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EVIDENCE FOR A Ca^{2+} CHANNEL WITHIN THE RYANODINE RECEPTOR COMPLEX FROM CARDIAC SARCOPLASMIC RETICULUM

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SUMMARY. The solubilized [^{3}H]ryanodine receptor from cardiac sarcoplasmic reticulum was centrifuged through linear sucrose gradients. A single peak of radioactivity with apparent sedimentation coefficient of ~30S specifically comigrated with a high molecular weight protein of apparent relative molecular mass ~400,000. Incorporation of the ryanodine receptor into lipid bilayers induced single Ca^{2+} channel currents with conductance and kinetic behavior almost identical to that of native cardiac Ca^{2+} release channels. These results suggest that the cardiac ryanodine receptor comprises the Ca^{2+} release channel involved in excitation-contraction coupling in cardiac muscle.

INTRODUCTION. Excitation-contraction (EC) coupling in muscle is accompanied by a rapid release of Ca^{2+} ions from the intracellular membrane system called sarcoplasmic reticulum (SR) (1). Ion flux studies have indicated that, as for isolated skeletal muscle SR vesicles (2-5), rapid Ca^{2+} release from isolated cardiac SR vesicles is Ca^{2+} dependent, and can be modulated by Mg^{2+}, adenine nucleotides and calmodulin (6). Single channel recordings obtained upon incorporation of cardiac SR vesicles into planar lipid bilayers have shown the presence of a high conductance Ca^{2+} channel (73 pS in 53 mM

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ABBREVIATIONS: SR, sarcoplasmic reticulum; CHAPS, 3-[(3 cholamidopropyl) dimethylammonio]-1-propanesulfonate; EGTA, [ethylenebis(oxyethylenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulphate; EC, excitation-contraction; DIFP, diisopropylfluorophosphate; transverse tubule, T.

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Ca^{2+}), which could be stimulated by μM Ca^{2+} and mM ATP, and inhibited by mM Mg^{2+} (7). An identical mode of regulation has previously been observed in single channel recordings of the skeletal SR Ca^{2+} release channel (8,9).

The neutral plant alkaloid, ryanodine, has been the subject of much recent attention due to its specific, high-affinity interaction with the Ca^{2+} release channel from SR. Radioligand binding studies using [^{3}H]ryanodine have shown that the ryanodine receptor is concentrated in the SR vesicle fractions also enriched in Ca^{2+} release activity (6,10-12). In single channel recordings, ryanodine induced an irreversible, subconductance state of the cardiac and skeletal SR Ca^{2+} release channel, which then became no longer sensitive to regulation by other ligands (13).

Isolation of the cardiac (14,15) and skeletal (16,17) SR ryanodine receptor has suggested them to be associated with a high molecular weight protein of apparent relative molecular mass (M_r) ~350,000 (~350K). Similarly, a ~350K protein was identified using a monoclonal antibody capable of immunoprecipitating the skeletal [^{3}H]ryanodine receptor (18). More direct evidence has recently shown (17) that the reconstituted skeletal ryanodine receptor contains a high conductance, Ca^{2+} and adenine nucleotide gated Ca^{2+} channel very similar to the native SR Ca^{2+} release channel (8,9). Using [^{3}H]ryanodine as a Ca^{2+} release channel-specific probe, we report here the isolation of a large complex comprising polypeptides of ~400K, and which contains a cation-conducting channel with properties congruent with those of the native cardiac SR Ca^{2+} release channel.

MATERIAL AND METHODS. Heavy SR vesicles enriched in Ca^{2+} release activity, were isolated from canine cardiac muscle (6) in the presence of 2.5 mM EGTA, rapidly frozen, and stored at -135°C. Membranes were suspended in buffer A (0.5 M NaCl, 20 mM $Na_4P_2O_7$, 0.1 mM EGTA, 0.1 mM $CaCl_2$, 1 mM diisopropylfluorophosphate (DIFP), 20 mM NaH_2PO_4 pH 7.0), incubated for 60 minutes at 4°C, then centrifuged through 0.5 M sucrose, 10 mM NaPIPES pH 7.0 for 30 minutes at 100,000 x g. The pelleted membranes were resuspended to 3 mg protein/ml in buffer B (1 M NaCl, 0.1 mM EGTA, 0.15 mM $CaCl_2$, 1 mM DIFP, 25 mM NaPIPES pH 7.0) containing 0.2% soybean phosphatidylcholine (Avanti Polar Lipids Inc.,

