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Identification and Characterization of the High Affinity [^{3}H]Ryanodine Receptor of the Junctional Sarcoplasmic Reticulum Ca^{2+} Release Channel*

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Kevin P. Campbell‡§, C. Michael Knudson‡, Toshiaki Imagawa‡¶, Albert T. Leung‡‖, John L. Sutko‡‡, Steven D. Kahl‡, Carol Reynolds Raab‡, and Linda Madson‡**

*From the ‡Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242 and the **Department of Pharmacology, University of Nevada Medical School, Howard Medical Science Building, Reno, Nevada 89557*

The high affinity ryanodine receptor of the Ca^{2+} release channel from junctional sarcoplasmic reticulum of rabbit skeletal muscle has been identified and characterized using monoclonal antibodies. Anti-ryanodine receptor monoclonal antibody XA7 specifically immunoprecipitated [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads in a dose-dependent manner. [^{3}H]Ryanodine binding to the immunoprecipitated receptor from unlabeled digitonin-solubilized triads was specific, Ca^{2+}-dependent, stimulated by millimolar ATP, and inhibited by micromolar ruthenium red. Indirect immunoperoxidase staining of nitrocellulose blots of various skeletal muscle membrane fractions has demonstrated that anti-ryanodine receptor monoclonal antibody XA7 recognizes a high molecular weight protein (~350,000 Da) which is enriched in isolated triads but absent from light sarcoplasmic reticulum vesicles and transverse tubular membrane vesicles. Thus, our results demonstrate that monoclonal antibodies to the ~350,000-Da junctional sarcoplasmic reticulum protein immunoprecipitated the ryanodine receptor with properties identical to those expected for the ryanodine receptor of the Ca^{2+} release channel.

The mechanism by which the depolarization of the transverse tubular membrane triggers Ca^{2+} release from the sarcoplasmic reticulum is the least understood process in skeletal muscle excitation-contraction coupling (1-3). Ca^{2+} release from isolated sarcoplasmic reticulum vesicles has been extensively studied in recent years (4-15). Ruthenium red (4, 9, 11) or high concentrations of ryanodine (8, 11, 13, 15) have been shown to inhibit Ca^{2+} release while millimolar adenine nucleotides (5, 9) or low concentrations of ryanodine stimulate Ca^{2+} release (12, 13). [^{3}H]Ryanodine has been shown to bind with high affinity and specificity to heavy or junctional sarcoplasmic reticulum vesicles (12, 15-17). The high affinity ryanodine receptor appears to be an integral component of the sarcoplasmic reticulum Ca^{2+} release channel since ruthenium red, an inhibitor of Ca^{2+} release, inhibits [^{3}H]ryanodine binding while adenine nucleotides, activators of Ca^{2+} release, stimulate [^{3}H]ryanodine binding (15-17). Sarcoplasmic reticulum Ca^{2+} release channels have been recently identified in planar lipid bilayers on the basis of their activation by adenine nucleotides, blockade by ruthenium red, and selectivity for divalent cations (18, 19). Although the properties of the sarcoplasmic reticulum Ca^{2+} release channels are currently being examined, little is known about the protein component(s) or the structure of the Ca^{2+} release channel.

In this report, monoclonal antibodies have been used to identify and characterize the high affinity ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. Our results demonstrate that monoclonal antibodies capable of specifically immunoprecipitating the high affinity [^{3}H] ryanodine-labeled receptor from digitonin-solubilized triads recognize a high molecular weight protein (~350,000 Da) which is enriched in the junctional sarcoplasmic reticulum membrane.

