High Molecular Weight Proteins Purified From Cardiac Junctional Sarcoplasmic Reticulum Vesicles Are Ryanodine-Sensitive Calcium Channels

David P. Rardon, Dominic C. Cefali, Robert D. Mitchell, Steven M. Seiler, and Larry R. Jones

The cardiac high molecular weight proteins/ryanodine receptors were purified to homogeneity from junctional sarcoplasmic reticulum membranes and shown to exhibit large conductance calcium channel activity. High molecular weight proteins were solubilized from junctional sarcoplasmic reticulum in zwitterionic detergent and purified by size-exclusion chromatography followed by sucrose density gradient centrifugation. The purified proteins exhibited an apparent $M_\text{r} = 400,000-350,000$, and bound $[^3\text{H}]$ryanodine with a $K_\text{d}$ of 4.6 nM and a $B_{\text{max}}$ of 140–280 pmol/mg protein. High molecular weight proteins demonstrated divalent cation channel activity after incorporation into planar lipid bilayers. Two channel types were identified. Large conductance channels had a slope conductance of $96\pm13$ pS and a $E_{\text{m}}$ of $42\pm9$ mV ($n=5$); small conductance channels had a slope conductance of $5.5\pm1$ pS [1.0 µM cis CaCl$_2$; 50 mM trans Ba(OH)$_2$]. Reducing cis calcium from 1 µM to 1 nM reduced the large conductance channel open time from $7\pm1\%$ to 0.1% (holding potential, $-100$ mV). Adding ATP (1 mM) to the cis chamber increased channel open time from $6\pm1\%$ to $52\pm4\%$ (holding potential, $-100$ mV); 10 nM ryanodine increased and 100 nM ryanodine decreased percent of open time of the 96 pS channel, without altering unitary channel conductance. The large conductance channel was similar to the calcium release channel detected in native canine cardiac junctional sarcoplasmic reticulum vesicles. Our data suggest that the ryanodine receptor, the calcium-release channel, and the high molecular weight proteins are all identical proteins containing allostereic regulatory sites for calcium, ATP, and ryanodine. (Circulation Research 1989;64:779–789)
cardiac and skeletal muscle. Using the planar lipid bilayer technique, calcium-activated, adenine-nucleotide-sensitive calcium or barium channels have been demonstrated in junctional SR vesicles isolated from rabbit skeletal and canine cardiac muscle. These observations have provided key functional and quantitative biochemical assays for ultimate purification of the ryanodine receptor/calcium-release channel from both cardiac and skeletal muscle.

Recently, Inui et al purified the ryanodine receptor from rabbit skeletal muscle SR by a combination of column chromatography steps. The purified receptor consisted of three major polypeptides of \( M_r = 360,000, 330,000, \) and 175,000. Campbell et al immunoprecipitated the ryanodine receptor protein solubilized from skeletal muscle tissue and found it to be a single polypeptide of \( M_r = 350,000 \). Further work from this latter group demonstrated that the immunoprecipitated ryanodine receptor exhibits calcium channel activity when reconstituted into planar lipid bilayers. The channel had a mean slope conductance of 35 pS in 54 mM barium or calcium, but channel opening was not regulated by ryanodine or micromolar calcium, prerequisites essential for demonstrating that the ryanodine receptor and the calcium-release channel are identical proteins. Very recently, Lai et al reported that the purified ryanodine receptor from skeletal muscle exhibits channel activity that is regulated by calcium, ATP, and ryanodine. Inui et al isolated the cardiac ryanodine receptor and found it to be similar in biochemical characteristics to the skeletal muscle receptor. The purified cardiac ryanodine receptor exhibited channel activity with a slope conductance of 72 pS [50 mM Ca(OH)\(_2\) trans; 2.5 \( \mu \)M CaCl\(_2\) cis]. Ryanodine binding as well as single-channel activities of cardiac and skeletal muscle ryanodine receptor complexes in intact SR vesicles have been shown to exhibit subtle differences in allosteric regulation. This suggests that distinct protein isoforms of the ryanodine receptor may exist in cardiac and skeletal muscle.

