Purified Ryanodine Receptor from Rabbit Skeletal Muscle Is the Calcium-Release Channel of Sarcoplasmic Reticulum

JEFFREY S. SMITH, TOSHIAKI IMAGAWA, JIANJIE MA, MICHAEL FILL, KEVIN P. CAMPBELL, and ROBERTO CORONADO

From the Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030, and the Department of Physiology and Biophysics, University of Iowa, College of Medicine, Iowa City, Iowa 52242

ABSTRACT The ryanodine receptor of rabbit skeletal muscle sarcoplasmic reticulum was purified as a single 450,000-dalton polypeptide from CHAPS-solubilized triads using immunoaffinity chromatography. The purified receptor had a [^{3}H]ryanodine-binding capacity (B_{max}) of 490 pmol/mg and a binding affinity (K_d) of 7.0 nM. Using planar bilayer recording techniques, we show that the purified receptor forms cationic channels selective for divalent ions. Ryanodine receptor channels were identical to the Ca-release channels described in native sarcoplasmic reticulum using the same techniques. In the present work, four criteria were used to establish this identity: (*a*) activation of channels by micromolar Ca and millimolar ATP and inhibition by micromolar ruthenium red, (*b*) a main channel conductance of 110 ± 10 pS in 54 mM *trans* Ca, (*c*) a long-term open state of lower unitary conductance induced by ryanodine concentrations as low as 20 nM, and (*d*) a permeability ratio $P_{Ca}/P_{Tris} \simeq 14$. In addition, we show that the purified ryanodine receptor channel displays a saturable conductance in both monovalent and divalent cation solutions (γ_{max} for K and Ca = 1 nS and 172 pS, respectively). In the absence of Ca, channels had a broad selectivity for monovalent cations, but in the presence of Ca, they were selectively permeable to Ca against K by a permeability ratio $P_{Ca}/P_K \simeq 6$. Receptor channels displayed several equivalent conductance levels, which suggest an oligomeric pore structure. We conclude that the 450,000-dalton polypeptide ryanodine receptor is the Ca-release channel of the sarcoplasmic reticulum and is the target site of ruthenium red and ryanodine.

INTRODUCTION

Release of Ca from the sarcoplasmic reticulum (SR) of skeletal muscle is initiated by and coupled to a transient depolarization of the transverse-tubular (t-tubule) membrane (Melzer et al., 1986*a, b*; Brum et al., 1987*a, b*; Berwe et al., 1987). The exact nature of this coupling is presently unknown. Current hypotheses invoke chemical messengers, participation of the dihydropyridine receptor as t-tubule voltage sen-

Address reprint requests to Dr. Roberto Coronado, Dept. of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.

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sor, and the possibility of direct physical communication via feet structures that bridge the 100-200-Å gap between the t-tubule and SR membranes (Vergara et al., 1985; Schneider, 1986; Rios and Brum, 1987). Recently, ryanodine, a plant alkaloid known to interfere with excitation-contraction coupling, was shown to bind with high affinity to a receptor localized in the junctional SR membrane (Fleischer et al., 1985; Pessah et al., 1985, 1986; Meissner, 1986; Lattanzio et al., 1987; Inui et al., 1987). Junctional SR is derived by disruption of triad structures formed between t-tubules and the SR (Campbell et al., 1980; Cadwell and Caswell, 1982; Kawamoto et al., 1986). Using radiolabeled ryanodine, several laboratories have reported partial or near-homogeneous purification of a large-molecular-weight ryanodine receptor protein complex from skeletal muscle (Pessah et al., 1986; Inui et al., 1987; Campbell et al., 1987; Imagawa et al., 1987). Imagawa et al. (1987) have shown that the immunoaffinity-purified ryanodine receptor is a single polypeptide chain of 450,000 mol wt, which functions as a Ca-permeable pore. Inui et al. (1987) suggested that the purified receptor is the major protein responsible for forming the feet structures that in intact muscle appear to anatomically connect the t-tubule and SR membranes (Ferguson et al., 1984).

Reconstitution studies in planar bilayers have shown that native SR is endowed with a novel type of Ca channel that has a characteristically large unitary conductance (>100 pS in 50 mM Ca or Ba) and a low divalent-over-monovalent ionic selectivity ($P_{Ca}/P_{Cs} < 10$), and is gated into the open state by key cellular ligands such as Ca and ATP (Smith et al., 1985, 1986*a, b*). "Native" refers to SR purified by conventional cell fractionation procedures in the absence of detergent solubilization steps. Because this channel (*a*) is only present in Junctional SR, (*b*) is present in >90% of the vesicle population, and (*c*) mediates Ca-induced Ca release, it has been named the Ca-release channel (Meissner, 1984). Two known blockers of the release channel are the polycationic dye ruthenium red and Mg (Smith et al., 1985, 1986*a, b*). The channel is also the target of the alkaloid ryanodine, which induces a long-term open state of reduced unit conductance (Imagawa et al., 1987; Rousseau et al., 1987). In this report, we demonstrate that purified ryanodine receptor forms monovalent- and divalent-selective channels when incorporated into planar bilayers. On the basis of a detailed characterization, we conclude that the purified ryanodine receptor channel is identical to the Ca-release channel of native SR. Comparisons between the two channels were made on the basis of (*a*) single-channel conductance, (*b*) ionic selectivity, (*c*) activation by micromolar Ca and millimolar ATP, and (*d*) sensitivity to ryanodine and ruthenium red. In addition, we describe permeation properties of the purified channel that, because of the large background of K and Cl currents present in the SR, could not be studied in the native membrane. From our findings, we propose that the 450,000-dalton polypeptide ryanodine receptor is the Ca-release channel of the SR and that this protein, in intact muscle, is the functional target site of ruthenium red and ryanodine. A preliminary account of this work has been presented in abstract form (Smith et al., 1988).

MATERIALS AND METHODS

Membrane Preparations

Triads were isolated from adult rabbit skeletal muscle by a modification of the method of Mitchell et al. (1983) as described in Sharp et al. (1987). The following protease inhibitors

were used: aprotinin (76.8 nM), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and phenylmethylsulfonyl fluoride (PMSF) (0.23 mM). 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. (1951) as modified by Peterson (1977), with bovine serum albumin as a standard.

Purification of Ryanodine Receptor

Ryanodine receptor was purified from isolated triads by immunoaffinity column chromatography as previously described (Imagawa et al., 1987) with several modifications. Anti-ryanodine receptor monoclonal antibody (Mab-XA7) was purified and the immunoaffinity absorbent (Mab-XA7-Sepharose) was prepared as described previously (Imagawa et al., 1987). Isolated triads (200 mg) were solubilized with 1% (16 mM) 3-[(3-cholamidopropyl)dimethylammonio]1-propane sulfonate (CHAPS), 0.5% asolecithin (L-α-phosphatidylcholine from soybean; type II-S, Sigma Chemical Co., St. Louis, MO) in the presence of 0.5 M Nad 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), antipain (7.2 μg/ml), chymostatin (7.2 μg/ml), and pepstatin A (0.6 μg/ml). Solubilized triads were applied to a Mab-XA7-Sepharose column (20 ml) and recycled overnight. The column was washed with 20 ml of buffer A containing 1% CHAPS, 0.5% asolecithin, and 0.5 M KCl, followed by 40 ml of 0.3% (5.3 mM) CHAPS, 0.15% asolecithin, and 0.5 M KCl in buffer A. Ryanodine receptor was eluted from an XA7-Sepharose column with 0.3% CHAPS, 0.15% asolecithin, and 0.5 M KSCN in buffer A. Immediately after 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 PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ) pre-equilibrated with 0.3% CHAPS, 0.15% asolecithin, 5 mM dithiothreitol, and 0.5 M KCl in buffer A to remove KSCN. Protein was measured using the method of Lowry et al. (1951) as modified by Peterson (1977) after the protein was precipitated with 6% trichloroacetic acid in the presence of 0.5 mg sodium deoxycholate.

The amount of [^{3}H] ryanodine binding to the purified receptor was determined as previously described (Imagawa et al., 1987). Samples (5-100 μg/ml of protein) were incubated with 1-30 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), 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 (Imagawa et al., 1987). Nonspecific binding was determined in the presence of 1-10 μM unlabeled ryanodine.

The purity of the preparation was determined by sodium dodecyi sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 3-12% polyacrylamide gradient gels in the buffer system of Laemmli (1970). After electrophoresis, gels were stained with Coomassie blue. Molecular weight standards were: nebulin, M_r ~500,000; myosin, M_r 200,000; phosphorylase, M_r 97,400; bovine albumin, M_r 68,000; ovalbumin, M_r 43,000.