EXPERIMENTAL PROCEDURES

Preparation of Skeletal Muscle Membranes—Rabbit skeletal muscle triads were isolated according to the procedure of Mitchell *et al.* (20). Light and heavy sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle as described by Campbell *et al.* (21). Transverse tubular membrane vesicles were isolated from skeletal muscle triads using the French press treatment of Lau *et al.* (22). All of the membrane preparations were carried out 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) and stored frozen at −135 °C in 0.25 M sucrose, 10 mM histidine (pH 7.4). Protein was determined by the method of Lowry *et al.* (23) as modified by Peterson (24). [^{3}H]Ryanodine (25) binding to isolated membranes was measured as previously described (12, 15).

Preparation of Junctional-specific Monoclonal Antibodies—Five- to six-week-old female BALB/c mice were immunized intraperitoneally with 0.5 mg of rabbit skeletal muscle triads emulsified in Freund's complete adjuvant. After 4 weeks, the immunization was repeated three or four times at 2-week intervals with the same amount of triads in Freund's incomplete adjuvant. Hybridoma cells were produced by fusion of spleen cells with NS-1 myeloma cells (26). Hybridoma supernatants were screened against light sarcoplasmic reticulum vesicles, skeletal muscle triads, and transverse tubular vesicles using an immunodot assay (27) with Millititer plates (Millipore). Positive colonies were those producing antibodies against the isolated triads (enriched in junctional sarcoplasmic reticulum membrane) and not with light sarcoplasmic reticulum or transverse tubular membranes (devoid of junctional sarcoplasmic reticulum membrane). Positive hybridomas were grown, dilution-cloned, and passaged in RPMI 1640 medium supplemented with 10% fetal bovine serum. Ascites fluid was produced by injecting 5×10^6 hybridoma cells intraperito-

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§ Established Investigator of the American Heart Association. To whom correspondence should be addressed.

¶ Muscular Dystrophy Association Postdoctoral Fellow.

‖ American Heart Association Medical Student Research Fellow (1985-86) and Iowa Graduate Fellow.

‡‡ Established Investigator of the American Heart Association.

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; pristane, 2,6,10,14-tetramethylpentadecane; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; GAM, goat anti-mouse; PBS, 50 mM NaH_2PO_4, 0.9% NaCl, pH 7.4; mAb, monoclonal antibody; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

neally into Pristane-primed BALB/c mice. The ascites fluid was delipidated with Lipoclean reagent from Behring Diagnostics.

Immunoprecipitation Assay for Anti-ryanodine Receptor Antibodies—Goat anti-mouse IgG Sepharose beads (Cooper Biomedical, Inc.) were diluted with Sepharose CL-4B beads to an IgG binding capacity of 1 mg/ml and then washed with 1% BSA in Buffer A (100 mM NaCl, 50 mM Tris, pH 7.4). Hybridoma supernatants (15 bed volumes) were incubated with the beads overnight at 4 °C with gentle mixing. The supernatant was then removed and the beads were washed twice with Buffer A. Triads (2 mg/ml) were pre-equilibrated with 20 nM [^{3}H]ryanodine at 37 °C for 60 min in a solution containing 150 mM KCl, 10 mM HEPES (pH 7.4), 5 μM PMSF, 3 mM ATP, and 20 μM Ca^{2+} (free) and then solubilized at 1.0 mg/ml with 1% digitonin in 0.5 M NaCl, 50 mM Tris-HCl (pH 7.4) for 45 min at 0 °C. The suspension was then centrifuged at 160,000 × *g* for 30 min to obtain the digitonin-solubilized [^{3}H]ryanodine-labeled triads. The solubilized [^{3}H]ryanodine-labeled receptor was assayed by precipitation with PEG in the presence of carrier proteins as described for the dihydropyridine receptor by Glossman and Ferry (28). Immunoprecipitates were obtained by incubation of 100 μl of digitonin-solubilized [^{3}H]ryanodine-labeled triads with 100 μl of monoclonal antibody beads for 2 h at 0–4 °C. The mixture was then centrifuged in an Eppendorf centrifuge and the supernatants were removed and assayed for the disappearance of [^{3}H]ryanodine binding activity using the PEG precipitation assay. Sepharose beads with bound immunoprecipitates were washed three times with binding buffer containing 0.3% digitonin and then counted in a liquid scintillation counter. [^{3}H] Ryanodine binding to the immunoprecipitated receptor from unlabeled digitonin-solubilized triads was measured in a solution containing 150 mM KCl, 10 mM HEPES (pH 7.4), 5 μM PMSF, 3 mM ATP, 20 μM Ca^{2+} (free), and 10 nM [^{3}H]ryanodine for 1 h at 37 °C. The immunoprecipitated receptor bound to Sepharose beads was washed as described above for prelabeled receptor and then counted. All assays were run in triplicate.