Birmingham, AL) and 5 nM [^{3}H]ryanodine (18.4 Ci/mmol), and CHAPS added to 1.5%. After a 2.5 hour incubation at room temperature (23°C) and centrifugation for 30 minutes at 100,000 x g, the supernatant (1 ml of 1.6 mg/ml) was loaded onto 5-20% linear sucrose gradients in buffer B containing 0.2% soybean phosphatidylcholine and 1% CHAPS, then centrifuged at 26,000 rpm, 2°C for 16 hours in a SW41 rotor. Gradient fractions were collected from the top, and analyzed for (i) radioactivity by liquid scintillation counting, (ii) protein concentration (19), and (iii) protein composition on 5-12% linear polyacrylamide gradient gels (20) followed by silver staining (21). E. coli ß-galactosidase (16S), bovine catalase (11.2S) and yeast alcohol dehydrogenase (7.6S) (Sigma Chemical Co., St. Louis, MO), were centrifuged in parallel sucrose gradients to obtain a sedimentation calibration curve. Apparent sedimentation coefficient (uncorrected for bound detergent) of the [^{3}H]ryanodine receptor was determined by extrapolation of the enzyme calibration curve. The skeletal muscle SR ryanodine receptor was purified as described previously (17,22). Single channel recordings were performed (7) by direct incorporation of the purified ryanodine receptor (17), isolated in the absence of [^{3}H]ryanodine, or by fusion of heavy SR vesicles into Mueller-Rudin planar lipid bilayers containing phosphatidylethanolamine, phosphatidylserine and dioleoylphosphatidylcholine, 50:30:20 (Avanti Polar Lipids Inc.). Bilayer currents were measured and analyzed as described previously (9,13).

RESULTS. Figure 1 shows the sedimentation profile of the CHAPS-solubilized proteins from cardiac SR, and of the [^{3}H]ryanodine receptor, upon centrifugation through a linear sucrose gradient. A single peak of radioactivity comigrating with a minor protein peak was

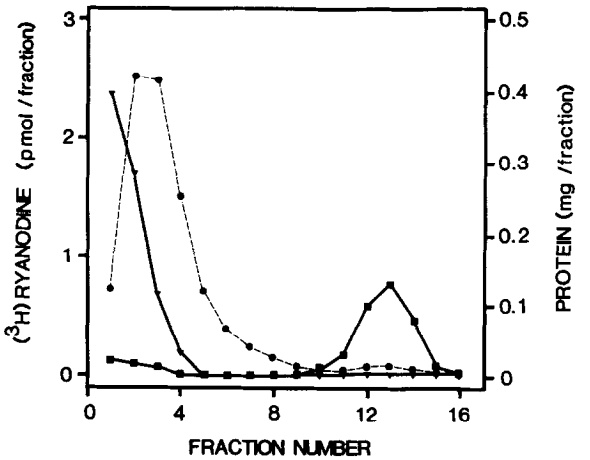

FIGURE 1. Sedimentation profile of the CHAPS-solubilized [^{3}H]ryanodine receptor from cardiac SR, in a linear sucrose gradient. Samples were labelled with [^{3}H]ryanodine in the presence (▼), or absence (■) of 10 µM unlabelled ryanodine (solid lines). Following solubilization and centrifugation (see Materials and Methods), sucrose gradients (12 ml) were fractionated (0.75 ml/fraction) and aliquots taken for analysis of radioactivity (100 µl) and protein content (50 µl). The protein profile (●) is shown by a dashed line. Recovery of protein and radioactivity loaded onto the gradient was > 90%.

observed in the lower half of the gradient. The small amount of radioactivity present in the top fractions of the gradient was found to be free unbound [^{3}H]ryanodine (17). When membranes were preincubated with an excess of unlabelled ryanodine, no radioactivity was present in the lower half of the gradient. Centrifugation of soluble enzyme standards in parallel sucrose gradients gave a linear calibration curve with a correlation coefficient (r) of 0.99, and enabled estimation of the apparent sedimentation coefficient of the cardiac [^{3}H]ryanodine receptor (by linear extrapolation). The resulting value of ~30S, suggests that the cardiac ryanodine receptor is a very large macromolecular complex with a molecular weight in excess of 10^6 daltons, as has been similarly found for the skeletal ryanodine receptor (17,22).

