3 mM ATP, 260 μM $CaCl_2$ (20 μM free Ca^{2+}), 10 mM Na-HEPES, pH 7.4. The amount of [^{3}H]ryanodine bound was determined by membrane filtration as previously described in Refs. 11 and 13. Solubilized samples (5–100 μg/ml of protein) were incubated with 20 nM [^{3}H]ryanodine for 1 h at 37 °C in a solution containing 0.5 M KCl, 0.5 M sucrose, 2 mM dithiothreitol, 5 μM PMSF, 10 mM ATP, 1.1 mM $CaCl_2$ (60 μM free Ca^{2+}), and 50 mM Tris-HCl, pH 7.4. The amount of [^{3}H]ryanodine bound was determined by precipitation with polyethylene glycol as previously described (17). Nonspecific binding was determined in the presence of 1–10 μM unlabeled ryanodine.

SDS-Polyacrylamide Gel Electrophoresis—Gel electrophoresis was performed on 3–12 or 2–10% polyacrylamide gradient gels in the buffer system of Laemmli (22). After electrophoresis, gels were stained with Coomassie Blue and scanned by using a Hoefer GS 300 Scanning Densitometer. Molecular weight standards were: nebulin, ~500,000; myosin 200,000; phosphorylase *b*, 97,400; bovine albumin serum, 68,000; and ovalbumin, 43,000.

Planar Bilayer Methods—Calcium release channels from isolated sarcoplasmic reticulum membranes were incorporated into Mueller-Rudin planar bilayer membranes as described previously by Smith *et al.* (7). Briefly, planar lipid bilayers composed of phosphatidylethanolamine:phosphatidylserine (1:1, 50 mg/ml) in decane were formed on a 0.3-mm hole in a Lexan polycarbonate partition separating two aqueous filled chambers. Heavy sarcoplasmic reticulum vesicles stored in 0.3 M sucrose were added to one chamber, designated cis, containing 0.25 M choline chloride, 5 mM $CaCl_2$, 10 mM HEPES/Tris, pH 7.4. The other chamber, designated trans, was held at virtual ground and contained 50 mM choline chloride, 10 mM HEPES/Tris, pH 7.4. Fusion of membranes was monitored at 0 mV as discrete increases in Cl^--specific membrane current. After membrane insertion, both chambers were perfused with buffers containing impermeant monovalent cations and anions with Ca^{2+} or Ba^{2+} present as current carriers. The cis perfusion buffer contained 0.95 mM $CaCl_2$, 1 mM EGTA (1.2 μM free Ca^{2+}), 125 mM Tris base/250 mM HEPES, pH 7.4. The trans perfusion buffer was composed of 54 mM $Ca(OH)_2$ or $Ba(OH)_2$/250 mM HEPES, pH 7.4. Under these bi-ionic conditions, E_{Tris} is nominally minus infinity, $E_{divalent}$ is nominally plus infinity, and E_{HEPES} = 0 mV. Divalent conducting channels could be easily recognized as negative current deflections at 0-mV holding potential. Purified ryanodine receptor in buffer containing 0.3% digitonin was incorporated into bilayers formed from phosphatidylethanolamine:phosphatidylserine:cholesterol in 6:6:1 (w/w) ratio. Bilayer solutions used for incorporation and recording were identical to the perfusion buffers used for recording the native sarcoplasmic reticulum calcium release channels.

Ryanodine receptor channels appeared spontaneously after dilution of purified receptor into one or both of the bilayer chambers and were identified as divalent conducting channels by their positive reversal potential; *i.e.* channels conducted negative current at 0 mV (trans to cis cation flux) which extrapolated to zero current at positive potentials. Single channels were recorded from three separate preparations of purified receptor. The total recorded time digitally stored and analyzed was 2 h. Channel activity was recorded in virtually every attempt, provided that purified receptor and cholesterol containing bilayers were allowed to incubate together for 2 min or more. Breakage and reformation of the bilayer in the presence of purified receptor was found to facilitate insertion of channels during the incubation period. However, this technique was used only when channels did not appear after several minutes of incubation as it usually resulted in the incorporation of many channels simultaneously.

Recordings of native sarcoplasmic reticulum calcium release channels were filtered through an 8-pole low-pass Bessel filter at 700 or 500 Hz and digitized at 2 kHz for storage on hard disk. Ryanodine-modified native channels and purified receptor-derived channels were filtered at 200 and 100 Hz, respectively, and digitized at 500 Hz for storage on hard disk. Handling of single channel data has been described in detail elsewhere (Smith *et al.* (9)).

Materials—Ryanodine was obtained from Penick Corp. (Lindhurst, NJ). Cyanogen bromide-activated Sepharose 4B, Sepharose CL-4B, and molecular weight standards were purchased from Sigma. Digitonin was from Sigma or Fisher. Soluble digitonin was prepared by heating a 1.5% solution of digitonin to 95–100 °C for 10 min, allowing the solution to stand at room temperature for 2–3 days, and then removing the precipitation by filtration through a 0.22-μm filter. The filtrate was lyophilized and stored at room temperature. Bovine brain phosphatidylethanolamine and phosphatidylserine were obtained from Avanti Polar Lipids, Inc. All other reagents were analytical grade or the highest purity available.

RESULTS

Immunoaffinity Purification of Ryanodine Receptor—The high affinity ryanodine receptor from junctional sarcoplasmic reticulum of rabbit skeletal muscle has been purified using a monoclonal antibody affinity column (Fig. 1 and Table I). Monoclonal antibody XA7 can specifically immunoprecipitate [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads in a dose-dependent manner (17) and therefore was used in the formation of the affinity column. Monoclonal antibody XA7, which is an IgM immunoglobulin, was purified from ascites fluid or culture medium by ammonium sulfate precipitation and Sepharose CL-4B column chromatography. The purity of the monoclonal antibody was >95% based on SDS-polyacrylamide gel electrophoresis. The purified monoclonal antibody was coupled to cyanogen bromide activated-Sepharose 4B to form the immunoaffinity adsorbent (XA7-Sepharose) which was able to immunoprecipitate [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads.