Immunoblot Assay—Light sarcoplasmic reticulum vesicles, transverse tubular membranes, and isolated triads (50 μg) were separated on 3–12% SDS-PAGE (29) gels and transferred to nitrocellulose membranes using a modification of the procedure of Towbin *et al.* (30). BLOTTO (Bovine Lacto Transfer Technique Optimizer/PBS/5% nonfat dry milk) (31) was used for blocking the nitrocellulose transfers and dilution of antibodies. Nitrocellulose blots were first incubated with hybridoma supernatant (1:20 dilution) and then incubated with goat anti-mouse IgG peroxidase linked secondary antibody (Cooper Biomedical, Inc.) at 1:1000 dilution. 4-Chloro-1-naphthol was used for color development.

RESULTS AND DISCUSSION

Monoclonal antibodies capable of specifically immunoprecipitating the [^{3}H]ryanodine-labeled receptor of the junctional sarcoplasmic reticulum from rabbit skeletal muscle have been produced. Mice were immunized with rabbit skeletal muscle triads which are a highly enriched source of the ryanodine receptor (10–20 pmol/mg of vesicles). Immunoprecipitation experiments with antiserum from mice immunized with isolated triads demonstrated the presence of antibodies that precipitated the [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads (Table I). Immunoblot analysis demonstrated that the mice were producing antibodies to all the major sarcoplasmic reticulum proteins (not shown). Since the [^{3}H]ryanodine receptor is enriched in the junctional sarcoplasmic reticulum membrane, hybridoma supernatants were initially screened for antibodies to the junctional sarcoplasmic reticulum membrane using a triple immunodot assay with light sarcoplasmic reticulum vesicles, transverse tubular membrane vesicles, and isolated triads (Fig. 1). Hybridoma supernatants that reacted with light sarcoplasmic reticulum vesicles and/or transverse tubular membrane vesicles (two preparations that are devoid of ryanodine receptor) were eliminated from further testing. A hybridoma supernatant was considered positive in the immunodot assay if it reacted with triad membranes, but showed no reactivity with light sarcoplasmic reticulum vesicles and transverse tubular vesicles (Fig. 1). The serum from the immunized mouse used for the fusion was used as a control in each screening and was shown to be strongly reactive against all the antigens (not shown).

TABLE I

Immunoprecipitation of [^{3}H]ryanodine-labeled receptor using various junctional-specific monoclonal antibodies

Controls		Amount of [^{3}H]ryanodine-labeled receptor[a]
		fmol
GAM-IgG beads		3.4 ± 0.2
GAM-IgG beads preincubated with immunized mouse serum		57.2 ± 5.1
Junctional-specific monoclonal antibody beads		**Amount of [^{3}H]ryanodine-labeled receptor**
mAb	Epitope	
	kDa	*fmol*
XA7	~350	194.8 ± 10.6
VIIH4$_2$	~350	91.4 ± 9.1
IIIB9	~300	3.4 ± 0.2
IXA12	~300	3.7 ± 0.2
IIG12	94	5.2 ± 0.3
VF1	90	8.1 ± 0.9
ID1	63	3.4 ± 0.7
VIE12	56	8.2 ± 1.6

[a] Monoclonal antibody beads were prepared as described under "Experimental Procedures" using various junctional-specific monoclonal antibodies. The epitope of each junctional-specific monoclonal antibody was determined by immunoblotting with isolated triads as described under "Experimental Procedures." Immunoprecipitation assays were performed as described under "Experimental Procedures," and the amount (femtomoles) of immunoprecipitated [^{3}H] ryanodine-labeled receptor ± S.E. is given. Total amount of [^{3}H] ryanodine receptor in the assay was 345 fmol.