We report here a method for the purification of the ryanodine receptor to homogeneity from canine cardiac ventricular muscle. The receptor is composed of a 400,000–350,000 dalton polypeptide doublet, which corresponds to the junctional SR high molecular weight (HMW) proteins previously characterized by our laboratory. When reconstituted into artificial planar lipid bilayers, the cardiac ryanodine receptors are shown to behave as large conductance calcium channels. The channels are regulated by calcium, ATP, and ryanodine, properties unique to the calcium-release channel. Our data suggest the identity of the ryanodine receptor, the calcium-release channel, and the HMW proteins in canine cardiac junctional SR vesicles.

**Materials and Methods**

**Membrane Preparation**

Junctional SR vesicles were isolated from canine cardiac left ventricular tissue essentially as described previously. Modifications included the omission of the calcium oxalate loading step and high ionic strength salts from the sucrose gradient solutions. Briefly, left ventricular tissue was minced and homogenized in 10 mM NaHCO\(_3\) three times for 30 seconds with a Polytron (Lucerne, Switzerland). The homogenate was centrifuged in a Beckman Model JA-14 rotor (Fullerton, California) at 8,700 rpm for 20 minutes. The supernatants were sequentially centrifuged in a Beckman Model JA-14 rotor at 19,000 rpm for 20 minutes and in a Beckman Model JA-20 rotor at 19,000 rpm for 30 minutes. Pellets from the 19,000 rpm spin were resuspended in 30 mM histidine (pH 7.0) and centrifuged in a Beckman Model JA-20 rotor at 19,000 rpm for 30 minutes. The sedimented material was resuspended in 0.25 M sucrose and 10 mM histidine (pH 7.0), and SR subfractions were separated by centrifugation through discontinuous sucrose gradients (0.25, 0.6, 0.8, 1.0, and 1.5 M sucrose steps in 10 mM histidine) at 27,000 rpm for 2 hours in a Beckman Model SW-27 rotor. The junctional SR fraction was recovered from the 1.0/1.5 M sucrose interface, resuspended in 0.25 M sucrose and 30 mM histidine (pH 7.4), and stored at -20° C. Calcium uptake by this fraction was stimulated fivefold to 10-fold by 100 \( \mu \)M ryanodine (S.P. Penick Co, West Lynnhurst, New Jersey).

**Purification of High Molecular Weight Proteins**

HMW proteins/ryanodine receptors were purified from 3-[2-cholamido-propyl]-dimethylammonio]-1-propane sulfonate (CHAPS) (Sigma Chemical Co, St. Louis, Missouri) solubilized junctional SR membranes. Junctional SR vesicles (35–40 mg of protein) were solubilized at pH 7.4 for 1 hour on ice in 5 ml of buffer containing 20 mM 3-(N-morpholino)propane sulfonic acid (MOPS), 250 mM sucrose, 2% CHAPS, 0.5 M KCl, 2 mM dithiothreitol; 0.06 mM CaCl\(_2\), and 10 \( \mu \)g/ml leupeptin. During solubilization, the membrane suspension was placed on ice and vortexed for 30 seconds every 10 minutes. Insoluble proteins were removed by ultracentrifugation for 5 minutes at 100,000 rpm using a Beckman Model TL 100.2 rotor and a Beckman Model TL 100 ultracentrifuge. The supernatant was fractionated on a Fractogel TSK HW 65 (F) (EM Science, Gibbstown, New Jersey) size exclusion column (90 x 1.6 cm) preequilibrated with the CHAPS solubilization buffer supplemented with 0.3% CHAPS and 5 \( \mu \)g/ml leupeptin. Three-milliliter fractions were collected at a flow rate of 15 ml/hr. Fractions enriched in HMW proteins were identified by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue or silver staining as well as by measuring \([3H]\)ryanodine (New England Nuclear, Boston, Massachusetts) binding to fraction aliquots. Protein standards for SDS-PAGE were obtained from Sigma Chemical Co and Boehringer Mannheim Corp, Indianapolis, Indiana.) Peak fractions were pooled and concentrated with an Amicon (Amico Div, WR
Grace & Co, Danvers, Massachusetts). Concentrated samples (500 µg protein/0.5 ml) were further purified by sedimentation through 12.2-mI 9-24% linear sucrose gradients prepared in 20 mM MOPS, 0.5 M KCl, 2 mM dithiothreitol, 0.06 mM CaCl₂, and 0.3% CHAPS at pH 7.4. Gradients were centrifuged in a Beckman Model SW 40 rotor at 35,000 rpm for 12.5 hours at 4°C. After centrifugation, 0.24-ml fractions were collected and aliquots subjected to SDS-PAGE and assayed for [³H]ryanodine binding. Selected fractions containing only HMW proteins were used for electrophysiological studies.