Isolated rabbit skeletal muscle triads were used for the purification of the ryanodine receptor for the following reasons: 1) skeletal muscle triads are enriched in high affinity [^{3}H]ryanodine binding activity (10–20 pmol/mg); 2) the ~450,000-Da protein is enriched in isolated triads and constitutes 4–5% of the total triad protein; and 3) there is a high yield of triads from rabbit skeletal muscle (600–1000 mg/kg of tissue). Table I summarizes the purification of ryanodine receptor from digitonin-solubilized triads. Isolated triads were solubilized with 1% digitonin and 0.5 M NaCl in the presence of protease inhibitors and the extract was then incubated with XA7-Sepharose. Under these conditions, 80–90% of the [^{3}H]ryanodine binding activity in the isolated triads was solubilized and 50–80% of the [^{3}H]ryanodine binding activity in the extract was bound to XA7-Sepharose. The ryanodine receptor was eluted with 0.5 M NaSCN and 0.3% digitonin. The NaSCN-eluted ryanodine receptor was applied to a Pharmacia PD-10 column to remove NaSCN. The recovery of [^{3}H]ryanodine binding activity from the XA7-Sepharose column was 20–30%.

The composition of the purified ryanodine receptor was analyzed by SDS-polyacrylamide gel electrophoresis and was consistent with a single high molecular weight protein (Fig.

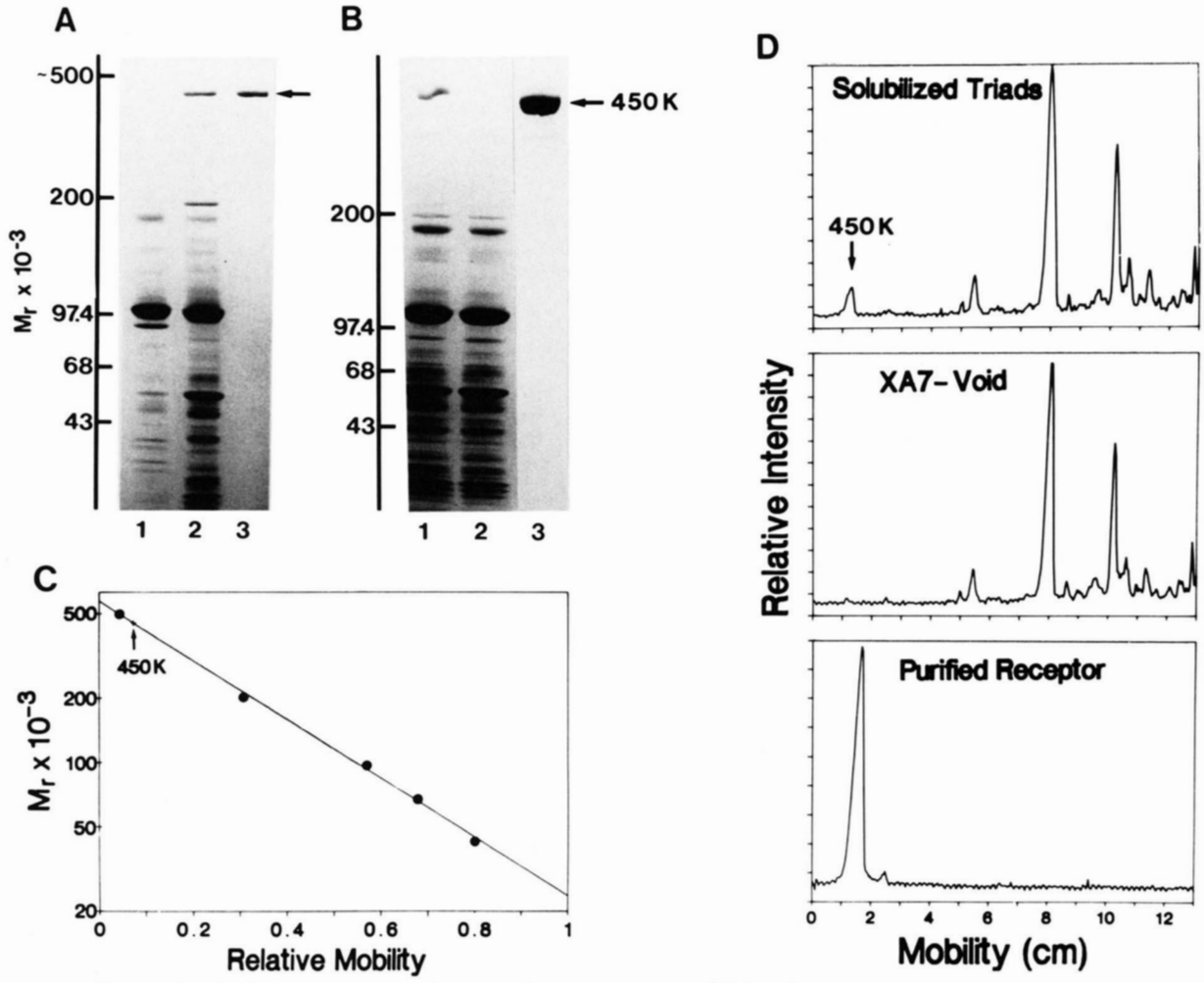

FIG. 1. **Analysis of the purified ryanodine receptor on SDS-polyacrylamide gel electrophoresis gradient gels.** Isolated membranes or purified receptor were subjected to 2–10% (*A*) or 3–12% (*B*) SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. *A*, 25 μg of light sarcoplasmic reticulum vesicles (*lane 1*); 25 μg of isolated triads (*lane 2*); and 2 μg of purified ryanodine receptor (*lane 3*). *B*, 30 μg of digitonin-solubilized triads (*lane 1*); 30 μg of void from XA7-Sepharose column (*lane 2*); and 8.5 μg of purified ryanodine receptor (*lane 3*). The ~450,000-Da protein (*450 K*) is indicated by an *arrow*. Specific [^{3}H]ryanodine binding activities of the samples were determined as described under "Experimental Procedures," and are: light sarcoplasmic reticulum vesicles, 0.2 pmol/mg; isolated triads, 10.7 pmol/mg; purified ryanodine receptor, 180 pmol/mg; and void from XA7-Sepharose, 1.6 pmol/mg. *C*, a plot of M_r of the molecular weight standards (nebulin, ~500,000; myosin, 200,000; phosphorylase b, 97,400; bovine serum albumin, 68,000; and ovalbumin, 43,000) *versus* relative mobility used to determine the M_r of the purified receptor proteins. *D*, densitometric scans of gel lanes 1, 2, and 3 in *B*.