Monoclonal antibodies specific for the junctional sarcoplasmic reticulum were then screened for their ability to immunoprecipitate the solubilized [^{3}H]ryanodine-labeled receptor (Table I). Monoclonal antibodies from hybridoma supernatants or ascites fluid were preincubated with goat anti-mouse IgG Sepharose beads (GAM-IgG beads) to form monoclonal antibody goat anti-mouse IgG Sepharose beads (mAb-GAM-IgG beads). These monoclonal antibody beads were then tested for their ability to immunoprecipitate the prelabeled digitonin-solubilized [^{3}H]ryanodine receptor. The radioactivity on the beads was counted to determine directly the amount of [^{3}H]ryanodine-labeled receptor bound by the monoclonal antibody beads. Monoclonal antibody XA7 and monoclonal antibody VIIH4$_2$ were found to immunoprecipitate the [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads (Table I). Goat anti-mouse IgG Sepharose by itself or other junctional-specific monoclonal antibodies immunoprecipitated a background level of less than 8% of the total [^{3}H]ryanodine-labeled receptor in the assay mixture (Table I).

Monoclonal antibody XA7 was shown to remove quantitatively the [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads (Fig. 2). A close inverse correlation was found between the amount of [^{3}H]ryanodine receptor immunoprecipitated by monoclonal antibody XA7 and the [^{3}H]ryanodine receptor remaining in the supernatant of the mAb-XA7-GAM-IgG Sepharose beads as determined by the PEG precipitation assay (Fig. 2). The maximum amount of [^{3}H]ryanodine-labeled receptor immunoprecipitated by anti-ryanodine receptor antibodies ranged from 80 to 95% of the total ryanodine receptor in the assay.

The specificity of the anti-ryanodine receptor monoclonal antibody XA7 was established by immunoprecipitating the ryanodine receptor from digitonin-solubilized triads that were prelabeled with [^{3}H]ryanodine in the absence of ATP, in the presence of ATP, in the presence of ATP and ruthenium red,