In some experiments, HMW proteins were purified from cardiac junctional SR membranes after initial solubilization in Zwittergent 3-14 (Calbiochem, San Diego, California). Fractionation by size-exclusion chromatography and sucrose density gradient centrifugation were performed as described above. Junctional SR vesicles (30 mg of protein) were solubilized on ice for 10 minutes at pH 7.4 in a buffer consisting of 3% Zwittergent 3-14, 0.5 M NaCl, 20 mM MOPS, 0.5 µg/ml leupeptin, 100 µM phenylmethylsulfonyl fluoride (PMSF), 100 µM N-tosyl-L-phenylalanine chloromethylketone (TPCK), and 100 µM N-p-tosyl-L-lysine chloromethylketone (TLCK). Size-exclusion chromatography of the supernatant was performed in a buffer consisting of 20 mM MOPS, 10 mM CaCl₂, 0.6 M NaCl, and 0.005% Zwittergent 3-14, at pH 7.1. Sucrose gradient centrifugation was conducted in a buffer consisting of 40 mM tris(hydroxymethyl)aminomethane (TRIS) (Sigma Chemical Co), 1.0 M NaCl, 0.4 mM CaCl₂, 0.1 mM [ethylenebis(oxyethyl)enitrilo] tetraacetic acid (EGTA), and 1% CHAPS at pH 7.1. Gradients were centrifuged in a Beckman Model SW 40 rotor at 20,000 rpm for 17 hours at 4°C. Fractions containing only HMW proteins, as identified both by Coomassie blue and silver staining, were used for electrophysiological experiments.

[³H]Ryanodine Binding

[³H]Ryanodine binding to soluble receptor proteins from column and sucrose gradient fractions was performed in 0.2 ml of incubation volume containing 20 mM MOPS, 0.25 M sucrose, 0.5 M KCl, 2 mM dithiothreitol, 0.6 mM CaCl₂, and 0.2 mg bovine serum albumin, at pH 7.0. Samples were incubated at 37°C for 1 hour in the presence of 20 nM [³H]Ryanodine, which was determined to be a saturating concentration. Nonspecific binding was measured in the presence of 10 µM nonradioactive ryanodine. Bound and free [³H]ryanodine were separated using the polyethylene glycol precipitation method of Glossman and Ferry.²⁷ [³H]Ryanodine retained on Whatman GF/C filters was quantitated using a Packard Model 2000 liquid scintillation counter (Downers Grove, Illinois). [³H]Ryanodine binding could be detected in samples initially solubilized in CHAPS but not in samples solubilized in Zwittergent 3-14. Binding of [³H]ryanodine to membrane fractions was performed as above except that the polyethylene glycol precipitation step was omitted.

SDS-PAGE and Protein Assay

SDS-PAGE was performed with 5% polyacrylamide gels [acrylamide:bis = 30:0.8 (wt/wt)] according to our modification²⁸ of the method of Porzio and Pearson.²⁹ Gels were stained with either Coomassie blue or by the silver method.³⁰ With the use of α₁-macroglobulin (Mr = 340,000) as the largest protein standard, we obtained calculated molecular weights of 400,000 and 350,000 for the cardiac HMW protein doublet. Protein contents were determined according to Schaffner and Weissman,³¹ using bovine serum albumin as the standard.