Planar Bilayer Methods

Native Ca-release channels from heavy SR membranes were incorporated into Mueller-Rudin planar bilayer membranes as described previously by Smith et al. (1985). Planar lipid bilayers composed of brain phosphatidylethanolamine:phosphatidylserine in a 1:1 weight ratio (Avanti Polar Lipids, Inc., Birmingham, AL), 50 mg/ml in decane (Aldrich Chemical Co., Milwaukee, WI), were formed on a 0.3-mm hole in a Lexan polycarbonate partition. Junctional SR vesicles were added to one chamber, designated *cis*, composed of 0.25 M choline Cl, 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 Cl, 10 mM HEPES/Tris, pH 7.4. Heavy SR was

purified as described elsewhere (Meissner, 1984). Fusion of membranes was monitored at 0 mV as discrete jumps of Cl conductance. After fusions, both chambers were perfused with buffers containing impermeant monovalent cations and anions with Ca or Ba present as the current carrier. This cis perfusion buffer was composed of 0.95 mM $CaCl_2$, 1 mM EGTA (1.2 μM free Ca), 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 (Tris/Ca-HEPES buffer). According to our convention of signs, E_{Tris} is nominally minus infinity, $E_{divalent}$ is nominally plus infinity, and E_{HEPES} = 0 mV. For me purpose of single-channel recording, purified ryanodine receptor was shipped frozen on dry ice in 0.3% CHAPS, 0.5 M sucrose, 0.5 M KCl, 0.15% asolecithin, 50 mM Tris-Cl, pH 7.4, 0.75 mM benzamidine, 0.1 mM PMSF, 5 mM dithiothreitol. The protein concentration was 0.3-1.2 mg/ml. Channels were incorporated into planar bilayers after dilution of purified receptor in 0.3% CHAPS buffer into one of the bilayer chambers. Two ionic conditions were used routinely. For comparison with release channels of native SR, purified receptor was incorporated into planar bilayers bathed in the same Ca/HEPES (*trans*) and Tris/HEPES (*cis*) buffers described above for native channels. In this case, protein was always added to the cis side and care was taken not to break the membranes once protein was present in the solution to avoid leakage of *trans* Ca into the cis chamber. For determination of ionic selectivity (K vs. Cl or K vs. alkali cations), channels were first incorporated in symmetrical solutions composed of0.25M KC1, 10 mM HEPES/Tris, pH 7.4, followed by exchange of the cis solution with the desired composition. A double push/pull syringe system was used (model 915, Harvard Apparatus, South Natick, MA). The composition of the solution in each experiment is indicated in the figure legends and text. Data were accumulated from 12 separate preparations of purified receptor. The total time of single-channel recording was >30 h, of which 6 h were digitally stored and analyzed.

Recordings of native release channels were filtered through an eight-pole low-pass Bessel (Frequency Devices, Inc., Haverhill, MA) at 0.5-0.7 kHz and digitized at 2 kHz. Recordings of ryanodine-modified native channels were filtered at 0.2 kHz and digitized at 0.5 kHz. Recordings of purified ryanodine receptor channels were filtered at 3-10 kHz and digitized at 71 kHz. Acquisition, storage, and analysis were done on a computer using Keithley-DAS 570 software (Cleveland, OH). The handling of single-channel data has been described in detail elsewhere (Coronado and Affolter, 1986; Smith et al., 19866).

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 Chemical Co. CHAPS was from Pierce Chemicals (Rockford, IL). Bovine brain phosphatidylethanolamine and phosphatidylserine were obtained from Avanti Polar Lipids, Inc. All other reagents were reagent or analytical grade.

RESULTS

Purification of Ryanodine Receptor and Single-Channel Recording

Ryanodine receptor was purified from isolated triads as previously described (Imagawa et al., 1987) with several modifications. Solubilization was carried out in 1% CHAPS and 0.5% asolecithin instead of 1% digitonin. The extract was applied to a Mab-XA7-Sepharose column and recycled overnight. After the column was washed with 0.3% CHAPS, 0.15% asolecithin, and 0.5 M KC1, ryanodine receptor was eluted with 0.5 M KSCN (instead of NaSCN) in the presence of 0.3% CHAPS, 0.15% asolecithin. The recovery of ['Hjryanodine-binding activity from the Mab-

XA7-Sepharose column was 40-50%, which is higher than that previously reported (20-30%).

The composition of the purified ryanodine receptor was analyzed by SDS-PAGE and was consistent with a single polypeptide of M_r ~450,000. This is shown in Fig. 1 *A*. The purity of the ~450,000-dalton protein determined by gel scans was >95%. These results were similar to the previous report (Imagawa et al., 1987). Scatchard analysis of [^{3}H] ryanodine binding to the purified receptor in the presence of 10 mM ATP and 60 μM Ca (free) yielded a straight line with apparent B_{max} and K_d values of 490 pmol/mg and 7.0 nM, respectively. The ryanodine receptor purified using CHAPS as a detergent for solubilization (Fig. 1 *B*) exhibited a higher B_{max} value compared with the receptor purified using digitonin (B_{max} = 280 pmol/mg). However, the K_d value for [^{3}H] ryanodine was similar to those of the receptor purified using digitonin and isolated triads (9.0 and 6.8 nM, respectively).

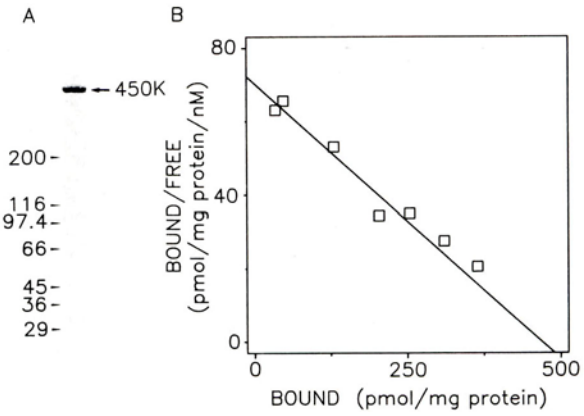

FIGURE 1. Analysis of the purified ryanodine receptor. (*A*) SDS-PAGE analysis of the purified ryanodine receptor- Purified receptor (3 μg) was subjected to SDS-PAGE on a 3-12% gradient gel and stained with Coomassie blue. Molecular-weight standards are indicated by the arrowheads. The ~450,000-dalton (450K) ryanodine receptor is indicated by an arrow. (*B*) Scatchard analysis of [^{3}H]ryanodine binding to purified receptor. Purified receptor (20 mg/ml) was incubated for 1 h at 37°C with 1-20 nM [^{3}H]ryanodine in a solution containing 0.5 M KCl, 0.5 M sucrose, 10 mM ATP, 1.11 mM $CaCl_2$ (60 μM free), 2 mM dithiothreitol, 5 μM PMSF, 0.3% CHAPS, 0.15% asolecithin, and 50 mM Tris-HCl, pH 7.4. The amount of [^{3}H]ryanodine bound was determined by the precipitation with polyethylene glycol. Nonspecific binding was determined in the presence of 100-fold excess unlabeled ryanodine and was 1.3-3% of the total binding activity.

The [^{3}H]ryanodine binding to the purified receptor was stimulated by ATP and Ca, and inhibited by ruthenium red and high concentrations of Ca. Ruthenium red inhibited the [^{3}H]ryanodine binding to the purified receptor. Micromolar concentrations of ruthenium red (IC_{50}, 4 μM) were effective in inhibiting [^{3}H]ryanodine binding in the presence of 0.5 M KCl, but submicromolar concentrations (IC_{50}, 0.4 μM) were effective in the presence of 0.15 M KCl (not shown).

Recording of channels from the purified receptor was achieved by direct dilution of the CHAPS-solubilized preparation into one of the bilayer chambers. Initial studies were done in KC1 and $BaCl_2$ solutions (Figs. 2 and 3) and a later comparison with native release channels was done in Cl-free, K-free buffers (Figs. 4 and 5). Typical chamber concentrations of protein and detergent that resulted in the incorporation

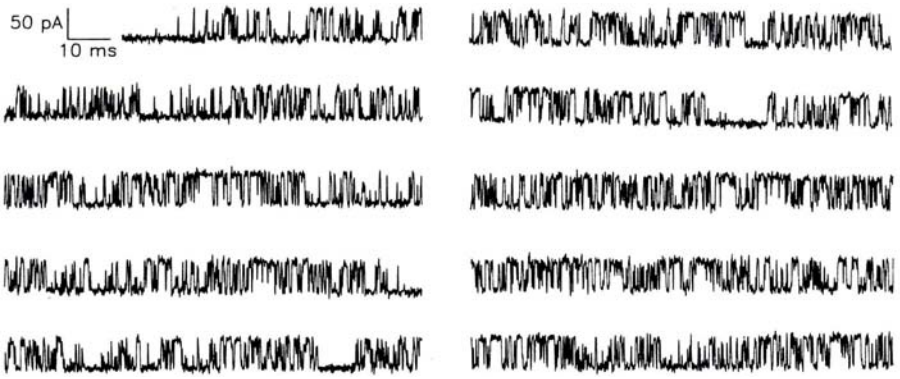

FIGURE 2. Purified ryanodine receptor channels. 1 s of continuous records (from top to bottom, left to right) of purified ryanodine receptor in 250 mM KCl, 10 mM HEPES/Tris, pH 7.4, is shown. Both *cis* and *trans* solutions have the same composition. Channels were incorporated after diluting 6 μl of receptor (0.8 mg/ml) into the 3-ml *cis* chamber. The holding potential was +100 mV; records were filtered at 3 kHz and digitized at 18 kHz.

of channels were 0.6 μg/ml and 8 μM, respectively. Extensive control experiments showed that buffer without protein or CHAPS detergent alone up to a concentration of 100 μM had no effect on bilayer conductance or on mechanical stability. In a few experiments, removal of CHAPS by extensive perfusion of the *cis* chamber or addition of up to 50 μM *cis* CHAPS had no obvious effect on channel activity. Continuous tracings of the purified receptor channel are shown in Fig. 2. In this experiment, the holding potential was 100 mV and the current carrier was 0.25 M KCl.