SDS-polyacrylamide gradient gel electrophoresis of the ryanodine receptor peak fractions indicated a specific comigration of a single high molecular weight polypeptide of M_r ~400K (Fig. 2), which was absent in all other gradient fractions. Variable, but small amounts (< 10% of silver stain) of the Ca^{2+}, Mg^{2+}-ATPase protein, M_r 110K (23), was often present in the ryanodine receptor peak, which could be removed by recentrifugation of the peak fraction on a second sucrose gradient (not shown). The specific comigration of a single protein band with the ~30S complex suggests that the cardiac ryanodine receptor comprises subunits of ~400K which stoichiometrically form into a large oligomeric complex. Whether this complex comprises a homo- or hetero-oligomer of ~400K subunits remains to be elucidated. Comparison of the protein composition of the cardiac ryanodine receptor with that of the skeletal receptor (Fig. 2), previously shown to also consist of high molecular weight polypeptides of ~400K (17), indicated that the mobility (R_f) of the cardiac receptor protein upon electrophoresis in the presence of SDS (0.105), was slightly higher than that of the skeletal receptor protein (0.094). Under identical

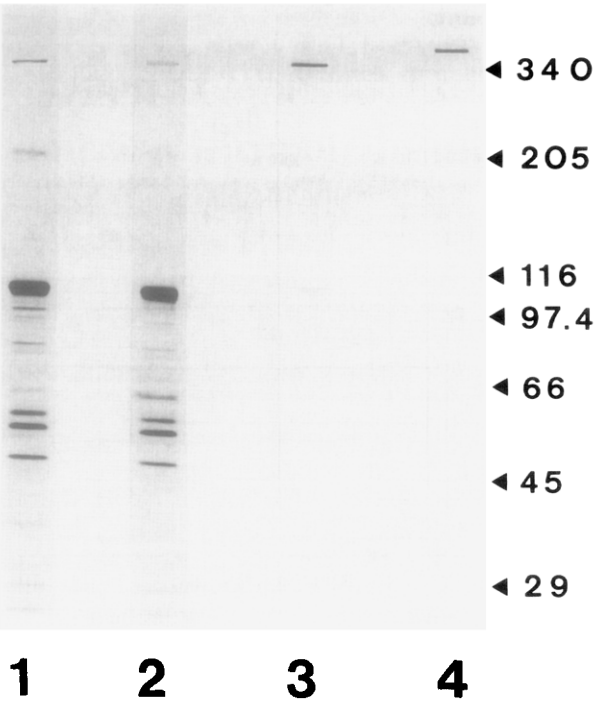

FIGURE 2. SDS-polyacrylamide (5-12%) gradient gel electrophoresis of cardiac heavy SR membranes prior to pyrophosphate extraction, 3 µg (lane 1), supernatant of CHAPS-solubilized cardiac heavy SR membranes, 2 µg (lane 2), cardiac ryanodine receptor gradient peak fraction, 0.3 µg (lane 3) and skeletal ryanodine receptor gradient peak fraction, 0.3 µg (lane 4). Molecular weight standards (Sigma Chemical Co.) carbonic anhydrase (29 K), ovalbumin (45 K), bovine serum albumin (66 K), phosphorylase b (97.4 K), ß-galactosidase (116 K), myosin (205 K) and α_2-macroglobulin (340 K, non-reduced) are shown by arrows. The high molecular weight ryanodine receptor protein subunits have been designated ~400K in the text, since they have a slightly lower mobility than that of laminin A-chain, a protein of 350-400K (see Results).

electrophoretic conditions, laminin A-chain, a protein of 350-400K (24) moved as a diffuse band with an R_f of ~0.110 (not shown).

Upon reconstitution of the cardiac ryanodine receptor (isolated in the absence of [^{3}H]ryanodine) into planar lipid bilayers, a Ca^{2+} conducting channel activity was observed (Fig. 3A). The slope conductance of the Ca^{2+} current induced by the reconstituted receptor protein, 72 pS, was very similar to that obtained for the native cardiac SR Ca^{2+} release channel, 83 pS, recorded under identical conditions (Fig. 3B), and to the value of 73 pS reported previously (7). Appearance of a Ca^{2+} current was specific to the ryanodine receptor peak fractions, since use of other gradient fractions failed