Planar Bilayer Measurements

Single-channel activities were recorded by the Mueller-Rudin lipid bilayer technique.³² A mixture of phosphatidylserine and phosphatidylethanolamine (4:5 weight ratio) in decane was painted over a 0.25-mm diameter hole in the Lexan partition that separated the cis and trans chambers of the bilayer apparatus. The cis chamber, to which vesicles or purified proteins were added, contained 1 mM CaCl₂, 1 mM EGTA, 125 mM TRIS, and 250 mM (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), at pH 7.4. The calculated free calcium concentration in the cis chamber was 1 µM. The trans chamber contained 50 mM Ba(OH)₂ and 250 mM HEPES, at pH 7.4. Under these bi-ionic conditions where E⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻柒

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Figure 1. Size exclusion chromatography of 3-{(3-cholamido-propyl)-dimethylammonio}-1-propane sulfonate (CHAPS) solubilized cardiac ryanodine receptors. Panel A: Results of $[^3H]$ryanodine binding assay conducted on column fractions. ---, absorbance at 280 nm; ——, total binding measured with 20 nM $[^3H]$ryanodine; O-O-O, nonspecific binding measured in the additional presence of 10 μM nonradioactive ryanodine. Panel B: Photograph of an SDS gel of the column fractions stained with Coomassie blue. The high molecular weight protein doublet (HMW) was comprised of protein bands of apparent M, s=400,000 and 350,000.

Results

Purification of High Molecular Weight Proteins

We observed that 100% of the $[^3H]$ryanodine binding activity was solubilized from cardiac junctional SR vesicles with the use of the zwitterionic detergent CHAPS. Two peaks of $[^3H]$ryanodine binding activity were recovered when CHAPS solubilized receptors were fractionated by size-exclusion chromatography (Figure 1A). The first, minor, peak of specific binding (fractions 3–11) coincided with the void volume of the column and probably contained aggregated receptors. The second, major, peak of specific binding (fractions 13–20) contained most of the recovered ryanodine receptors and was enriched in an HMW protein doublet (Figure 1B). The molecular weights of the two proteins comprising the doublet were estimated to be 400,000 and 350,000.

Nonspecific $[^3H]$ryanodine binding measured in the column fractions was negligible (Figure 1A). The major peak of $[^3H]$ryanodine binding recovered from the size exclusion column was further fractionated by sucrose density gradient centrifugation. Sucrose gradient fractions were then assayed for protein contents and $[^3H]$ryanodine binding. Fractions recovered toward the top of the gradient (fractions 2–19) showed no $[^3H]$ryanodine binding (Figure 2A) and contained mostly contaminant proteins (Figure 2B). A large peak of $[^3H]$ryanodine binding and a small peak of protein were recovered near the bottom of the sucrose gradient (Figure 2A, fractions 23–25), and the HMW proteins, purified to homogeneity as assessed by SDS-PAGE (Figure 2B), were localized to these fractions. Scatchard analysis of purified receptors (Figure 3) revealed a homogeneous population of binding sites with a $K_d$
FIGURE 2. Sucrose density gradient centrifugation of cardiac ryanodine receptors. Fractions 13–20 obtained from the sizing column (Figure 1) were subjected to sucrose density gradient centrifugation. Panel A: Distribution of \[^{3}H\]ryanodine binding and total protein throughout the gradient. Panel B: Photograph of a Coomassie blue stained gel of the gradient fractions in which 125 \(\mu\)l of each gradient fraction were applied per gel lane. This volume applied corresponded to 8 \(\mu\)g of protein applied to gel lane 24, which corresponded to the peak fraction of purified high molecular weight (HMW) proteins. The HMW proteins are concentrated in fractions 23–25. Gel lanes 26–31 contained no appreciable Coomassie blue staining material and are not shown in the photograph. The lower protein component of the doublet did not reproduce well with photography and is barely visible.

FRACTION NUMBER

Panel A: Distribution of \[^{3}H\]ryanodine binding and total protein throughout the gradient.