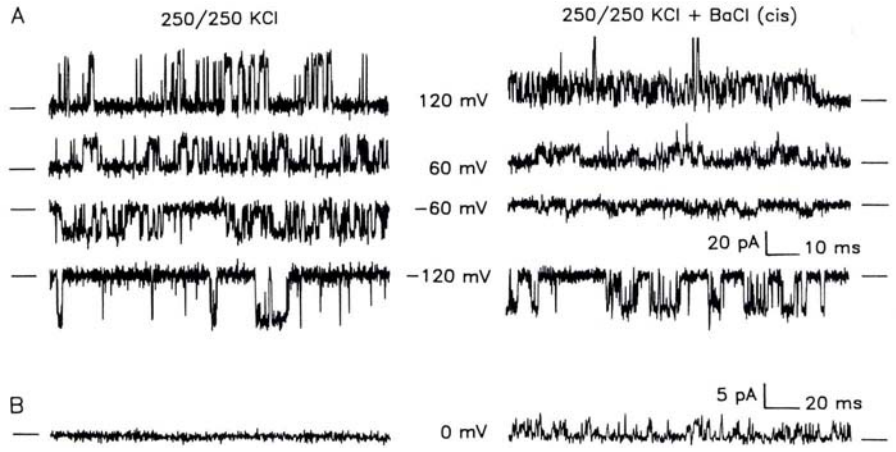

FIGURE 3. Current vs. voltage characteristics of ryanodine receptor channels in K and Ba solutions. All traces are from the same experiment at the indicated holding potentials and solutions. The horizontal bars indicate baseline current. (*A*) Recordings in symmetrical solutions composed of 0.25 M KCl (left) and after as addition of 0.1 M $BaCl_2$ (right). (*B*) Recordings on an expanded current scale of 0 mV, the reversal potential for K ions.

Three outstanding features of these channels found in each of the 12 preparations of receptors analyzed are the unit currents, which are extremely large, the kinetics of opening and closing, which are extremely fast, and the fact that monovalent ions can pass current. Permeation of divalent ions, as would be expected from a channel that is involved in Ca movement, is shown in Fig. 3. The left-hand panel shows records with 0.25 M KCl on both sides (symmetrical solutions) in the range of +120 to -120 mV. The single-channel conductance is ~360 pS, independent of voltage, and the reversal (Fig. 3 *B*) is close to 0 mV (E_K = 0 mv, E_{Cl} = 0 mV). The records in the right-hand panel of Fig. 3 are from the same channel above in symmetrical KCl plus 100 mM *cis* $BaCl_2$. At negative potentials, the slope conductance is slightly lower than for KCl alone (360 pS), while at positive potentials, there is a significant drop to ~110 pS. Fig. 3*B* (right panel) shows that in the presence of Ba, the channel reversal shifted toward the Ba equilibrium potential, and at 0 mV, there was a net positive Ba current (shown upward). In the presence of Ba, E_{Ba} « 0 (nominally minus infinity), E_K = 0 mV, and E_{Cl} = +15 mV. By extrapolation of data at positive and negative potentials, we estimate that channel reversal is in the vicinity of -26 mV. This corresponds to a Goldman permeability ratio P_{Ba}/P_K. = 4.3. The monovalent and divalent permeability under a variety of mixed salt and biionic conditions is described in detail in Figs. 10-12.

Comparison of Native and Purified Ryanodine Receptor Channels in Ca-Tris Solutions

The observation that ryanodine receptor protein formed divalent-conducting channels of large unitary conductance suggested a similarity to native release channels of SR described previously in planar bilayers (Smith et al., 1985, 1986*a, b*). Therefore, we decided to compare native and purified channels side by side in identical solutions. For this purpose, we used Ca as the intra-SR current carrier, Tris as the myoplasmic cation, and HEPES as the anion on both sides. The use of Tris and HEPES as the large impermeant cation and anion eliminated currents through K and Cl channels that are present in native SR vesicles and become incorporated into the bilayer along with the release channel (Smith et al., 1985). Fig. 4 shows native release channels and purified receptors in Cl-free, K-free buffers. Prolonged stationary activity of native channels was elicited by *cis* micromolar free Ca, *cis* millimolar ATP, or both (Smith et al., 1986*b*). In this particular case, channels were activated by 1.2 μM *cis* Ca in the absence of ATP. In the range of 0 to -80 mV, native channels displayed a single conductance state, which in 54 mM *trans* Ca had a unit value of ~110 pS. This value is the same as in previous reports (Smith et al., 1985). Records corresponding to this conductance are shown in Fig. 4 at a holding potential of 0 mV (labeled "native channel"). However, we identified at least two conductance states in the purified receptor (four states are actually resolved in KCl and CsCl; see Fig. 13). Like native release channels, the most frequent conductance of the purified receptor was 110 pS. At large negative potentials, a second conductance state of 50 pS was present in ~20% of the records. Both conductances are shown in Fig. 4 at a holding potential of -80 mV (labeled "purified receptor"). The occurrence of low-conductance states in native channels was rare at all potentials, and, when present, the states were not fully resolved at frequency bandwidths of up

to 1 kHz. The divalent cation selectivity measured in Ca-Tris was found to be the same for both native and purified channels. This is shown in the current-voltage curve at the bottom of Fig. 4, where the squares correspond to native release channels and the circles correspond to purified ryanodine receptor. Not only is the slope conductance the same (110 ± 10 pS), but the reversal of currents is also the same for both sets of data, approximately +38 mV. Thus, native release channels and purified ryanodine receptors are indeed very similar. For that voltage and salt concentration, the Goldman permeability ratio P_{Ca}/P_{Tris} is 13-7. We previously reported P_{Ca}/P_{Tris} = 8.7 for native and digitonin-purified ryanodine receptors under similar

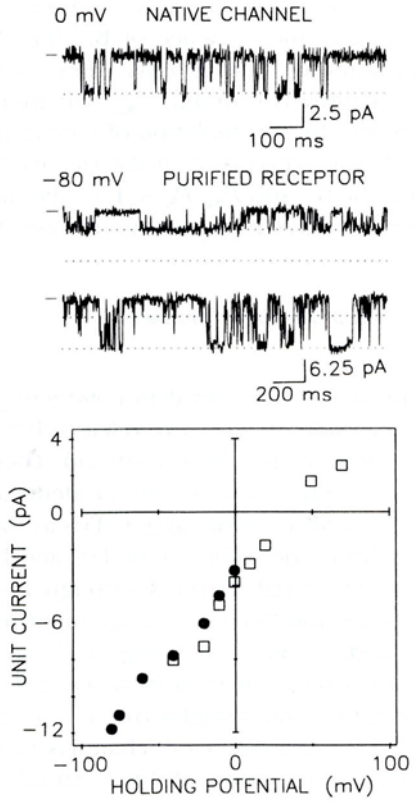

FIGURE 4. Comparison of ryanodine receptor and native Ca-release channels. The solid lines indicate baseline current and the dotted lines indicate open current levels. Native release channels were recorded in Tris/Ca-HEPES buffer with 54 mM *trans* Ca as the current carrier and 1.2 μM free Ca (0.95 mM $CaCl_2$ and 1 mM EGTA) in the *cis* chamber. Ryanodine receptor channels were recorded in the same solutions after diluting purified receptor (0.6 μg/ml protein) into the CM chamber. The upper recording of purified receptor shows a 50-pS conductance state; the lower recording shows the most common 110-pS conductance state. Single-channel current-voltage relationships are given for native release channels (squares) and ryanodine receptor channels (circles) in the solutions described above. Current reversal in both cases was approximately +38 mV.

conditions (Imagawa et al., 1987). The present data are more accurate, however, since we now report true reversal of single-channel currents, whereas before we relied on extrapolated reversals for calculations.

A second major similarity between the ryanodine receptor and native release channels was found in the effects of the alkaloid ryanodine. We have shown previously that micromolar ryanodine opens the native release channel into a conductance state of lower unitary value and much longer lifetime than controls (Imagawa et al., 1987). In the present work, we tested ryanodine in the micromolar and nanomolar ranges using two different protocols. We used micromolar concentrations in direct addition experiments (Fig. 5) in order to reduce the time between

drug addition and single-channel modification to no more than a few seconds. We were forced to use high concentrations because the forward rate constant for binding of ryanodine to its receptor is extremely slow. At 5 nM ryanodine, the time constant of association under optimal conditions is ~40 min (Pessah et al., 1986). Unfortunately, this waiting time (and perhaps longer) is beyond the average duration of our single-channel experiments. Effects in the nanomolar range, close to the equilibrium dissociation constant of radiolabcled ryanodine, were measured by preincubating the purified receptor with low concentrations of ryanodine for several hours before bilayer incorporation and channel recording (Fig. 6). In Fig. 5, we compare native release channels and purified receptors, both modified by exposure

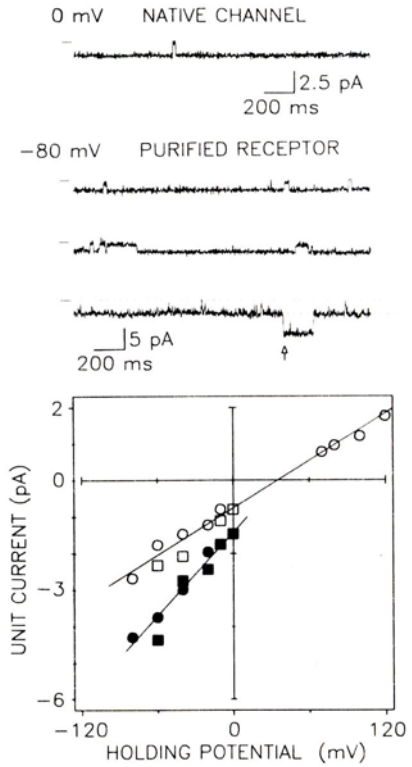

FIGURE 5. Effect of ryanodine on native release channel and purified ryanodine receptor. Native and purichannels were recorded in the Tris/Ca-HEPES buffers described in 4 in the presence of 7 μM ryanodine added to the *cis* side. The line indicates baseline current; holding potential is indicated in record. Single-channel current voltage relationships are for native (squares) and purified recep- (circles). The filled and open symare for the large and small conductance, respectively. The slopes drawn through data points are from linear regressions: 40 ± 2 pS (filled symbols) and 22 ± 3 pS (open sym- Current reversal is at approximately +35 mV.