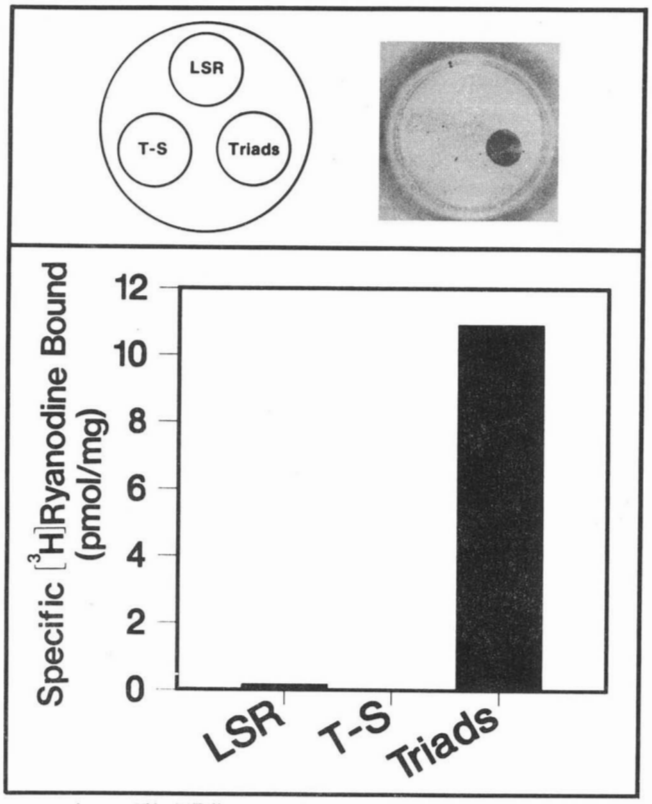

FIG. 1. **Immunodot screening assay for selection of hybridoma colonies producing monoclonal antibodies specific for the junctional sarcoplasmic reticulum membrane.** *Upper left,* light sarcoplasmic reticulum vesicles (*LSR*), skeletal muscle triads (*Triads*), and transverse tubular membrane vesicles (*T-S*) were dotted (~0.5 μl, 1 μg/μl) onto the nitrocellulose at 12, 4, and 8 o'clock in each well of a Millititer plate (Millipore) as diagrammed and allowed to dry. The plates were blocked with 3% BSA-TBS (20 mM Tris-HCl, 200 mM NaCl, pH 7.5) and allowed to react with hybridoma supernatants (50 μl). A peroxidase-conjugated goat anti-mouse IgG secondary antibody (Cooper Biomedical, Inc.) at 1:1000 dilution in 3% BSA-TBS was then used and the plates were developed using 4-chloro-1-naphthol as the substrate. *Upper right,* result of the immunodot assay using anti-ryanodine receptor hybridoma supernatant XA7. *Bottom,* specific [^{3}H]ryanodine binding activity (pmol/mg) for the light sarcoplasmic reticulum vesicles (*LSR*), transverse tubular membrane vesicles (*T-S*), and skeletal muscle triads (*Triads*) used in the immunodot assay. Isolated membranes were incubated for 1 h at 37 °C in a solution containing 150 mM KCl, 10 mM HEPES (pH 7.4), 5 μM PMSF, 3 mM ATP, 20 μM Ca^{2+} (free), and 10 nM [^{3}H]ryanodine (54 Ci/mmol) at a final protein concentration of 50 μg/ml (14, 16). Nonspecific binding was measured by the addition of 10 μM unlabeled ryanodine (Penick). Samples (0.5 ml) were rapidly filtered through Whatman GF/B glass fiber filters using a Brandel Model M-24R cell harvester. After two washes with 5 ml of ice-cold assay buffer, the filters were added to 10 ml of scintillation fluid and radioactivity was determined using a liquid scintillation counter. All experiments were performed in triplicate.

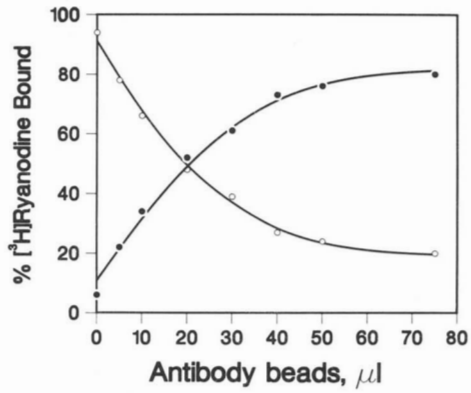

FIG. 2. **Immunoprecipitation of [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads with monoclonal antibody XA7-goat-anti-mouse IgG Sepharose 4B beads.** Immunoprecipitation assays were performed as described under "Experimental Procedures" except that various amounts of mAb-XA7-GAM-IgG Sepharose beads (μl) were used with the total volume of the beads kept constant using Sepharose CL-4B. The amount of immunoprecipitated [^{3}H]ryanodine-labeled receptor (●) correlated inversely with the amount of [^{3}H]ryanodine-labeled receptor remaining in the supernatant following immunoprecipitation (○).

