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MINI-REVIEW

The Muscle Ryanodine Receptor and Its Intrinsic Ca^{2+} Channel Activity

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Abstract

In skeletal and cardiac muscle, contraction is initiated by the rapid release of Ca^{2+} ions from the intracellular membrane system, sarcoplasmic reticulum. Rapid-mixing vesicle ion flux and planar lipid bilayer–single-channel measurements have shown that Ca^{2+} release is mediated by a high-conductance, ligand-gated Ca^{2+} channel. Using the Ca^{2+} release-specific probe ryanodine, a 30 S protein complex composed of four polypeptides of $M_r \sim 400{,}000$ has been isolated. Reconstitution of the purified skeletal and cardiac muscle 30 S complexes into planar lipid bilayers induced single Ca^{2+} channel currents with conductance and gating kinetics similar to those of native Ca^{2+} release channels. Electron microscopy revealed structural similarity with the protein bridges ("feet") that span the transverse-tubule–sarcoplasmic reticulum junction. These results suggest that striated muscle contains an intracellular Ca^{2+} release channel that is identical with the ryanodine receptor and the transverse-tubule–sarcoplasmic reticulum spanning feet structures.

Key Words: Skeletal muscle; cardiac muscle; ryanodine receptor; Ca^{2+} release channel; transverse tubule; sarcoplasmic reticulum; junctional feet.

Introduction

Activation of skeletal (voluntary) and cardiac (involuntary) muscle contraction results from a transient increase in the intracellular free Ca^{2+} ion concentration (Ebashi, 1976; Endo, 1977; Schneider, 1981; Stephenson, 1981; Fabiato, 1983). The chain of events that culminate in the contraction process is initiated by an action potential, originating at the neuromuscular

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synapse, which spreads rapidly over the muscle surface into transverse (T)-tubular infoldings of the plasma membrane (T-system). This electrical signal, upon reaching the T-system, triggers a rapid and massive release of Ca^{2+} ions from the sarcoplasmic reticulum (SR), an intracellular network of membranes derived from the endoplasmic reticulum, which functions as a myoplasmic Ca^{2+} store during the contraction– relaxation cycle. The specific mechanism of intermembrane signal transduction (from T-system to SR), commonly referred to as excitation– contraction(E–C) coupling is believed to occur at junctional locations where T-system and SR membranes abut, but has yet to be fully defined. At these T–SR junctions, large protein structures have been morphologically identified that span the $\sim 12\,\text{nm}$ gap separating the two membranes, and that have been variously termed feet (Franzini-Armstrong, 1970), bridges (Somlyo, 1979), and pillars (Eisenberg and Eisenberg, 1982). This article outlines recent progress made in characterization of the Ca^{2+} release properties of SR, the use of ryanodine as a specific ligand for the Ca^{2+} release channel, and its ultimate isolation and identification as the T–SR-spanning feet structures.

Calcium Release from SR

Electron microscopy has shown that the SR is a complex heterogeneous system of membranes with four typical structural regions: terminal cisternae, intermediate cisternae, longitudinal cisternae, and fenestrated cisternae (Peachey, 1965; Sommer and Johnson, 1979; Peachey and Franzini-Armstrong, 1983). Fragmentation of this intricate membranous network during homogenization of muscle tissue has enabled the separation and biochemical characterization of two distinct types of vesicles called "light" and "heavy" SR, with respect to their sedimentation properties (Meissner, 1975; Jones and Cala, 1981; Meissner, 1984). Heavy SR corresponds to the terminal cisternae (or "junctional") SR and light SR to the other types of nonjunctional (or "free") SR. The ability to separate junctional from nonjunctional SR, and correlation of their functional roles in SR Ca^{2+} release and reuptake, have had a major effect on subsequent approaches to the study of E–C coupling and Ca^{2+} release properties of SR. Another important approach to studying the mechanism of SR Ca^{2+} release has been to isolate and subfractionate "triad" structures, composed of pinched-off T-tubule vesicles sandwiched by two heavy SR vesicles (Caswell *et al.*, 1976; Brunschwig *et al.*, 1982; Mitchell *et al.*, 1983; Saito *et al.*, 1984; Costello *et al.*, 1986).