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to 7 μM ryanodine added to the *cis* side. Control channels before drug addition are shown in Fig. 4 at the same holding potentials. The onset of effects was within or immediately after the 30-s stirring period after *cis* addition. In native channels (Fig. 5, top records, 0 mV), ryanodine promotes openings of long duration, with an average conductance of 40 pS. The control conductance in the absence of ryanodine averaged 110 pS in the same solutions (Fig. 4). In the purified receptor, openings induced by ryanodine (Fig. 5, bottom records, -80 mV) were as long as in native channels but were associated with two unitary conductances of average slope value 22 ± 3 and 40 ± 2 pS (open and filled circles, respectively, in Fig. 5, bottom). The control conductances of the purified receptor averaged 110 and 50 pS (Fig. 4). The

opening and closing of a 40-pS-type channel, while a 22-pS-type remains open throughout the trace, is shown by the arrow in the bottom record of Fig. 5. In native and purified channels, a reduction in conductance was accompanied by dramatic increases in mean open time, as can be seen by comparing control (Fig. 4) and drug-modified (Fig. 5) records. Current-voltage curves for native (squares) and purified receptor (circles) are given in Fig. 5 (bottom). The solid lines correspond to linear regression of four sets of data, the two conductance states observed in the purified receptor (open and filled circles), and similar conductances observed in native channels (open and filled squares). In native channels, the 22-pS state was far less frequent that in the purified receptor. Both the 22-pS (open symbols) and the 40-pS (filled symbols) channels have reversal potentials that extrapolate to approximately +35 mV or to a Goldman permeability ratio P_{Ca}/P_{Tris} = 11. This value is slightly larger than previously reported in the digitonin-purified receptor (Imagawa et al., 1987) but slightly smaller than the same ratio determined in the absence of ryanodine in native channels (Smith et al., 1985). Rousseau et al. (1987) also reported

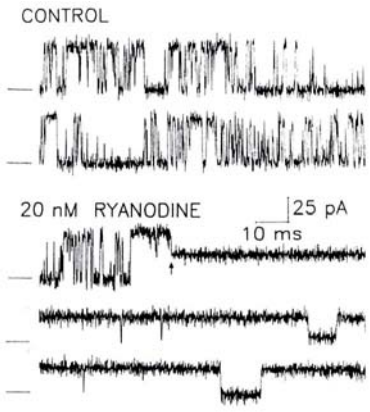

FIGURE 6. Modification of ryanodide receptor channel by a low concentration of ryanodine. Purified receptor at a final concentration of 2 μg/ml was added to the *cis* chamber after 2 h incubation without (control) or with alkaloid (20 nM ryanodine). The *cis* and *trans* solutions were composed of 0.25 M KCl, 10 mM HEPES/Tris, pH 7.4. The holding potential was +100 mV. The horizontal bars indicate baseline current. The arrow indicates the transition from control to the drug-modified condition.

a small decrease in the permeability ratio P_{Ca}/P_{Tris} of the native channel in the presence of ryanodine. This could reflect some alteration of pore selectivity induced by the alkaloid.

The effect of nanomolar concentrations of ryanodine on a purified receptor channel is shown in Fig. 6. The current carrier is 0.25 M K and the holding potential is +100 mV. In this experiment, 10 μg of receptor protein in CHAPS buffer (see Materials and Methods) was incubated on ice at final protein and ryanodine concentrations of 0.6 mg/ml and 20 nM, respectively. Aliquots were tested in planar bilayers at different times to determine whether long-term exposure to a low concentration of ryanodine could actually modify channels. The records of Fig. 6 correspond to receptor exposed to the alkaloid for 2 h (labeled "20 nM Ryanodine") and receptor incubated without alkaloid for the same time (labeled "control"). Control channels had the characteristic large conductance and fast gating of unmodified channels. The 2-h exposure was sufficient to induce, in most cases (8 recordings out

of 12), the lowered conductance and increased mean open time typical of ryanodine-modified channels. The ryanodine-exposed channel of Fig. 6 corresponds to a case in which the transition to the drug-modified state actually occurred during the recording period. This is indicated in Fig. 6 by the arrow. Transitions in the opposite direction, that is, from low-conductance slow kinetics to high-conductance fast kinetics, were never observed. Hence, the modification is essentially irreversible within the time scale of the experiment. Channel conductance was 360 pS in control and 190 pS in ryanodine-modified channels.

Activation of Ryanodine Receptor Channel by Co. and ATP and Inhibition by Ruthenium Red

In agreement with our report on native release channels (Smith et al., 1986*a, b*), we found that ryanodine receptors are activated by micromolar Ca and millimolar ATP

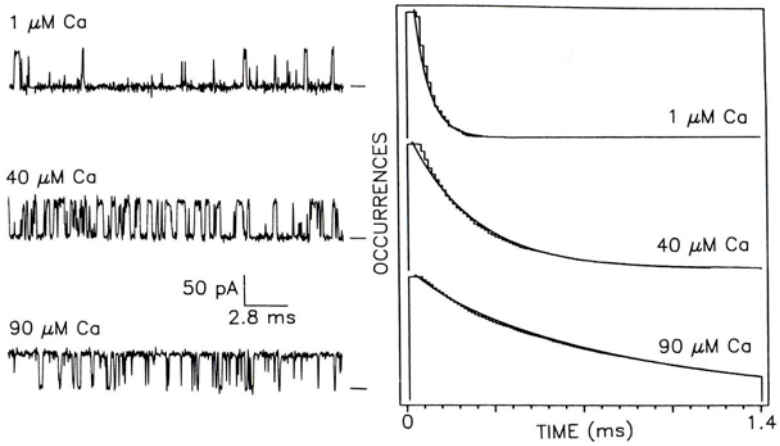

FIGURE 7. Activation of ryanodine receptor channels by Ca. Channels were recorded at +60 mV in symmetrical 245 mM KC1, 10 mM K-HEPES, pH 7.4. Openings are upward and the horizontal lines denote baseline current. Recordings were filtered at 10 kHz and digitized at 71 kHz. The left-hand side corresponds to recordings in 1, 40, and 90 μM *cis* Ca. The open probability was 0.014, 0.55, and 0.85 at 1, 40, and 90 μM *cis* Ca, respectively. The right-hand side corresponds to cumulative histograms of open times fitted with time constants (solid lines): 1 μM Ca (0.06 ms), 40 μM Ca (0.2 and 0.5 ms), 90 μM Ca (0.18 and 0.92 ms).

and inhibited by ruthenium red. The activating effects of Ca and ATP were studied using K as the current carrier in order to optimize our frequency resolution, given that K currents through the channel are much larger than Ca currents. In Fig. 7, a single ryanodine receptor channel was monitored at a holding potential of 60 mV in symmetrical 245 mM KCl. Stationary open probabilities were measured at constant *voltage and ligand (Ca, ATP) concentrations. Approximately 120 s of single-channel data were stored under each condition at 10 kHz bandwidth and 71 kHz sampling rate. A representative segment of 20 s was actually used in each calculation. In the presence of 1* μM free *cis* Ca, the open probability was 0.014. Raising *cis* Ca to 40

μM produced a dramatic increase in the number of openings as well as in the mean duration of each open event; the open probability (P_O) at this Ca concentration was 0.55. At 90 μM Ca, P_O was further increased to 0.85. Analysis of the Hill coefficient for Ca activation revealed a value n = 1.33 (not shown), which suggests that cooperative binding of Ca ions is required to optimally activate the channel. Fig. 8 shows that millimolar ATP also stimulates activity of the purified receptor. In the presence of 10 μM free Ca, P_O was 0.16 (Fig. 8, top). Addition of 5 mM ATP to the *cis* chamber increased P_O to 0.60 and increased the mean duration of openings- 10 mM ATP further increased P_O to 0.93. The activating effects of Ca and ATP were observed routinely with K or Ca as the current carrier; however, some channels responded to *cis* additions, while others responded to *trans*-side additions. We attribute this to a possible random orientation of purified receptors in the bilayer.