Panel B: Photograph of a Coomassie blue stained gel of the gradient fractions in which 125 \(\mu\)l of each gradient fraction were applied per gel lane. This volume applied corresponded to 8 \(\mu\)g of protein applied to gel lane 24, which corresponded to the peak fraction of purified high molecular weight (HMW) proteins. The HMW proteins are concentrated in fractions 23–25. Gel lanes 26–31 contained no appreciable Coomassie blue staining material and are not shown in the photograph. The lower protein component of the doublet did not reproduce well with photography and is barely visible.

equal to 4.6 nM; the \(B_{\text{max}}\) from this typical experiment was 185 pmol \(^{3}H\)ryanodine bound per milligram of protein. From multiple experiments we have observed the \(B_{\text{max}}\) to range between 140 and 280 pmol \(^{3}H\)ryanodine bound per milligram of protein. Repeated freezing and thawing of purified protein preparations reduces \(B_{\text{max}}\) by approximately 50%, a reduction similar to that observed by Seifert and Casida. Receptors were purified 20- to 30-fold relative to specific binding activity detected in the parent junctional SR vesicles (10 pmol/mg protein).

When cardiac junctional SR vesicles were first solubilized with Zwittergent 3-14 and fractionated by size-exclusion chromatography in the same detergent, a much greater initial purification of HMW proteins was achieved (Figure 4A). Exchange of Zwittergent 3-14 with CHAPS by sucrose density gradient centrifugation allowed purification of the HMW proteins to apparent homogeneity (Figure 4B, lane C), but no \(^{3}H\)ryanodine binding activity could be detected in the purified preparation, even though calcium channel activity was readily measured (see below).

Calcium Channel Activity of Cardiac Junctional Sarcoplasmic Reticulum Vesicles

Figure 5 is a typical recording of single barium channel activity obtained after addition of 2 \(\mu\)g of canine cardiac junctional SR vesicles to the cis chamber of the bilayer apparatus. In the presence of 50 mM trans barium, the channels had a slope conductance of 85 \(\pm\) 11 pS and a reversal potential of 37 \(\pm\) 18 mV (n=6). This slope conductance is similar to values previously reported for cardiac and skeletal muscle calcium release channels. The deviation of the reversal potential from the pre-
Large conductance channel in the purified preparation of high molecular weight proteins. 

**FIGURE 3.** Binding isotherm of $[^3H]$ryanodine to purified high molecular weight proteins. High molecular weight proteins were purified as depicted in Figures 1 and 2. •, total binding; ○, nonspecific binding, measured with additional 10 μM nonradioactive ryanodine. Values are averages of duplicate samples. The inset is the Scatchard plot for the experiment shown. The apparent $K_d$ and $B_{max}$ values are 4.6 nM and 185 pmol $[^3H]$ryanodine bound mg protein, respectively.

**Channel Activity of Purified High Molecular Weight Proteins**

HMW proteins, whether purified from CHAPS or Zwittergent 3-14 extracts of SR membranes, exhibited single-channel activity similar to that detected with native SR vesicles. Typical channel activity recorded from purified HMW proteins initially solubilized with CHAPS is depicted in Figure 6. The large conductance channel in the purified preparation appeared identical to the large conductance channel exhibited by native SR vesicles. The mean slope conductance of this channel was determined to be 96±13 pS; the reversal potential was 42±9 mV ($n=5$). The slope conductance and reversal potential of the purified HMW proteins and the calcium-release channel of native junctional SR vesicles were therefore similar. Figure 7B shows channel activity recorded from purified HMW proteins initially solubilized with Zwittergent 3-14. The slope conductance of Zwittergent 3-14 and CHAPS solubilized purified protein channels was similar. Openings of the Zwittergent 3-14 solubilized channel, however, were less frequent; and the open state amplitudes were less well resolved than with native SR vesicles. A second, small conductance channel (slope conductance~5.5 pS) was also frequently observed after reconstitution of HMW proteins purified from either Zwittergent 3-14 (Figure 7B) or CHAPS solubilized membranes (Figure 8).