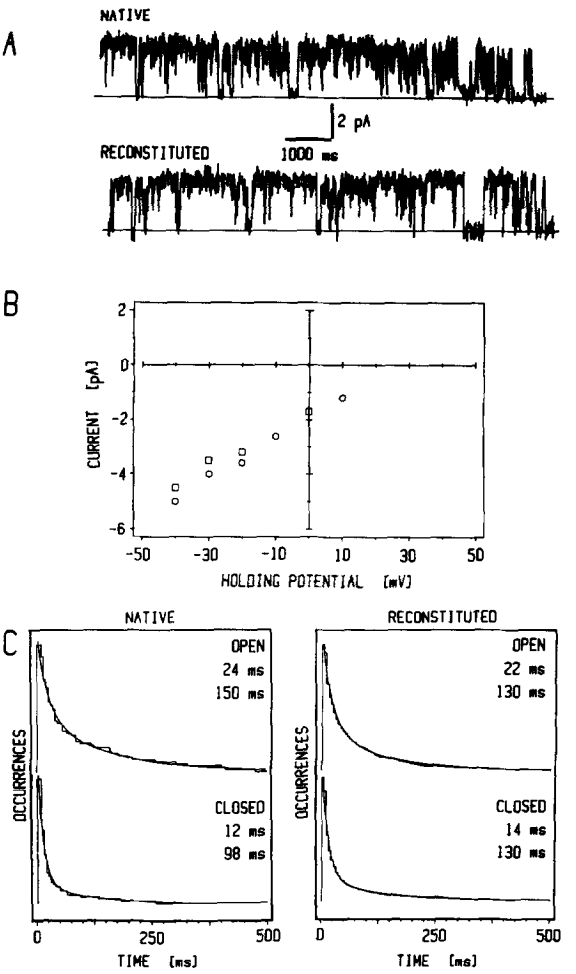

FIGURE 3. Conductance and kinetic properties of single Ca^{2+} channels from native cardiac SR and purified cardiac ryanodine receptor. A. Native cardiac Ca^{2+} release channel recorded after incorporating heavy SR vesicles (upper trace), and Ca^{2+} channel observed upon incorporation of the cardiac ryanodine receptor (lower trace) into planar lipid bilayers. The fraction of channel open time (P_o) was 0.60 and 0.58, respectively. Both recordings were made in 50 mM $Ca(OH)_2$/250 mM HEPES pH 7.4 trans, and 2.5 μM Ca^{2+} (0.1 mM EGTA, 0.1 mM $CaCl_2$), 125 mM Tris/250 mM HEPES pH 7.4 cis. Holding potential was -20 mV. B. Current-voltage relationship for the native cardiac SR Ca^{2+} release channel (o) and the reconstituted ryanodine receptor Ca^{2+} channel (□), giving slope conductance values of γ = 83 pS and γ = 72 pS, respectively. C. Cumulative time histogram analysis (9) for the open (upper) and closed (lower) duration states of the native cardiac SR Ca^{2+} release channel (left) and the reconstituted ryanodine receptor Ca^{2+} channel (right). The values shown for each event distribution correspond to the mean time constants (τ_1 and τ_2) obtained when the curve was fitted to the sum of double exponential functions.

to reveal any Ca^{2+} channel activity. Exponential fits to the open and closed single channel lifetimes of both the native cardiac SR Ca^{2+} release channel and the reconstituted ryanodine receptor Ca^{2+} channel,

suggested that both channels exist in distinct states (Fig. 3C). The clear presence, and close similarity, of the time constants of two open states of 24 + 150 msec and 22 + 130 msec, and two closed states of 12 + 98 msec and 14 + 130 msec, for both the native and reconstituted channel, respectively, strongly suggests that the channels are very similar if not identical.

DISCUSSION. The ryanodine receptor from skeletal muscle has recently been isolated as a ~30S complex comprising polypeptides of M_r ~400K (17). Reconstitution and structural studies have shown it to be equivalent to the high conductance SR Ca^{2+} release channel, and the "feet" structures which span the transverse tubule (T)-SR junctional gap (17). This identification is of physiological significance to the mechanism of EC coupling in muscle since it suggests that opening of SR Ca^{2+} release channels may be triggered directly via its specific association with the T-membrane.

Our present study provides the first indication that, as for the ryanodine receptor from skeletal muscle, the cardiac muscle ryanodine receptor also contains a Ca^{2+} conducting pathway with properties which match those of the native Ca^{2+} release channel. In addition, we have found that the cardiac and skeletal ryanodine receptors similarly exist as a large oligomeric ~30S complex, comprising a single high molecular weight polypeptide of ~400K. However, one noticeable difference is the greater mobility of the cardiac ryanodine receptor protein on polyacrylamide gels (Fig. 2), suggesting it to be of slightly lower molecular weight than that of the skeletal receptor (14). Additional differences which may be related to the latter are (i) the lower sensitivity of the cardiac Ca^{2+} release channel to inhibition by Mg^{2+} and ruthenium red, but its greater sensitivity to activation by Ca^{2+} (6,7), and (ii) the lower single channel unit

conductance of the cardiac (73 pS), relative to the skeletal (100 pS) Ca^{2+} release channel (7-9).

In conclusion, we suggest that the cardiac SR Ca^{2+} release channel is present within the ryanodine receptor complex, which comprises subunits of ~400K. Further studies of the purified cardiac ryanodine receptor complex to establish the adenine nucleotide, Mg^{2+} and ruthenium red sensitivity of its constituent Ca^{2+} conductance will be required to demonstrate retention of these regulatory sites of the native Ca^{2+} release channel.

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