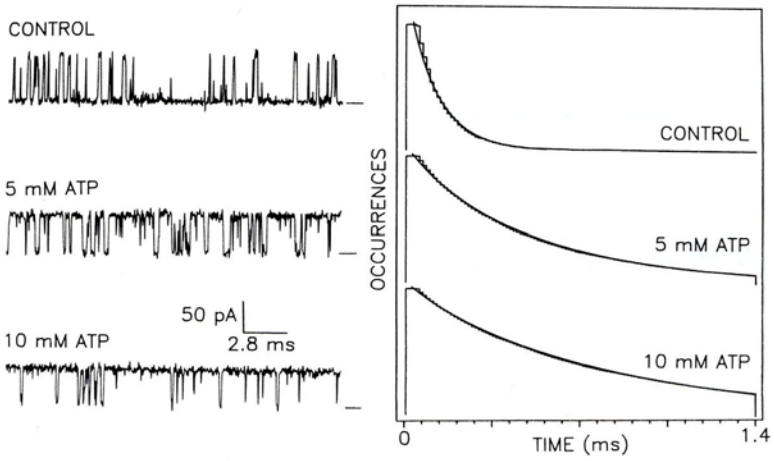

FIGURE 8. Activation of ryanodine receptor channels by ATP. Channels were recorded at +80 mV in symmetrical 245 mM KCl, 10 μM $CaCl_2$ 10 mM K-HEPES, pH 7.4. Openings are upward and the horizontal lines denote baseline current. Recordings were filtered at 10 kHz and digitized at 71 kHz. The right-hand side corresponds to control, plus 5 mM CM ATP and plus 10 mM *cis* ATP, respectively, all from the same channel. The left-hand side corresponds to cumulative histograms of open times fitted with time constants (solid lines): control, 10 μM Ca (0.12 ms), plus 5 mM ATP (0.18 and 0.84 ms), plus 10 mM ATP (0.18 and 0 92 ms).

Distributions of open times in Ca (Fig. 7) or in Ca plus ATP (Fig. 8) revealed channel kinetics that are ligand dependent and extremely fast, on the time scale of microseconds. Each histogram was constructed by collecting 2,000-2,500 events at a sampling rate of 14 μs/point and a cutoff filter frequency of 10 kHz. A 50% threshold detector was used to separate openings from baseline current. Inspection of the data in terms of a plot of event duration vs. event amplitude (Coronado and Affolter, 1986) indicated that no severe attenuation occurred for durations $\geq$ 42 μs (not shown). In 1 μM Ca (Fig. 7, top) and 10 μM Ca (Fig. 8, top), open-time distributions were monoexponential and could be adequately fitted with time constants of 60 and 120 μs, respectively. Increasing Ca to 40 μM (Fig. 7, middle) or addition

of 5 mM ATP (Fig. 8, middle) resulted in an increase in channel lifetime to ~200 μs and the appearance of a new population of channels with a mean duration of 0.5—0.8 ms. These results are in excellent agreement with single-channel kinetics reported for native release channels (Smith et al., 1986*b*) in that Ca, Ca-ATP, and ATP, in the same concentration range tested here, were shown to increase channel durations as well as P_O. However, the resolved kinetics of the native channel were significantly poorer than in the present report. For example, we previously reported (Imagawa et al., 1987) single-channel lifetimes in 2 μM free Ca of 32 and 180 ms, which are approximately three orders of magnitude slower than the present measurements.

Finally, the blocking action of ruthenium red, a functionally specific blocker of the native release channel (Smith et al., 1985), is shown in Fig. 9. Ruthenium red was found to block either *cis* or *tram*, varying with each channel tested. As shown in the center record of Fig. 9, 1 μM ruthenium red is sufficient to block channels in an all-or-none fashion. This observation is similar to that described in native SR, where

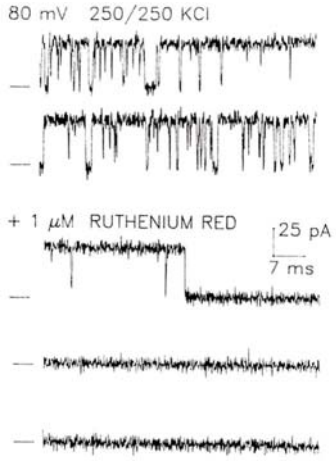

FIGURE 9. Inhibition of ryanodine receptor channels by ruthenium red. A single ryanodine receptor was recorded at +80 mV in symmetrical 250 mM KCl, 10 mM HEPES/Tris, pH 7.4. 1 μM ruthenium red was added *tras*; inhibition occurred ~10 s after addition (middle trace). The horizontal bars indicate baseline current.

the dye shifted the distribution of channels from all open to all closed (Smith et al., 1986*a*). The specificity of ruthenium red at the functional level is supported by the observation that dihydropyridine-sensitive Ca channels from the same source of muscle are insensitive to this agent in the range 1-5 μM (unpublished).

Permeability of Ryanodine Receptor to Monovalent and Divalent Ions

Taking advantage of the purified receptor preparation, we attempted to estimate the Ca and K conductance of the channel in physiological salts. In resting muscle, K ions are approximately at equilibrium across the SR membrane (Somlyo et al., 1981), whereas Ca is several orders of magnitude higher inside the SR compared with the cytoplasm. Fig. 10 shows that channels in symmetrical 150 mM KC1 have an extremely large unit conductance, 639 ± 14 pS. Openings are spontaneous, given that the buffer contains micromolar levels of contaminating Ca, which are sufficient to activate the channel. Open-time histograms in symmetrical 150 mM KCl could be

fitted with a single-exponential time constant of 0.1 ms (not shown). Addition of 5 mM $CaCl_2$ to the *trans* chamber (Fig. 10, middle) caused a drop in the slope conductance to 242 ± 9 pS. There was also a shift in the reversal potential from 0 to +5 mV, which indicates that Ca was indeed permeable. Single-channel currents in the presence of 5 mM $CaCl_2$ were longer in duration than those measured in KCl alone. Open-time histograms could be fitted with two time constants of 0.2 and 5 ms (not shown). Assuming a zero Cl permeability (demonstrated in Fig. 11 A), we calculated that the 5-mV shift in reversal in Fig. 10 (bottom) is consistent with a P_{Ca}/P_K. of 4. Thus, in physiological salts, in addition to Ca current, there is a substantial contribution of K current. This result, obtained at low concentrations of K and Ca, is consistent with the reversal potential of Fig. 12 *A* measured at a high salt concentration.

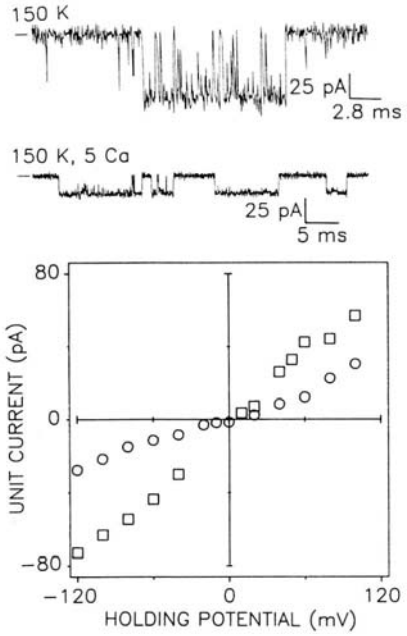

FIGURE 10. Currents through the ryanodine receptor channel in mixed K and Ca solutions. (*Upper recording*) Single ryanodine receptor channels recorded in symmetrical 150 mM KCl solutions. Currents were 63 ± 2 pA at — 100 mV. (*Lower recording*) Same channel after 5 mM $CaCl_2$ was added *trans*. Single currents at — 100 mV were 22 ± 1.5 pA. (*Bottom*) Current vs. voltage curve in symmetrical 150 mM KCl (squares) and symmetrical 150 mM KCl plus 5 mM *trans* $CaCl_2$ (circles). The slope conductances close to 0 mV were 638 ±14 pS (squares) and 242 ± 9 pS (circles). Current reversal in symmetrical 150 mM KCl was 0 mV. With addition of 5 mM *trans* $CaCl_2$, reversal shifted to +5mV.