1, *A* and *B*). The apparent molecular mass of ryanodine receptor protein on a 2–10% gradient gel was estimated at 450,000 Da using nebulin (~500,000), myosin (200,000), phosphorylase *b* (97,400), bovine serum albumin (68,000), and ovalbumin (43,000) as standards (Fig. 1*C*). The ~450,000-Da ryanodine receptor protein was enriched in isolated triads and absent in light sarcoplasmic reticulum vesicles, which are devoid of ryanodine binding activity (Fig. 1*A*). The ~450,000-Da protein and [^{3}H]ryanodine binding activity were specifically removed from the digitonin-solubilized triads by the XA7-Sepharose column (Fig. 1, *B* and *D*). Finally, the purity of the ryanodine receptor determined by gel scans was greater than 95% with a small amount (less than 5%) of contamination from a ~300,000-Da protein which is probably a proteolytic fragment of the ~450,000-Da protein (Fig. 1*D*).

[^{3}H]Ryanodine Binding to Isolated Triads and Purified Receptor—Binding of [^{3}H]ryanodine to the purified receptor was examined and compared to that in isolated triads (Fig. 2). Scatchard analysis of [^{3}H]ryanodine binding to isolated triads in the presence of 3 mM ATP and 20 μM Ca^{2+} (free) yields a straight line with an apparent B_{max} and K_d values of 15.9 pmol/mg and 6.8 nM, respectively. Scatchard analysis of [^{3}H]ryanodine binding to the purified receptor in the presence of 10 mM ATP and 60 μM Ca^{2+} (free) also yields a straight line with apparent B_{max} and K_d values of 280 pmol/mg and 9.0 nM, respectively. Thus, purification of the ryanodine receptor from digitonin-solubilized triads using the monoclonal antibody affinity column resulted in a 17.5-fold purification without any significant change in affinity for [^{3}H]ryanodine. Inasmuch as the size of the functional ryanodine binding unit is unknown (monomer, dimer, etc), the predicted enrichment of binding activity is best determined from gel scans of total triad proteins. Based on this argument, the enrichment of ryanodine binding from isolated triads should be 20–25-fold, because the content of ~450,000-Da protein in isolated triads is estimated to be 4–5% (Fig. 1*D*). Since isolated triads have

TABLE I

Purification of ryanodine receptor

The ryanodine receptor was purified from digitonin-solubilized triads as described under "Experimental Procedures." The values indicated here are from a typical experiment.

Fraction	Protein	Ryanodine binding[a]		
		Specific activity	Total activity	Yield
	mg	*pmol/mg*	*pmol*	%
Solubilized triads	64.9	12.0	782	100
XA7-Sepharose void fraction	61.5	2.6	158	20.3
NaSCN eluted fractions[b]				
Fraction 1	0.43	185	78.8	10.1
Fraction 2	0.64	162	104.0	13.2
Fraction 3	0.19	32.9	6.1	0.8

[a] The [^{3}H]ryanodine binding was measured as described under "Experimental Procedures."

[b] The protein concentrations and [^{3}H]ryanodine binding activities of these fractions were measured after the removal of NaSCN using a Pharmacia PD-10 column.

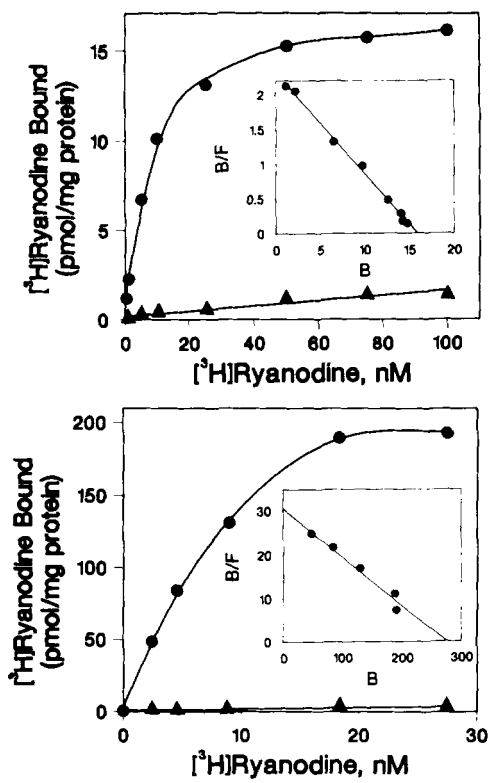

FIG. 2. **[^{3}H]Ryanodine binding to isolated triads and purified receptor.** Isolated triads (50 μg/ml, *upper panel*) or purified receptor (8.8 μg/ml, *lower panel*) were incubated for 1 h at 37 °C with 0.1–100 nM [^{3}H]ryanodine. Total [^{3}H]ryanodine binding (●) or nonspecific binding in the presence of 10 μM unlabeled ryanodine (▲) were determined as described under "Experimental Procedures." All experiments were performed in triplicate. Scatchard analysis of bound/free (B/F; pmol/mg/nM) *versus* bound (B; pmol/mg) for isolated *triads* (*upper panel, insert*) and purified receptor (*lower panel, insert*) yields apparent B_{max} and K_d values of 15.9 pmol/mg and 6.8 nM, respectively, for isolated triads and 280 pmol/mg and 9.0 nM, respectively, for the purified receptor.

a B_{max} ~16 pmol/mg for [^{3}H]ryanodine binding, a purified receptor would be predicted to bind 320–400 pmol of [^{3}H] ryanodine/mg of receptor. This assumption suggests that our purified receptor contains some inactive receptor. Analysis of the [^{3}H]ryanodine binding activity of the NaSCN eluted receptor indicated that initial fractions of eluted receptor were the most active while later fractions contained less active receptor (Table I). The purity of the ~450,000-Da protein in all eluted fractions determined by SDS-polyacrylamide gel electrophoresis was above 95%. Therefore, it appears that ryanodine receptor is being denatured with time of exposure to NaSCN.