The probability of the purified calcium channel being in the open state was found to be dependent upon the cis calcium concentration (Figure 7A). Lowering the cis calcium concentration from 1 μM to 1 nM by addition of EGTA decreased the percent of channel open time from 7±1% to 0.1% at a holding potential of -100 mV (Figure 7A). In the presence of 1 μM cis calcium, ATP (1 mM) in the cis chamber further increased the channel open time from 6±1% to 52±7% at a holding potential of -100 mV (Figure 7B). No differences were noted in ATP or calcium effects between channels purified from Zwittergent 3-14 or CHAPS extracts.

Ryanodine regulated the probability of channel opening in a complex fashion. Purified proteins from Zwittergent 3-14 extracts that did not bind $[^3H]$ryanodine, as well as purified proteins from CHAPS extracts that did bind ryanodine (Figure 2), exhibited similar responses to ryanodine after incorporation into planar bilayers and are discussed together. Figure 8 illustrates a typical experiment in which two large conductance channels purified from a CHAPS extract were incorporated into the bilayer. One channel is open much of the time, and the second exhibits frequent transitions between the open and closed states. A low concentration of ryanodine (10 nM) increased channel open time without altering the unitary channel conductance, such that both large channels were continuously open. A 10,000-fold higher concentration of ryanodine (100 μM) increased the probability that the two channels were closed. After the addition of this high concentration of ryanodine to the cis chamber, only rare brief openings were consistently seen. Channel conductance, however, was not altered.

**Discussion**

We report here the purification of canine cardiac junctional SR HMW proteins, which are demonstrated to bind ryanodine and to exhibit divalent cation channel activity that is regulated by ATP,
Ryanodine and micromolar calcium. Purification of the ryanodine receptor from cardiac\cite{16,23} and skeletal\cite{15,20,22} muscle has been recently reported by other laboratories, but this study is the first demonstrating that the protein purified from heart exhibits ryanodine-sensitive cation channel activity. Depending on the gel system used and protein standards analyzed, the apparent molecular weight of the ryanodine receptor purified from either tissue has been reported to range between 320,000 and 450,000.\cite{15,16,20,22,23} It has now become apparent that the purified ryanodine receptor is identical to the so-called "high molecular weight proteins" previously characterized in skeletal muscle by Caswell and Brunschwig\cite{30} and in cardiac muscle by Seiler et al.\cite{10} Our earlier results\cite{10} demonstrated that the HMW proteins are readily degraded by calcium-activated protease. The significance of the lower molecular weight protein component of the doublet remains uncertain, but it is possible that it is a proteolytic fragment of the higher $M_r$ protein component. Morphological studies\cite{22} have suggested the identities of the ryanodine receptor, the HMW proteins, and the feet structures of junctional SR. The purified ryanodine receptor from skeletal and cardiac muscle has been demonstrated to bind 280–650 pmol [\textsuperscript{3}H]ryanodine/mg protein.\cite{15,16,20,22} The binding activity reported here for the purified cardiac receptor falls within the range of these previously reported values.

Smith et al\cite{17} have established criteria for the identification of calcium-release channels in SR
vesicles fused with planar lipid bilayers. Calcium-release channels should exhibit relatively large divalent cation conductance and should be regulated by micromolar calcium, adenine nucleotides, and ryanodine. Our single-channel results on the purified cardiac HMW proteins fulfill all of these criteria. The purified channel had a slope conductance of 96 pS and a reversal potential of +42 mV with 50 mM trans barium. Similar values were obtained with intact junctional SR vesicles. Moreover, channel opening required micromolar calcium in the cis chamber, was stimulated by ATP, and was differentially regulated by ryanodine. Imagawa et al. recently measured the single-channel activity of the digitonin solubilized ryanodine receptor isolated from skeletal muscle SR vesicles. Only subconductance states (of 35 and 22 pS) were detected, and the channels were not regulated by calcium or ATP. Lai

**Figure 5.** Calcium-release channel activity recorded from a native junctional sarcoplasmic reticulum preparation. Panel A: Calcium-release channel activity recorded after incorporation of a channel from native junctional sarcoplasmic reticulum in a planar lipid bilayer (cis and trans solutions as outlined in "Materials and Methods"). The closed state of the channel is noted by the solid dark line to the left of each current record. The opening of the channel is a downward deflection that represents negative current flow. Panel B: I-V (current-voltage) relationship of the channel (values are mean±SEM from six experiments). Similar conductances were obtained when 50 mM calcium was substituted for barium in the trans chamber.