In buffers containing Cl salts of monovalent cations, receptor channels were found to behave as Nernst-selective for cations over Cl. Fig. 11 *A* is a current vs. voltage relation constructed in 50 mM *cis*/250 mM *trans* KCl. Under these conditions, the Nernst equilibrium potential for K is +39 mV and the Cl equilibrium potential is -39 mV. Single-channel currents revert at approximately +40 mV, which indicates that the Cl permeability was zero. As a function of the symmetrical concentration of KCl, conductance through the channel saturates after what is apparently a single Langmuir isotherm. However, deviations from one-site conductance behavior at low salt cannot presently be discarded. In Fig. 115, the slope conductance has been plotted as a function of K activity and fitted with a rectangular hyperbola having the parameters K_m = 47 mM and γ_{max} = 1.03 nS. The inset in Fig. 11 *B* is an Eadie-Hofstee plot of the conductance-activity data. A straight line

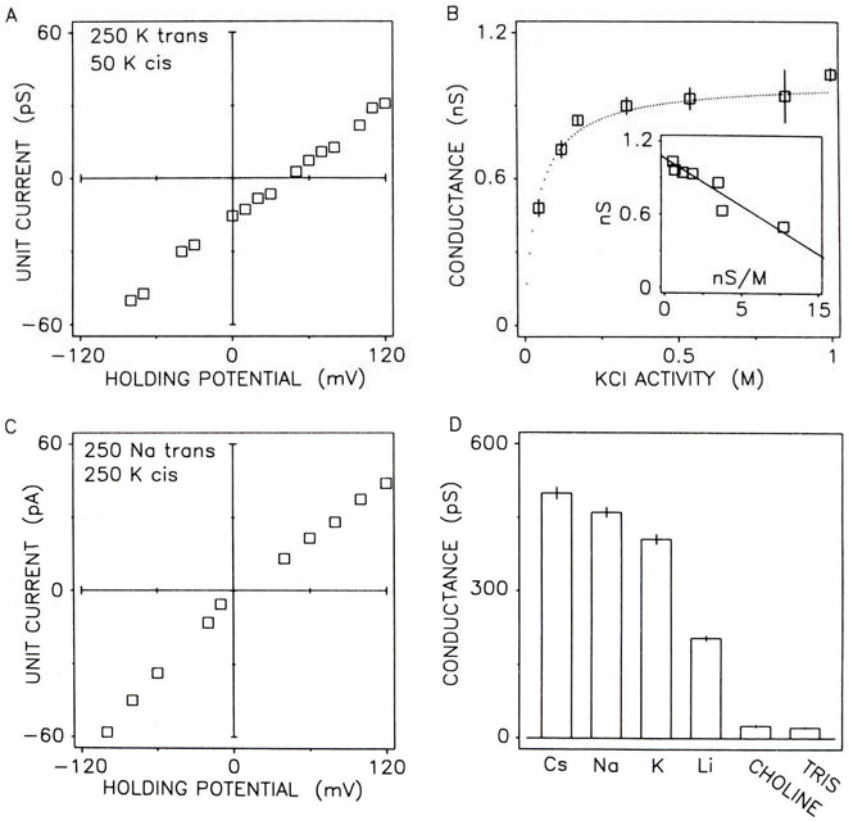

FIGURE 11. Monovalent cation current through ryanodine receptor channels. (*A*) Single-channel current-voltage relationship for channels recorded in 50 mM CM KC1 and 250 mM *trans* KCl; 10 mM K-HEPES, pH 7.4, was present in both chambers. Current reversal was at +40 ± 2 mV. The Nemst reversal for K is +39 mV. (*B*) Saturation of K conductance according to a rectangular hyperbola with K_s = 47.3 mM and γ_{max} = 1.032 nS. The inset is an Eadie-Hofstee plot of K conductance vs. concentration. The line drawn through data points has a slope of -0.0473 M and a *y*-intercept at 1.032 nS. The concentration of KCl was increased symmetrically in both solutions. (*C*) Single-channel current-voltage curve for channels recorded in 250 mM *cis* KCl and 250 mM *trans* NaCl; 10 mM K-HEPES or Na-HEPES, pH 7.4, was present in the *cis* and *trans* sides, respectively. Current reversal was at 0 mV. (*D*) Single-channel conductance for selected monovalent cations recorded in 50 mM cis and 250 mM *trans* test cation concentration. Unit conductances were 500 ± 12, 461 ± 10, 406 ± 10, 205 ± 5, 26 ± 2, and 22 ± I pS for Cs, Na, K, Li, choline, andTris, respectively. Cl salts were used in all experiments.

with a slope of -0.047 M and a *y*-intercept at 1.03 fitted the data with a correlation coefficient >0.95.

Even though receptor channels conduct K ions, they show no specificity for this cation. Fig. 11 *C* is a current-voltage relation obtained in biionic 250 mM KCl and 250 mM NaCl. Channels revert at ~0 mV, which indicates an equal permeability to both K and Na. All biionic pairs of Cs, Na, K, and Li were tested and selectivity was

found to be lacking in each case. However, single-channel conductance does vary significantly among monovalents. Fig. 11 *D* compares the slope conductance of Cs, Na, K, Li, and two organic cations, choline and Tris, in experiments with each of the test cations separately. Among the alkali cations, Cs had the highest (490 pS) and Li had the lowest (200 pS) conductance. A dramatic decrease in conductance is seen for the larger organic cations, which probably reflects the sieving properties of the pore. The Tris and choline conductances are ~ 15 times lower than the K conductance.

In large gradients of divalent salts, receptor channels displayed selectivity for divalents; however, permeability ratios were not found to be exceedingly large. A current-voltage curve under biionic conditions with *trans* 50 mM Ca and *cis* 50 mM K is shown in Fig. 12 *A*. Reversal is at +39 mV, corresponding to a permeability ratio P_{Ca}/P_K = 6.6. The slope conductance with 50 mM Ca as the current carrier was 123 ± 3 pS, a value that agrees well with the 110 ± 10 pS obtained in Tris/Ca-HEPES buffer using 54 mM Ca as the current carrier (Fig. 4). Like the K conductance described previously, the Ca conductance in the activity range of 7-100 mM saturates according to a Langmuir isotherm. Additional data are needed in the low concentration range, however, to decide whether single-site behavior is significant. In Fig. 12*B*, the $CaCl_2$ concentration was varied symmetrically and conductance vs. activity data were fitted to a rectangular hyperbola with parameters K_m = 3 mM and γ_{max} =172 pS. When data were plotted in an Eadie-Hofstee relation, a straight line drawn with slope –0.003 M and *y*-intercept = 0.172 nS included all but the data point at the lowest concentration (4 mM). Other divalents, such as Mg and possibly Ba (see Fig. 3), can pass current through the purified receptor with conductances similar to that of Ca. The case of Mg is particularly interesting because this ion is highly conductive in native release channels when present in the *trans* intra-SR compartment, but it blocks open channels when present in the *cis* myoplasmic compartment (Smith et al., 1986*b*). We tested Ca vs. Mg permeability under biionic conditions (Fig. 12 *C*), that is, with only one current carrier present in each side. This simplified interpretation of the results as well as the numerical computation of permeability ratios. Using *cis* 50 mM $MgCl_2$ against *trans* 50 mM $CaCl_2$, the slope conductance averaged 140 pS and the reversal potential was extrapolated to ~10 mV or a Goldman permeability ratio P_{Ca}/P_{Mg} = 2.3. This value is close to the ratio of mobilities of the two cations in solution, namely 1.21 (Parsons, 1959). The purified receptor thus achieves little if any discrimination among the tested divalents. The fact that the current-voltage relationship in Fig. 12 *C* is approximately linear at positive and negative potentials implies that *cis*-to-*trans* Mg current flows with equal ease as *trans*-to-*cis* Ca current, i.e., that there is no *cis* block by Mg as described in native release channels (Smith et al., 1986*b*). This discrepancy could be explained in several ways, none of which can be formally discarded at the present time. One possibility is that the purified receptor lacks intrinsic modulation by Mg. We think this is not the case, based on the results shown in Fig. 12 *D*. A second explanation may be related to the orientation factor. Because the orientation of each channel in the bilayer is more or less random, the ionic conditions themselves would tend to select as active those channels in which the Mg blocking site faces into the *trans* chamber. Those with the Mg site facing into the *cis* chamber would not conduct current since they would be