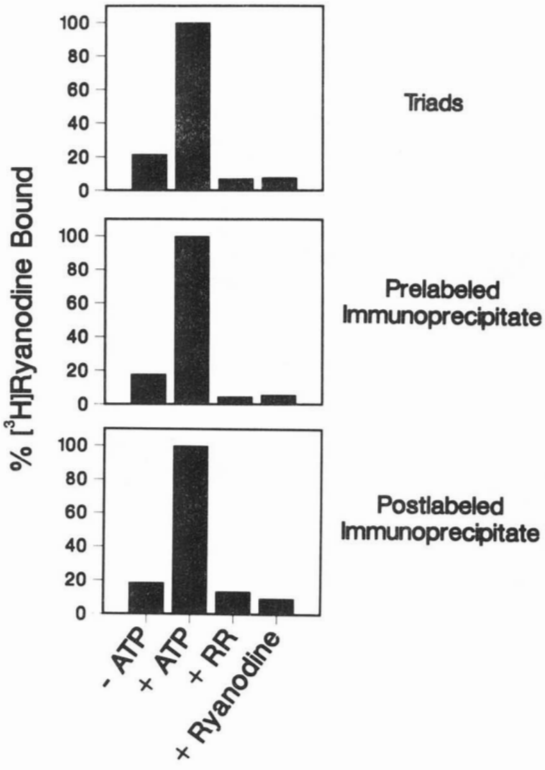

FIG. 3. **Specificity of the ryanodine receptor immunoprecipitated with monoclonal antibody XA7.** [^{3}H]Ryanodine binding to isolated triads (*top*) was performed as described in Fig. 1. Immunprecipitation of [^{3}H]ryanodine-labeled receptor from digitonin-solubilized triads with mAb-XA7-GAM-IgG Sepharose beads (*middle*) and [^{3}H]ryanodine postlabeling of the ryanodine receptor immunoprecipitated from digitonin-solubilized triads with mAb-XA7-GAM-IgG Sepharose Beads (*bottom*) were performed as described under "Experimental Procedures." [^{3}H]Ryanodine binding was performed in the absence of ATP (*−ATP*), in the presence of 2 mM ATP (*+ATP*), in the presence of 2 mM ATP and 2 μM ruthenium red (*+RR*), and in the presence of 2 mM ATP and 50 μM ryanodine (*+Ryanodine*). Free Ca^{2+} was maintained at 20 μM and [^{3}H]ryanodine binding in the presence of ATP was set at 100%. All experiments were performed in triplicate.

or in the presence of ATP and excess unlabeled ryanodine (Fig. 3, *middle*). Millimolar ATP was able to stimulate [^{3}H]ryanodine binding to isolated triads while micromolar ruthenium red or excess unlabeled ryanodine were able to inhibit the [^{3}H]ryanodine binding to isolated triads. Fig. 3 (*middle*) shows that monoclonal antibody XA7 immunoprecipitates the ryanodine receptor which exhibits binding properties similar to isolated triads (Fig 3, *top*).

We have also examined the ability of the ryanodine receptor immunoprecipitated from unlabeled digitonin-solubilized triads to bind [^{3}H]ryanodine. Fig. 3 (*bottom*) shows the postlabeling of the immunoprecipitated receptor with [^{3}H]ryanodine. Nonspecific binding (in the presence of 10 μM unlabeled