**Figure 6.** Channel activity recorded from a purified high molecular weight protein preparation. Panel A: Channel activity recorded after incorporation of high molecular weight proteins purified from 3-(3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate (CHAPS) extracts of junctional sarcoplasmic reticulum membranes. Closed state of the channel is noted by solid dark line to left of each current record. Opening of the channel is downward deflection that represents negative current flow. Panel B: I-V (current-voltage) relationship of the large conductance channel (values are mean±SEM for five experiments).
et al.\textsuperscript{22} on the other hand, purified the CHAPS solubilized ryanodine receptor from skeletal muscle SR and observed the appropriate channel conductance (91 pS) as well as channel regulation by calcium, ATP, and ruthenium red. Thus, our results and those of Lai et al.\textsuperscript{22} suggest that all of the allosteric regulatory sites identified to date for the calcium-release channel reside in the same HMW protein components.

HMW proteins purified after initial solubilization in Zwittergent 3-14 did not bind ryanodine but did exhibit ryanodine-sensitive channel activity after incorporation into lipid bilayers. We believe the most likely explanation for this apparently paradoxical result is that Zwittergent 3-14 denatured most of the ryanodine receptors. Because the planar bilayer method is so sensitive and only selects for active channels, only a small percent of residually active receptors would be required for detection of single-channel activity. Alternatively, it is possible that Zwittergent 3-14 solubilized receptors “renatured” after incorporation into phospholipid bilayers. Arguing against this, however, we could not restore any significant \textsuperscript{3}Hryanodine binding to Zwittergent 3-14 purified receptors when phospholipids were added directly to binding assays (data not shown). It therefore seems most likely that a high percent of ryanodine receptors were irreversibly denatured after solubilization in Zwittergent 3-14. Our results point out some of the potential pitfalls involved when correlating macroscopic biochemical results (\textsuperscript{3}Hryanodine binding data) with microscopic single-channel results.

Ryanodine regulated the purified cardiac calcium-release channel in complex fashion. Nanomolar concentrations of the alkaloid increased the proba-
Figure 8. Effects of ryanodine on channel activity recorded from a purified high molecular weight protein preparation. Channel activity is recorded after incorporation of purified high molecular weight proteins from a 3-[(3-cholamido-propyl)-dimethylammonio]-1-propane sulfonate (CHAPS) extract of junctional sarcoplasmic reticulum.

Panel A: Control record recorded at a holding potential of 0 mV. The bilayer conductance (closed state) is marked C. Channel openings are upward deflections that reflect negative current flow. In the initial portion of the current trace, two channels are incorporated into the bilayer. One channel is open continuously, the second undergoes frequent transitions between the open and closed states. As marked, 10 nM ryanodine (RY) was added to the cis chamber. In 10 nM ryanodine both channels were continuously open without any change in unitary channel conductance.

Panel B: Continuous record. As marked, 100 μM ryanodine was added to the cis chamber. During the artifact caused by stirring the chamber, one large conductance channel closes (at point 1). At point 2 a small conductance (55 pS) channel opens. At point 3 the second large conductance channel closes, and at point 4 the small conductance channel closes. All channels remained closed throughout the remainder of this experiment.

The existence of several calcium-release channel substrates has been suggested by different groups. In addition to the 96 pS channel, we observed a 5.5 pS conductance channel. However, we obtained no evidence for interconversion of the 96 pS channel and the 5.5 pS channel by ryanodine. The significance of the small conductance channel and its relation to the large conductance channel is not clear but is presently under investigation. Our results do establish the identity between the cardiac ryanodine receptor, the HMW proteins, and the calcium-release channel with its associated regulatory sites for calcium, ATP, and ryanodine. Detailed electrophysiological comparisons of the properties of the calcium-release channel purified from cardiac muscle and skeletal muscle, as well as sequence information on the two channel proteins, will be required to establish definitively whether different protein isoforms are expressed in the two tissues.

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