The [^{3}H]ryanodine binding activity in isolated triads and purified receptor was specific, stimulated by millimolar ATP, inhibited by ruthenium red, and was Ca^{2+}-dependent (Fig. 3). Ryanodine inhibited [^{3}H]ryanodine binding to isolated triads and the purified receptor with an IC_{50} of 22 and 18 nM, respectively. ATP, an activator of Ca^{2+} release from junctional sarcoplasmic reticulum, stimulated [^{3}H]ryanodine binding to both isolated triads and the purified receptor. A half-maximal stimulation was observed at 1 mM ATP and the maximal stimulation was at 3 mM for both isolated triads or the purified receptor. Ruthenium red, an inhibitor of Ca^{2+} release from junctional sarcoplasmic reticulum, inhibits [^{3}H]ryanodine binding to isolated triads and the purified receptor. Nanomolar concentrations of ruthenium red (IC_{50} 8.0 nM) were effective in inhibiting [^{3}H]ryanodine binding to isolated triads but micromolar concentrations (IC_{50} 2.5 μM) were required for the purified receptor. [^{3}H]Ryanodine binding to both isolated triads and the purified receptor shows a dependence on free Ca^{2+}. Approximately 2–3 or 8–9 μM Ca^{2+} (free) is required for half-maximal stimulation of [^{3}H]ryanodine binding to isolated triads or the purified receptor, respectively. High concentrations of Ca^{2+} (free) inhibit [^{3}H]ryanodine binding to isolated triads with an IC_{50} of 55 μM but greater than 1 mM Ca^{2+} was required for inhibition of [^{3}H]ryanodine binding to the purified receptor. Finally, submillimolar concentrations of tetracaine, another inhibitor of Ca^{2+} release from the sarcoplasmic reticulum (23, 24), inhibits [^{3}H]ryanodine binding to both isolated triads and the purified receptor (not shown). Therefore, these results demonstrate that the purified ryanodine receptor exhibits all the binding properties expected for the high affinity ryanodine receptor of the Ca^{2+} release channel (7–9, 11, 12), but concentrations for ruthenium red and Ca^{2+} required for half-maximal inhibition of [^{3}H]ryanodine binding were higher than those required for isolated triads.

Single Ca^{2+} Release Channels Treated with Ryanodine—The single channel properties of the purified ryanodine receptor were compared to those of Ca^{2+} release channels reconstituted from native membranes. Fig. 4 shows typical gating behavior of native sarcoplasmic reticulum calcium channels in planar bilayers. Channels are only active in the presence of either micromolar free *cis*-(myoplasmic side) Ca^{2+} or millimolar *cis*-ATP. The unit conductance is approximately 100 pS with 54 mM Ca^{2+} as the current carrier (see current voltage curve in Fig. 6). As shown in Fig. 4, ryanodine added to the cytoplasmic face of the channel causes an abrupt change in both gating kinetics and unit conductance. In this study and others, ryanodine in the nanomolar range is shown to bind slowly to its receptor. Thus, in equilibrium binding experiments the alkaloid and receptor must be preincubated for long periods (11, 13, 16). However, we have found in single channel experiments, as was shown previously in $^{45}Ca^{2+}$ flux measurements (12), that micromolar ryanodine is required to induce these changes within 10–30 s after exposure. For example, following treatment with 7 μM ryanodine in Fig. 4, the channel shifts into a state characterized by long openings, brief closures, and reduced unitary conductance. The ryanodine modified channel has a main conductance state of approximately 38 pS (see current voltage curve in Fig. 6) but another lower conductance