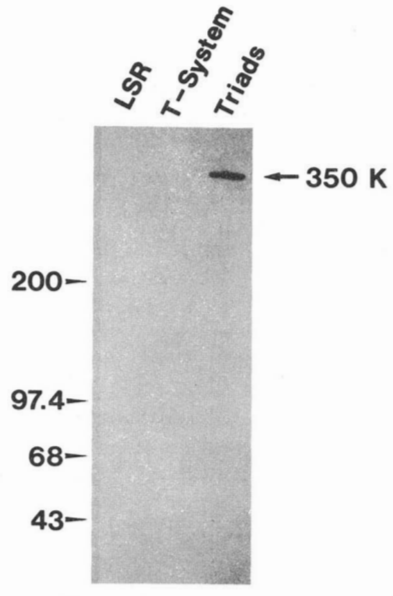

FIG. 4. **Immunoblot staining of light sarcoplasmic reticulum vesicles, transverse tubular membrane vesicles, and isolated triads with anti-ryanodine receptor monoclonal antibody XA7.** Fifty micrograms of light sarcoplasmic reticulum vesicles (*LSR*), transverse tubular membranes (*T-System*), and rabbit skeletal muscle triads (*Triads*) were subjected to SDS electrophoresis on a 3–12% polyacrylamide gradient gel (29) and transferred electrophoretically (30) to nitrocellulose. Indirect immunoperoxidase staining of the membrane fractions with monoclonal antibody XA7 was performed as described under "Experimental Procedures." Monoclonal antibody XA7 recognized a junctional-specific sarcoplasmic reticulum protein of ~350,000 Da (*350K*). Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the *left*.

ryanodine) was less than 10%, ATP stimulated [^{3}H]ryanodine binding 5-fold, and ruthenium red inhibited binding by greater than 85%. [^{3}H]Ryanodine binding was also Ca^{2+}-dependent (not shown). These results demonstrate that the binding of [^{3}H]ryanodine to the immunoprecipitated receptor is allosterically regulated by ATP and ruthenium red, and therefore the immunoprecipitated ryanodine receptor also contains the binding sites for ATP and ruthenium red.

The epitope of each junctional-specific monoclonal antibody was examined by indirect immunoperoxidase staining of nitrocellulose blots of skeletal muscle membranes and the results are reported in Table I. Monoclonal antibodies XA7 and $VIIH4_2$ recognized a high molecular weight protein (~350,000 Da) while the other junctional-specific monoclonal antibodies did not recognize the 350,000-Da protein. Fig. 4 shows that monoclonal antibody XA7 stained the high molecular weight protein (~350,000 Da) in isolated triads and the 350,000-Da protein was absent from both light sarcoplasmic reticulum vesicles and transverse tubular membrane vesicles (two preparations devoid of the ryanodine receptor). A comparison of the stained immunoblot with a Coomassie Blue-stained gel of light sarcoplasmic reticulum vesicles and isolated triads (not shown) has demonstrated that monoclonal antibody XA7 binds to the major high molecular weight protein of isolated triads. This high molecular weight protein of ~350,000 Da has been previously identified as a junctional-specific sarcoplasmic reticulum protein (8, 32–35). Caswell and co-workers (32, 33) have suggested that this protein spans the gap of the triad junction. Seiler *et al.* (8) have identified this high molecular weight protein in junctional sarcoplasmic reticulum isolated from both cardiac and skeletal muscle. Zorzato *et al.* (34) have shown that [^{14}C]doxorubicin, an activator of Ca^{2+} release, photolabels the 350,000-Da protein.

Excitation-contraction coupling in skeletal muscle is the least understood process in muscle contraction. Since the identification of the sarcoplasmic reticulum as the ATP-dependent vesicular relaxing factor, a wealth of information has been accumulated about the mechanism of Ca^{2+} transport by the sarcoplasmic reticulum and the proteins responsible for Ca^{2+} transport. In contrast, much less is known about the proteins which function directly or indirectly in excitation-contraction coupling and/or Ca^{2+} release from the junctional sarcoplasmic reticulum.

The results of our experiments have shown that monoclonal antibodies which are capable of specifically immunoprecipitating the [^{3}H]ryanodine-labeled receptor recognize the high molecular weight, ~350,000 Da, junctional-specific protein. The immunoprecipitated receptor has exhibited all the properties expected for the high affinity ryanodine receptor of the Ca^{2+} release channel. Our results strongly suggest that the 350,000-Da protein is a component of the ryanodine receptor. However, the exact composition of the ryanodine receptor and the relationship between the 350,000-Da protein and the Ca^{2+} release channel will require purification of ryanodine receptor and reconstitution of the purified receptor in phospholipid vesicles and/or planar lipid bilayers.

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