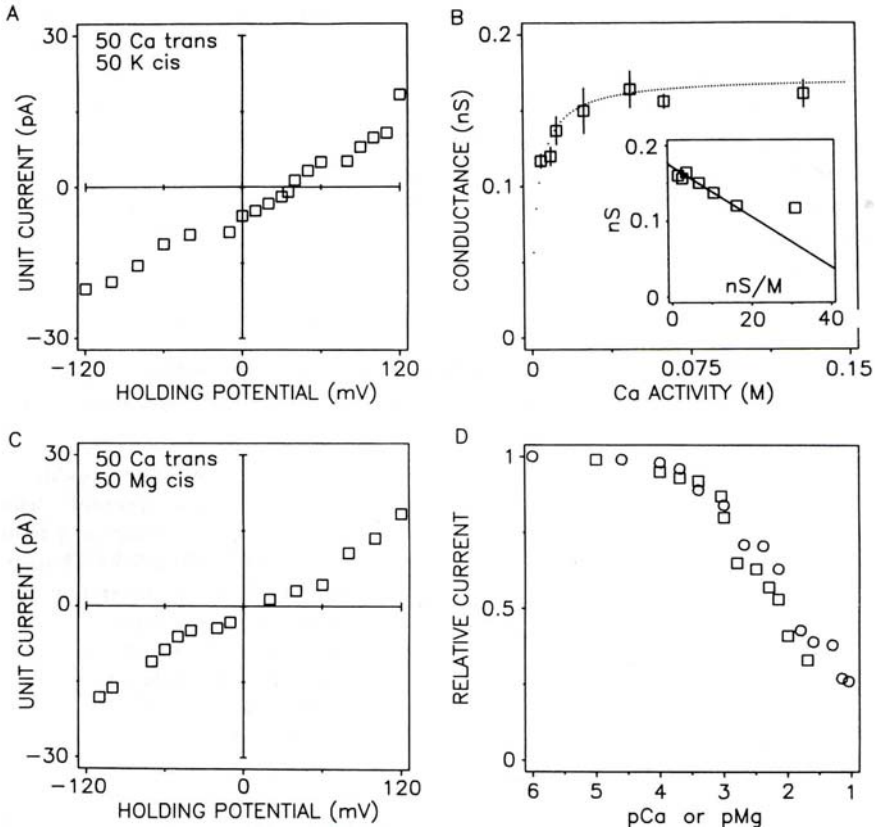

FIGURE 12. Divalent cation current through ryanodine receptor channels. (*A*) Single-channel current-voltage curve for channels recorded in 50 KCl, 10 mM K-HEPES, pH 7.4 CM, and 50 $CaCl_2$, 10 mM Ca-HEPES, pH 7.4 *trans*. The slope conductance is 123 ± 3 pS. The current reversal is +39 mV, which gives a permeability ratio P_{Ca}/P_K = 6.6. (*B*) Saturation of Ca conductance according to a rectangular hyperbola with fitted parameters K, = 3 mM and γ_{max} = 172 pS. The inset is an Eadie-Hofstee plot of Ca conductance vs. concentration. The line drawn through data points has a slope of —0.003 M and a *y*-intercept at 0.172 nS. The concentration of $CaCl_2$ was increased symmetrically in both solutions. (*C*) Single-channel current-voltage curve for channels recorded in 50 mM $MgCl_2$, 10 mM Mg-HEPES, pH 7.4 cis, and 50 mM $CaCl_2$, 10 mM Ca-HEPES, pH 7.4 *trans*. Current reversal was at 0 mV. (*D*) Blockade of K current by Ca and Mg. Channels were recorded at +60 mV in symmetrical 245 KCl, 10 mM K-HEPES, pH 7.4. $CaCl_2$ (squares) or $MgCl_2$ (circles) was added to the *cis* chamber. The IC_{50} for both Ca and Mg is 2.5 mM.

actually blocked by *cis* Mg. Finally, it is also possible that high *trans* Ca actually prevents blockade by *cis* Mg. We tested the latter by measuring K current in the presence of 1 μM to 100 mM Mg or Ca. Fig. 12 *D* plots single-channel current at a holding potential of + 60 mV as a function of divalent added to the *cis* chamber. In this experiment, the current carrier was 245 mM K on both sides. Control conductance in the absence of added divalent was taken as unity. As can be seen, Ca

(squares) and Mg (circles) are equally effective in decreasing K current through the channel, with an apparent K_d of ~2.5 mM. A similar curve was obtained when titrations were performed on the trans chamber, which implies either that divalent binding sites can be found on both ends of the protein or that a *cis*-located site is accessible by *trans*-added divalent and vice versa. The apparent K_d is in good agreement with measurements of Mg block in native channels (Smith et al., 1986*b*) and with the decrease in channel conductance shown in Fig. 10, where 5 mM Ca reduced conductance 2.6-fold. A Langmuir isotherm with a K_d of 2.5 mM would have predicted a 3.3-fold reduction. Hence, the qualitative agreement is clear.

Subconductance States of the Ryanodine Receptor Channel

In almost all instances, we found that the purified receptor channel displayed several conductance states. Fig. 13 shows the most commonly observed case of transi-

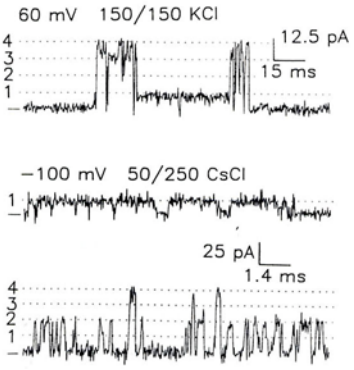

FIGURE 13. Substate conductances of the ryanodine receptor channel. (*A*) Channels recorded at a holding potential of +60 mV in symmetrical 150 mM KCl. The maximum unitary current was 44 ± 4 pA. The subconductance unit current was 11 ± 1 pA. (*B*) Channels at -100 mV in 50 mV *cis*/250 mM tram CsCl with 10 mM Cs-HEPES, pH 7.4, in both chambers. The upper recording shows a "monomer" channel with a unit current of 14 ± 1 pA. The lower recording shows "dimer" and "tetramer" channels. Currents were 31 ± 2 pA and 63 ± 2 pA for "dimers" and "tetramers," respectively. The horizontal bars indicate baseline current and the dotted lines denote open current levels.

tions of channels among four equally spaced conductance levels. We have referred to these levels as the monomer state, dimer state, trimer state, etc., given the mounting evidence that the functional receptor is a large-sized aggregate (Inui et al., 1987). In monovalent cation solutions, the channels most frequently appear as "dimers" or "tetramers." For instance, in 50/250 mM CsCl, dimers and tetramers would correspond to 250-pS and 500-pS states, respectively (Fig. 13, lower recordings). Less frequently seen are transitions to monomer or trimer states. Fig. 13 (upper recording) shows a channel in symmetrical 150 mM KCl, which opens into a tetramer of full conductance state. It then gates between tetramer and trimer states until it closes to a monomer conductance. The channel finally opens to the full conductance and flickers between fully open and fully closed a few times before completely closing for a long period. Such multistate openings that appear coupled in

time were fairly common in all records. In symmetrical 250 mM KCl, the most frequent states had average conductances of 92, 190, and 390 pS.

DISCUSSION

Ryanodine Receptor Preparation

In a recent report (Imagawa et al., 1987), we showed Ca channel activity in a digitonin-solubilized preparation of ryanodine receptor. However, in digitonin, channels did not reproduce the single-channel conductance or ligand-sensitive states of the native Ca-release channel. For example, under conditions similar to those in Figs. 4 and 5, the largest unit conductance in digitonin preparations was 35 pS and the lowest was 22 pS. Here we have shown that the largest conductance in the CHAPS preparation, similar to native channels, is 110 pS and the lowest is 50 pS. Equally significant, digitonin-purified channels were not responsive to ryanodine, nor did they activate in the presence of millimolar ATP and micromolar Ca. However, binding of ryanodine to the digitonin-purified receptor had a high affinity (K_d = 9 nM, B_{max} = 280 pmol/mg), similar to that seen in native SR, and it was enhanced by ATP and inhibited by ruthenium red (Imagawa et al., 1987). Thus, in the digitonin preparation, the receptor is functional with respect to ligand binding parameters but the channels lack some of the properties associated with functional release channels.

In this study, we have modified the purification procedures by using CHAPS and KSCN as the detergent and eluant, respectively. The purified receptor consisted of a single ~450,000-dalton polypeptide. The purity of the ~450,000-dalton polypeptide in purified receptor was >95%, the same as the previous report using digitonin and NaSCN as the detergent and eluant, respectively (Imagawa et al., 1987). The purified receptor exhibits higher [^{3}H]ryanodine-binding activity and a similar affinity for [^{3}H]ryanodine as compared to the previous purified receptor (Imagawa et al., 1987). Inui et al. (1987) have reported the purification of the ryanodine receptor using CHAPS as a detergent. Their purified receptor contains three major polypeptides of M_r 360,000, 330,000, and 175,000, and exhibits high [^{3}H]ryanodine-binding capacity (B_{max} = 393 pmol/mg, K_d = 78 nM). Our previous purification using digitonin as a detergent demonstrated that ryanodine receptor consists of a single ~450,000-dalton polypetide. However, the [^{3}H]ryanodine-binding capacity of our previous purified receptor (280 pmol/mg) was lower than theirs. The results shown in this report indicate that the low [^{3}H]ryanodine-binding capacity of our previous purified receptor is not due to a missing subunit, but to the inactivation of [^{3}H]ryanodine-binding activity during the elution from the affinity column. [^{3}H] Ryanodine binding to the purified receptor was inhibited by micromolar ruthenium red, as in the previous reports. Under low salt conditions (150 mM KCl), which were used in triads, IC_{50} decreased to 0.4 μM. These results indicate that the receptor purified in the presence of CHAPS and asolecithin exhibits a high [^{3}H]ryanodine-binding capacity and the same affinity for [^{3}H]ryanodine as that in triads, but a lower affinity for ruthenium red than that in triads. Previously, we identified the digitonin-purified receptor as the pore component of the native Ca-release channel primarily based on ionic selectivity data. We also suggested, among several possibilities, that

other junctional proteins may contribute to the formation of functional release channels (Imagawa et al., 1987). We now believe that this is not the case inasmuch as the 450,000-dalton receptor alone, when purified using CHAPS, is capable of reconstituting the complete repertoire of pharmacological, selectivity, and gating properties associated with the Ca-release channel of SR.

Comparison of Ryanodine Receptor and Ca-Release Channels

A characteristic feature of purified ryanodine receptor channels is fast gating kinetics composed of two, and possibly more, open and closed states. We previously concluded that, at a bandwidth of 0.3 kHz, ATP, in the presence of micromolar Ca, increased the lifetime of native SR release channels (Smith et al., 1986*b*). Adenine nucleotide—dependent activation of Ca release has been reported in ^{45}Ca efflux experiments in junctional SR vesicles (Meissner, 1986; Sumbilla and Inesi, 1987) and in skinned fiber tension experiments (Endo, 1981). The present data on the purified receptor confirm this observation at a much higher frequency resolution than previously achieved. We find that, at 10 kHz bandwidth, ATP shifts the equilibrium distribution of channels from closed to open and also lengthens the average open time. Evidently, ATP induces a new population of channels (or a new open state) with a lifetime of ~1 ms, not seen in controls (Fig. 8). The increase in time resolution is mostly due to the 10-times-larger channel conductance that is obtained when monovalents instead of divalents are used as current carriers. In previous measurements in native SR, we were forced to exclude monovalent ions from solution in order to prevent currents from background K and Cl channels.