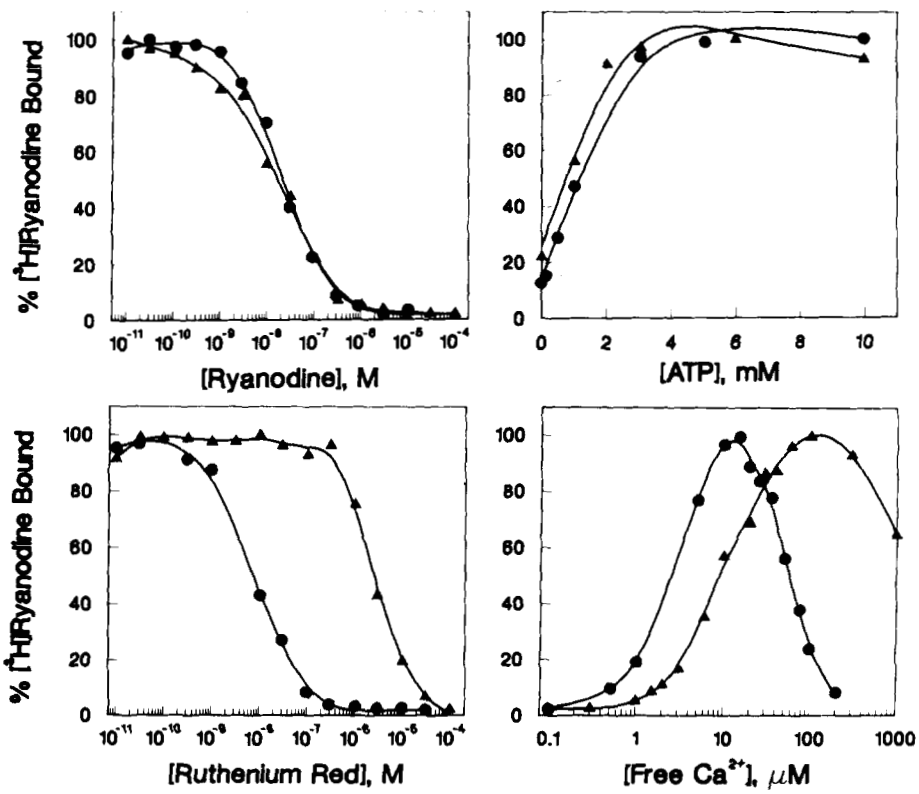

FIG. 3. **Effect of ryanodine, ruthenium red, ATP, and Ca^{2+} on ryanodine binding of isolated triads and purified receptor.** *Upper* and *lower left*: isolated triads (●) were incubated with 10 nM [^{3}H]ryanodine, 3 mM ATP, and 260 μM $CaCl_2$ (20 μM free Ca^{2+}) for 1 h at 37 °C in solution A (150 mM KCl, 5 μM PMSF, and 10 mM Na/HEPES, pH 7.4) in the absence or presence of various amounts of unlabeled ryanodine (*upper left*) or ruthenium red (*lower left*). Purified receptor (▲) was incubated with 10 nM [^{3}H]ryanodine, 10 mM ATP, and 1.1 mM $CaCl_2$ (60 μM free Ca^{2+}) for 1 h at 37 °C in solution B (0.5 M KCl, 0.5 M sucrose, 2 mM dithiothreitol, 5 μM PMSF, and 50 mM Tris-HCl, pH 7.4) in the absence or presence of various amounts of unlabeled ryanodine (*upper left*) or ruthenium red (*lower left*). *Upper right*: isolated triads (●) or purified receptor (▲) were incubated with 10 nM [^{3}H]ryanodine for 1 h at 37 °C in solution A for isolated triads or in solution B for purified receptor with various amounts of ATP. The concentration of free Ca^{2+} was maintained at 20 μM for isolated triads or 60 μM for purified receptor. *Lower right*: isolated triads (●) or purified receptor (▲) were incubated for 1 h at 37 °C with 10 nM [^{3}H]ryanodine and various concentrations of free Ca^{2+} in solution A containing 3 mM ATP or in solution B containing 10 mM ATP, respectively. The protein concentration in the incubation mixture was 50 μg/ml for isolated triads or 8.8 μg/ml for purified receptor. The [^{3}H]ryanodine binding was determined as described under "Experimental Procedures." All experiments were performed in triplicate.

substate of 24 pS is seen in the single channel records albeit less frequently (Fig. 4*B*). Although, *cis*-Mg^{2+} and ruthenium red are effective blockers of the native channels (7–9), the ryanodine-modified channels show almost no inhibition by micromolar ruthenium red and millimolar Mg^{2+}. Table II summarizes the effects of activating and inhibitory ligands on open probability in single channel recordings from native, ryanodine-treated, and receptor-derived sarcoplasmic reticulum calcium channels.

Single Channel Measurement of the Purified Receptor—Similar to the effects of ryanodine on native channels, Fig. 5 shows that purified ryanodine receptor exhibits spontaneous calcium channel activity when incorporated in planar lipid bilayers. Ryanodine receptor calcium channels were inserted into planar bilayers by the simple addition of digitonin-solubilized receptor to one chamber on either side of the planar bilayer. Detergent and protein concentrations in the aqueous phase that yielded channels were 0.0015–0.003% w/v and 0.5–1.0 μg/ml, respectively. Extensive control experiments showed that, at these levels, detergent without receptor protein was electrically silent. In bi-ionic recording solutions composed of 125 mM $Tris^+$ (cis myoplasmic side) and 54 mM Ca^{2+} or Ba^{2+} (trans intra sarcoplasmic reticulum ground side), channels were recognized as negative current deflections about 1 pA in amplitude at 0 mV. Ryanodine receptor channels were insensitive to inhibition by *cis*-Mg^{2+} and ruthenium red at concentrations of 10 mM and 25 μM, respectively (Table II). Purified receptor channels were characterized by the predominance of long open states with infrequent transitions into closed nonconducting states (see Fig. 5*A*). In Fig. 5*A*, single ryanodine receptor channels are shown inserting sequentially into the bilayer membrane. Channels displayed several subconductance states, the most common of which was a 22-pS state (0.5 pA at 0 mV) shown in Fig. 5, *A* and *B*. The last channel to become inserted in Fig. 5*A* first opens into a substate level which is the same showing in Fig. 5*B*, *lower O-mV record*. When Ba^{2+} was used as the current carrier, the occurrence of the 22-pS subconductance state was more frequent. A single channel current voltage relation constructed from the most frequently observed amplitudes resulted in slope conductance of 35 pS in either Ca^{2+}- or Ba^{2+}-containing buffers (Fig. 6, *circles*). Using an expression derived from constant field theory, the observed current reversal at +30 mV indicated a permeability ratio P_{Ba} or P_{Ca}/P_{Tris} = 8.74. In the same plot we have included mean amplitudes of native Ca^{2+} release channels (*squares*, 100 pS) and ryanodine modified native channels (*triangles*, 38 pS). The conductance of ryanodine-modified native channels and that of ryanodine receptor channels were indistinguishable. Both channels also appear to reverse at the same equilibrium potential, thus endowed with the same ionic selectivity. Selectivity data on native and purified channels are summarized in Table III. It is clear from Table III that all three channels, ryanodine receptors, ryanodine-modified, and native channels reverse at about 30 mV in 54 mM Ca^{2+}/125 mM Tris buffers. Thus the permeability ratio P_{Ca}/P_{Tris} is approximately 9 for all data.