The present work shows that two agents that interfere with Ca release in SR, namely ruthenium red and ryanodine, interact directly with the channel formed by the 450,000-dalton receptor protein. Ruthenium red in the micromolar range inhibits ryanodine binding to the purified receptor (Imagawa et al., 1987), inhibits ^{45}Ca efflux from junctional SR vesicles (Smith et al., 1985), blocks Ca-release channels in planar bilayer experiments (Smith et al., 1985, 1986*a*), and reduces fiber tension in skinned fiber experiments (Voipe et al., 1986). Our results (Fig. 9) confirm the most straightforward explanation, i.e., that ruthenium red shuts the release channel by direct interaction of the dye with the ryanodine receptor protein. Ryanodine, on the other hand, over prolonged periods, has also been shown to reduce fiber tension. However, contrary to earlier interpretations (Cannell et al., 1985; Fabiato, 1985; Sutko et al., 1985; Wier et al., 1985), we find that ryanodine opens the release channel, i.e., it does not inhibit Ca release. This was shown rather dramatically in Fig. 6, where 20 nM alkaloid transformed the fast kinetics of the unmodified channel into a slow, almost always open conformation. We find this result significant for two reasons. First, because effects could be observed at concentrations close to the equilibrium dissociation of the ryanodine-receptor complex (K_d = 7 nM; Fig. 1), we conclude that it is the receptor protein, and not a minor contaminant of the purified preparation, that makes the channel. A more quantitative argument is possible, but not without a painstaking determination of the electrophysiological drug-receptor affinity. Second, because K ions were used as current carriers, the result suggests that K conduction is intrinsic to the release channel, an observation that is novel and remains to be confirmed in release channels in situ.

Finally, our finding that ryanodine opens the release channel is in agreement with two recent single-channel reports on release channels (Imagawa et al., 1987; Rousseau et al., 1987), as well as vesicle flux experiments (Meissner, 1986; Lattanzio et al., 1987) and more recent tension experiments in skinned and intact skeletal muscle (Hwang et al., 1987). The most likely explanation for inhibition of Ca release reported earlier in muscle fibers treated with ryanodine is that ryanodine opens the population of release channels at a slow rate and this results in a subsequent depletion of Ca in the SR. Consistent with this hypothesis, Pessah et al. (1986) have shown that at low concentrations (<1 μM), the association half-life of ryanodine as well as the dissociation half-life are extremely slow, on the time scale of many hours.

Ion Permeation Properties of the Purified Co-Release Channel

Single-channel recording of the purified release channel allowed us for the first time to study in detail its permeability characteristics to many small ions, including K and Cl. It was not previously possible to do this work in native membranes because of the large background of currents from K and Cl conducting channels present in the SR (Miller, 1978). In gradients of Cl salts, the ryanodine receptor channel is perfectly cation selective and it does not conduct Cl under any of the conditions tested. The channel is essentially nonselective among alkali monovalent cations (Fig. 11), but small differences in single-channel conductance are measurable. The conductance sequence for monovalent cations is Cs > Na > K > Li » choline > Tris. Choline and Tris show significantly lower conductances than alkali cations. The low conductance for choline and Tris is probably due to their large cross-sectional areas (~38 Å^2), which may be near the exclusion size for charge carriers in the pore. K and Ca conductance saturates at high concentration as though ion-binding site(s) in the pore limited the rate of ion passage. At saturation, the K conductance is high, ~1 nS, and the Ca conductance is ~ 170 pS. Even though K is more conductive than Ca, the release channel does not have features in common with K channels of native SR (Coronado et al., 1980; Labarca et al., 1980). For example, we have not seen effects of ryanodine or ruthenium red on the SR K channel (unpublished), nor does the SR K channel have any Ca permeability (Coronado et al., 1980) or ligand-dependent activation by Ca or ATP (unpublished). Significant differences are also found in their cellular distributions. Recording of release channels and ryanodine-binding activity is restricted to junctional SR (Campbell et al., 1987; Imagawa et al., 1987), while SR K channels are distributed throughout the SR membrane and are abundant in light as well as in heavy fractions (Garcia and Miller, 1984). This simply rules out the possibility, albeit remote, that the purified ryanodine receptor may represent a functional or altered form of the SR K channel. Instead of K channels, the large conductance and lack of selectivity of the ryanodine receptor is reminiscent of gap junction channels recorded from purified lens junctional proteins (Zampighi et al., 1985).

The measured permeability ratio $P_{Ca}/P_K \simeq 6$ clearly defines the ryanodine receptor as a divalent-selective channel when both ions are present. This selectivity, however, is low compared with that of voltage-sensitive Ca channels recorded from t-tubule membranes that have a permeability ratio $P_{Ca}/P_{Na} > 25$ (Coronado and

Affolter, 1986). With respect to a conduction mechanism (one or multi-ion), it is interesting to note that the conductance affinity permeability condition (Lauger, 1973) for K and Ca suggests that the release channel may accommodate several ions at the same time. In one-ion pores, the saturation conductance ratio for any two permeant ions (X, Y), $g(Y)/g(X)$, and the binding affinity ratio (ratio of half-saturation constants), $K(X)/K(Y)$, and the Goldman permeability, $P(X)/P(Y)$, are linked by the relation:

$$g(Y)/g(X)\cdot K(X)/K(Y)\cdot P(X)/P(Y)=1 \quad (1)$$

For the Ca, K pair, Eq. 1 gives a value of 2.5, as defined by a conductance ratio $g(\mathrm{K})/g(\mathrm{Ca}) = 6$, a binding affinity ratio $K(\mathrm{Ca})/K(\mathrm{K}) = 0.063$ (Figs. 11 and 12), and a permeability ratio $P_{Ca}/P_K = 6.6$. This value deviates significantly from the experimental range described in one-ion channels (Coronado et al., 1980). Simultaneous occupancy of the channel by several ions could help to explain not only discrepancies between the conductance and permeability ratios, but also the large unitary conductance of the ryanodine receptor channel. For example, it has been shown in multi-ion pores (Hille and Schwarz, 1978) that conductance can be boosted one or more orders of magnitude by loading two or more ions in one end of the channel so that repulsion between loaded ions may contribute to increasing the ion exit rates at the other end. Our data are numerically consistent with this idea. The ascending part of the K conductance vs. activity curve has a slope of ~8 nS/M (Fig. 11 *B*), a value that is even steeper than that of two well-known multi-ion pores, the gramicidin channel (0.01 nS/M; Hladky and Haydon, 1972) and the Ca-activated K channel (2.5 nS/M; Latorre and Miller, 1983).

The large K conductance and low selectivity of the ryanodine receptor may have important consequences for the release process in muscle. The lack of K gradients across the SR (Somlyo et al., 1981) implies that, in the muscle cell, the ryanodine receptor channel will operate most of the time close to 0 mV, the K equilibrium potential. The SR Ca load, as Indicated in Fig. 10, contributes only modestly (~ 5 mV) to the channel reversal potential, which, in symmetrical K and 5 mM intra-SR Ca, is also close to 0 mV. Consequently, the physiological release process will take place essentially under zero net current conditions. That is, because the channel opens near its reversal potential, flow of Ca out of the SR driven by the Ca chemical gradient must be compensated by K ions moving into the SR, both through the same channel. Other permeant species such as Mg (Fig. 12 *C*) and possibly protons (not shown) may contribute as well. Thus, one of the important functions of this large-conductance channel, other than being the Ca efflux path, could be that of being the pathway for charge compensation during release.

Multiple Conductance States and Effects of Ryanodine

Still unexplained is the gating of the purified receptor into multiple conductance states. The conductance states of the purified protein were readily apparent in solutions of both divalent or monovalent cations and in the presence or absence of ryanodine. In solutions containing millimolar Ca, the channel dwells in two conductance states (Fig. 4). It became obvious that in monovalent solutions there were at least four conductance states (Fig. 13). Statistically, however, channels dwell ≥80%

of the time in a single state (the 360-390-pS conductance; Fig. 2). We believe that each of the conductance states can be considered to be a multiple of an elementary conductance unit. Biochemical evidence obtained from electron microscopy of purified receptor preparations suggest that the ryanodine receptor purified from CHAPS-solubilized triads is tetrameric in structure (Inui et al., 1987). One hypothesis is that each 450,000-dalton subunit or monomer forms an ion channel that can be physically and functionally linked to other subunits to form the pharmacologically competent, large-conductance ryanodine receptor/Ca-release channel. Within this framework, it is possible to hypothesize a mechanism for the observed effects of ryanodine. Ryanodine binding to the tetrameric ~400-pS channel would cause a blockade of one of the two "dimer" conductances and uncouple the normal linkage between subunits. The result of this would be a single-"dimer," ~200-pS channel with half the normal conductance and disrupted gating properties. Negative cooperativity in the binding of ryanodine could alter the affinity of the unligated "dimer" channel, locking it open and preventing complete blockade until very high concentrations are reached. Such a mechanism would explain the long openings as well as the biphasic effects of ryanodine seen in ^{45}Ca flux experiments, where low (~10 nM) concentrations stimulated and high (>10 μM) concentrations inhibited ^{45}Ca release (Meissner, 1986).

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