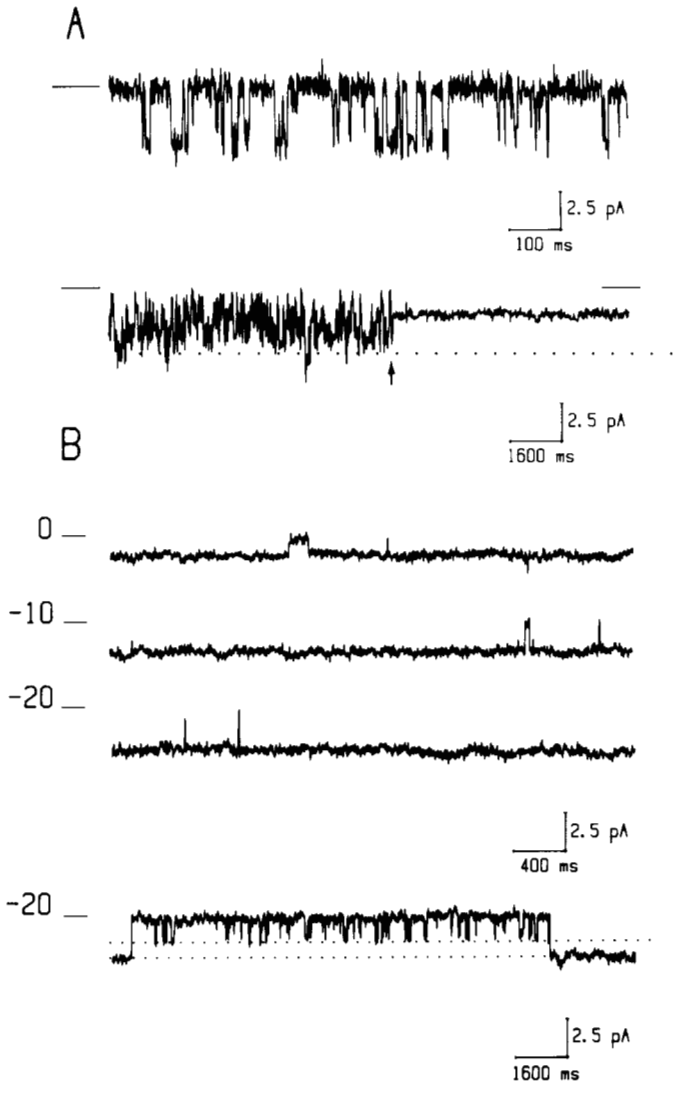

FIG. 4. **Ryanodine induces a long lived open channel substate in native sarcoplasmic reticulum calcium release channels.** Single sarcoplasmic reticulum channels were incorporated into bilayers as described under "Experimental Procedures." *Horizontal bars* indicate base-line current and *dotted lines* indicate open current level. *A, upper recording,* native sarcoplasmic reticulum calcium channel recorded at 0 mV with 54 mM *trans*-Ca^{2+} as the current carrier and 1.2 μM free Ca^{2+} (0.95 mM $CaCl_2$ and 1 mM EGTA) in the cis chamber. Openings are downward and have a unit amplitude of 3.7 ± 0.4 pA. This recording was filtered at 500 Hz. *A, lower recording,* in a different experiment a channel was recorded as above except that 1.2 μM free Ca^{2+} and 2 mM ATP were present in the cis chamber. Ryanodine (7 μM) was added to the cis chamber 10 s before the recording shown here. Conversion to the ryanodine-modified channel is indicated by the *arrow.* This recording was filtered at 80 Hz to clearly show the transition from normal to ryanodine-modified state. *B,* ryanodine-modified channel from experiment in *A* recorded at 0, −10, and −20 mV. *Lowermost record* (−20 mV) shows ryanodine-modified channel gating into a lower conductance substate. Ryanodine-modified channels were recorded at 200-Hz filter.

DISCUSSION

In this study we have purified the ryanodine receptor as a single ~450,000-Da polypeptide from digitonin-solubilized isolated triads using immunoaffinity column chromatography. The purified ryanodine receptor exhibits all the binding properties expected for the high affinity ryanodine receptor of the Ca^{2+} release channel. This purification results in a 17.5-fold purification without any significant change in affinity for [^{3}H]ryanodine. Our results demonstrated that the purified ~450,000-Da protein contains the high affinity binding site for ryanodine. The allosteric regulation of [^{3}H]ryanodine binding to the purified receptor strongly suggests that the sites for ATP and Ca^{2+}, which stimulate [^{3}H]ryanodine binding, are contained in the ~450,000-Da protein. The sites for ruthenium red and Ca^{2+}, which inhibit [^{3}H]ryanodine binding, also appear to be present in the ~450,000-Da protein but their affinities are lower. Finally, reconstitution of the purified ~450,000-Da protein in planar lipid bilayers revealed that this protein has Ca^{2+} channel activity.

The purified ryanodine receptor appears to be identical to the high molecular weight sarcoplasmic reticulum protein which we have previously identified as a junctional specific protein (17). The apparent molecular mass of the purified ryanodine receptor has been determined to be ~450,000 Da using several molecular weight standards including 500,000-Da chicken skeletal muscle nebulin (25). The high molecular weight protein in the junctional sarcoplasmic reticulum has been refered to as a ~350,000-Da protein by previous investigators (16, 26–29) including our laboratory (17). We believe that the correct molecular weight of the ryanodine receptor (and the high molecular weight sarcoplasmic reticulum protein) is closer to 450,000 since the purified receptor has a slightly faster mobility than nebulin and because previous molecular weight studies were probably inaccurate since a molecular weight standard larger than 350,000 Da was not used.

The high molecular weight junctional sarcoplasmic reticulum protein has been identified and characterized by several groups (26–29). Zorzato *et al.* (26) have shown that [^{14}C]doxorubicin, an activator of Ca^{2+} release, photolabels two high molecular weight junctional sarcoplasmic reticulum proteins (~170,000 and 350,000 Da). Caswell and co-workers (27, 28) suggested that this protein spans the gap of the triad junction. Seiler *et al.* (29) have identified this high molecular weight protein in junctional sarcoplasmic reticulum isolated from both cardiac and skeletal muscle. They have also reported that this protein is very sensitive to Ca^{2+}-activated protease. Recently, Inui *et al.* (16) have reported the 20-fold purification (B_{max} = 393 pmol/mg, K_d = 78 nM) of the ryanodine receptor from junctional terminal cisternae of rabbit skeletal muscle sarcoplasmic reticulum using heparin-agarose and hydroxylapatite chromatography in the presence of CHAPS. Their purified ryanodine receptor contains three major polypeptides of M_r 360,000, 330,000, and 175,000, and resembles the "sarcoplasmic reticulum feet" structures. In our studies we have found that monoclonal and polyclonal antibodies against the ~450,000-Da protein stained proteins of ~300,000 and 170,000 Da in addition to 450,000-Da protein on nitrocellulose transfers of some isolated triad preparations. However, the ~300,000- and 170,000-Da proteins were not detected in fresh triads prepared using a mixture of protease inhibitors. Therefore, it is possible that the 360,000-Da protein in their purified ryanodine receptor is identical to the ~450,000-Da protein, and the lower molecular weight proteins (330,000 and 170,000 Da) in their preparations of receptor are proteolytic fragments of the ~450,000-Da protein. This would imply that the ~450,000-Da protein is responsible for the sarcoplasmic reticulum feet structures.

Single calcium channels derived from purified ryanodine receptor appear to be similar in conductance and selectivity to ryanodine-modified Ca^{2+} release channels but are kinetically and pharmacologically different from the native Ca^{2+} release channel described previously (7–10). Ryanodine receptor channels open spontaneously in the absence of Ca^{2+} or

TABLE II

Ligand-dependent activation and inhibition of ryanodine receptor and native Ca^{2+} release channels

P_o (control) corresponds to the fraction of open time measured in the absence of added ATP and Ca^{2+} and in the presence of 1–10 mM EGTA. P_o (test) corresponds to the fraction of open time measured in the indicated concentration range of activator ligands, free Ca^{2+}, ATP and inhibitors, free Mg^{2+}, and ruthenium red (RR). Tabulated is the range of P_o (test) for the low and high range of ligands. All additions are to the cis side, 54 mM Ca/HEPES or Ba/HEPES trans. Holding potential, 0 mV.

	P_o (control) ATP (0 mM) + Ca^{2+} (<10 nM)	P_o (test) ATP (1–10 mM) + Ca^{2+} (1.2–2.5 μM)	P_o (test) ATP (1–10 mM) + Ca^{2+} (1.2–2.5 μM) + Mg^{2+} (4–10 mM)	P_o (test) ATP (1–10 mM) + RR (1–25 μM)
Ryanodine receptor channel[a]	0.9–0.98	0.9–0.98	0.9–0.98	0.9–0.98
Ca^{2+} release channel[a,b,c,d]	0.002–0.15	>0.99	0.1–0.25	0.01–0.083
Ca^{2+} release channel treated with ryanodine[a,e]	0.93–0.95	>0.99	(0.9[e])	(>0.99[e])

[a] Present data. [b] Ref. 7. [c] Ref. 8. [d] Ref. 9. [e] Ref. 30.

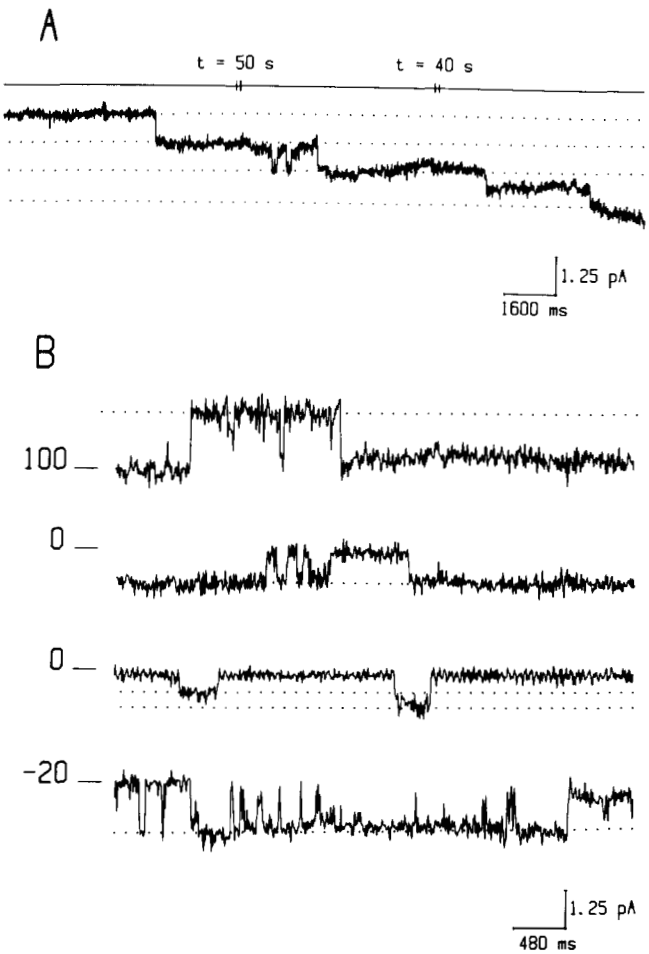

FIG. 5. **Ryanodine receptor calcium channels recorded in planar lipid bilayers.** *Horizontal bars* indicate closed current and *dotted lines* indicate open channel levels. *A*, sequential incorporation of ryanodine receptor channels at 0 mV in 125 mM Tris/54 mM Ca^{2+} buffer plus 1 mM EGTA, 2 mM ATP, and 10 mM $MgCl_2$ added to the cis chamber. Interruptions in base line indicate periods of time elapsed between channel insertions. *B*, selected single channel records show channels recorded in 125 mM Tris/54 mM Ba^{2+} buffer plus 1 mM EGTA added to the cis chamber, at 100, 0, and −20 mV. *Lower* 0-mV recording shows channel gating in two substates. Records were filtered through a low-pass filter at 100 Hz and digitized at 500 Hz.

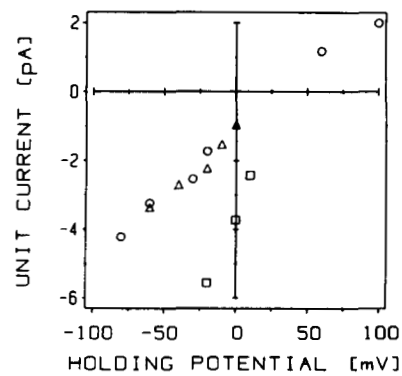

FIG. 6. **Single channel current *versus* voltage relationship of native, ryanodine-treated, and ryanodine receptor-derived sarcoplasmic reticulum calcium channels.** Single channel current *versus* voltage relationship of native sarcoplasmic reticulum calcium channels (□) are recorded as in Fig. 4*A*, *upper recording*, ryanodine-modified native channels (△) as in Fig. 4*B*, and purified ryanodine receptor (○) as in Fig. 5*B*. Linear regression of the three data sets gives slope conductance of 100 ± 9 pS (□), 38 ± 4 pS (△), and 35 ± 2 pS (○). Reversal potential for all data is +30 mV.

TABLE III

Ion selectivity properties of ryanodine receptor and native Ca^{2+} release channels

Single channel conductance corresponds to the slope value at 0 mV, 54 mM *trans*-Ca^{2+}, 125 mM *cis*-$Tris^+$, 1–2.5 μM free Ca^{2+}; * denotes the main conductance level; i_{Ca}/i_{Ba} corresponds to the single channel current ratio in 54 mM *trans* divalent, 125 mM *cis*-$Tris,^+$ at 0 mV; P_{Ca}/P_{Ba} corresponds to the Goldman permeability ratio calculated assuming P (anion) = 0, *cis* $[Ca^{2+}] = 0$.

	Single channel conductance	i_{Ca}/i_{Ba}	P_{Ca}/P_{Tris}
	pS		
Ryanodine receptor channel[a]	22; 35* ± 2	1.0	9 ± 2
Ca^{2+} release channel[a,b,c,d]	100* ± 9	0.7–0.75	8.7
Ca^{2+} release channel treated with ryanodine[a,e]	24; 38* ± 4	1.0	9 ± 2

[a] Present results. [b] Ref. 7. [c] Ref. 8. [d] Ref. 9 [e] Ref. 30.

adenine nucleotide. Although the binding data of Fig. 3 shows that regulatory sites for Ca^{2+}, ATP, and ruthenium red are present in the purified receptor, clearly these sites are not expressed functionally in the purified receptor channel. Previous work has shown that ryanodine opens release channels and renders $^{45}Ca^{2+}$ flux insensitive to activation by Ca^{2+} and ATP as well as to inhibition by Mg^{2+} and ruthenium red (11, 12). In planar bilayers, micromolar ryanodine induced a long-lived subconductance state that was unresponsive to additions of Ca^{2+}, Mg^{2+}, ATP, or ruthenium red (see Figs. 4 and 5, Table II, and Ref. 30). At first sight it would appear from the binding data (Fig. 3) that ruthenium red in the concentration range used here (up to 25 μM, see Table II) should have blocked ryanodine-opened native channels because 10 μM ruthenium red is sufficient to inhibit 50% of ryanodine binding in native triads. However, there are several factors that

make binding data (Fig. 3) and single channel data (Table II) not quantitatively comparable. First, ryanodine in the micromolar range was used in single channel recordings since, as mentioned under "Results," the association rate of the alkaloid is too slow to be of practical use when present in the nanomolar range (11, 13, 16). This high concentration of ryanodine offsets the rest of the competing ligands. Second, the dissociation rate of bound ryanodine is also exceedingly slow. The time constant of bound ryanodine is about 30 h (15). Hence, when ryanodine is bound to a channel, the probability of observing effects of other ligands, *i.e.* the probability of displacing ryanodine, are virtually null. Third, and most important, protocols in binding and single channel experiments are necessarily different. In single channel experiments, ryanodine was bound first and ligands were added afterwards. In binding experiments, ruthenium red and radiolabeled ryanodine were simultaneously added. Therefore, in binding experiments ligands compete for occupancy of ryanodine-free receptors while in single channel experiments ligands compete for occupancy of ryanodine-bound receptors.

Quantitatively, the purified receptor mimics the effects seen with ryanodine on native release channels. As summarized in Table III, both channels share the same set of conductance states which are lower than that of the native channel, a reduced Ba^{2+}/Ca^{2+} selectivity ratio when compared to native channels, and a similar Ca/Tris permeability. In addition, both channels exhibit long-term open kinetics which is ligand-insensitive (Table II). This similarity is present in spite of the fact that, for single channel recording experiments, receptor was purified from freshly prepared triads by using an antibody affinity column in the absence of radiolabeled ryanodine. Therefore, it appears that, in our purified receptor preparation, the low conductance, ligand-insensitive state (usually associated to bound ryanodine) has become stabilized in the absence of ryanodine.

There are several explanations we have considered in order to account for the fact that the conductive properties of ryanodine-modified and purified receptors are very similar (Table III). One hypothesis is that the ryanodine receptor forms the elementary calcium permeable pore in the sarcoplasmic reticulum Ca^{2+} release channel, and contributions from closely associated junctional proteins are necessary to confer the ligand sensitivity associated with native channels. Bound ryanodine would strip the receptor from regulatory components and thus the purified receptor and ryanodine treatment of native channels would elicit the same unregulated activity. Proteins, triad-specific or otherwise, that could fulfill this role have been described (17, 19, 27, 28, 31). A second explanation is that the ligand-insensitive state induced by ryanodine represents a low aggregation number of the receptor, perhaps a monomer, which is the same aggregate most easily inserted into planar bilayers under the conditions used here. Evidence obtained from gel filtration and electron microscopy indicates that the ryanodine receptor protein may form large molecular weight aggregates but the size of the ryanodine binding unit or the channel unit is presently unknown (15, 16). The third explanation is that the presence of low concentrations of digitonin (not removed by the dilution procedure) and/or partial denaturation of the receptor when eluted from the antibody affinity column may have contributed to stabilizing the ligand-insensitive state. The latter possibility is not exclusive of the aggregation hypothesis for it is clear that high ionic strength (used during elution of the receptor) and mild detergent conditions may change the average aggregate size. In spite of these uncertainties, the biophysical evidence gathered in Tables II and III strongly supports the conclusion that the purified ryanodine receptor, by being similar to ryanodine-modified native channels, is the calcium-permeable pore component of the sarcoplasmic reticulum release channel (7–10) and not a separate unrelated channel. A definite answer must await full recovery of conductive and gating properties.

In conclusion, the data presented demonstrate that the purified ryanodine receptor consists of a single ~450,000-Da polypeptide which is capable of forming the Ca^{2+}-conducting pore of the sarcoplasmic reticulum Ca^{2+} release channel. Although further experiments are required to identify the exact role of the ~450,000-Da protein in excitation-contraction coupling, our results strongly suggest that the ~450,000-Da protein is an important component of the sarcoplasmic reticulum Ca^{2+} release channel. Finally, the relatively high molecular weight of the purified ryanodine receptor presents the possibility that the ~450,000-Da protein could be the gene product in Duchenne muscular dystrophy since the gene responsible for Duchenne muscular dystrophy (32) most likely encodes for a very high molecular weight protein (~500,000 Da).

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