Light and heavy SR vesicles purified by sucrose gradient centrifugation have been shown to be capable of Ca^{2+} uptake in the presence of ATP. Sodium dodecyl sulfate (SDS)-gel electrophoresis of isolated light and heavy rabbit skeletal muscle SR vesicles revealed that the major protein component, which accounts for ~90% and ~60% of the vesicle protein composition, respectively, is an M_r ~ 110,000 protein (Meissner, 1975). This component has been identified as the Ca^{2+}, Mg^{2+}-ATPase, which functions as a Ca^{2+} pump, translocating cytoplasmic Ca^{2+} into the SR compartment, and has been extensively characterized (Martonosi, 1984; Inesi, 1985; Brandl *et al.*, 1986). Two protein components that are characteristic of heavy SR vesicles are calsequestrin (M_r ~ 60,000), a Ca^{2+} storage protein (MacLennan and Holland, 1975), and a high-molecular-weight protein (M_r ~ 400,000) that has been immunologiclly related to the feet structures (Kawamoto *et al.*, 1986). Ion channels permeable to both monovalent cations and anions have also been demonstrated both in light and heavy SR vesicles (Meissner, 1983). However, in addition to the specific presence of distinct protruding feet structures upon morphological analysis of heavy SR vesicles (Campbell *et al.*, 1980; Brunschwig *et al.*, 1982; Saito *et al.*, 1984; Ferguson *et al.*, 1984; Smith *et al.*, 1986*b*), one major functional feature that distinguishes the two vesicle populations is the ability of the heavy SR vesicles to release their internal Ca^{2+} stores on a very fast (millisecond) time scale. Vesicle ion flux studies using $^{45}Ca^{2+}$ have suggested that rapid Ca^{2+} release from heavy SR is a complex process that is activated by external μM Ca^{2+} and mM ATP, and inhibited by mM Mg^{2+} and μM calmodulin and ruthenium red (Nagasaki and Kasai, 1983; Ikemoto *et al.*, 1985; Meissner, 1986*b*; Meissner *et al.*, 1986; Moutin and Dupont, 1988). Incorporation of heavy SR vesicles into planar lipid bilayers has enabled single-channel measurements showing that the release of Ca^{2+} is mediated by a high-conductance Ca^{2+} channel, which can be activated or inhibited with the same pharmacological effectors as has been observed in vesicle $^{45}Ca^{2+}$ flux studies using heavy SR (Smith *et al.*, 1985, 1986*a*; Rousseau *et al.*, 1986) and that, by implication of its function during E–C coupling, is now generally referred to as the SR Ca^{2+} release channel.

Effects of Ryanodine on Ca^{2+} Release from SR

Ryanodine is a neutral plant alkaloid that is isolated from the stems of the South American shrub, *Ryania speciosa*, and that comprises two major active components: ryanodine and 9,21-didehydroryanodine (Fig. 1) (Jenden and Fairhurst, 1969; Waterhouse *et al.*, 1984; Sutko *et al.*, 1986). The two components display equal toxicity when administered to mice (Pessah *et al.*, 1985).

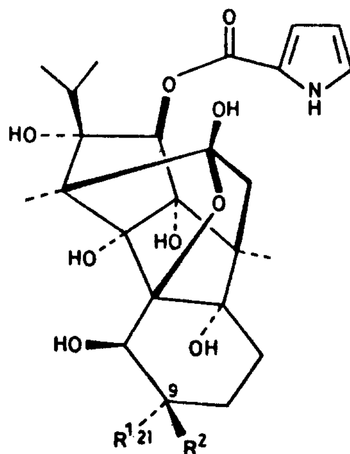

Fig. 1. Structure of ryanodine (R^1 = CH_3, R^2 = H) and 9,21-didehydroryanodine (R^1, R^2 = CH_2).

The pharmacological effects of ryanodine have been most clearly shown in muscle, where, depending on muscle type and activity, it can either cause contracture or a decline in contractile force (Jenden and Fairhurst, 1969; Sutko *et al.*, 1985). Localization of the site of interaction in muscle was determined using radiolabeled ryanodine, and by studying the effects of ryanodine on SR Ca^{2+} uptake and release using isolated membrane fractions.

The first radiolabeling of ryanodine was reported by Fairhurst using the bromination method to label the pyrrole ring (Fairhurst, 1971; Fleischer *et al.*, 1985). An alternative procedure, in which 9,21-didehydroryanodine is reduced to [^{3}H]ryanodine, has been more recently employed by several laboratories (Pessah *et al.*, 1985; Sutko *et al.*, 1986; Alderson and Feher, 1987) and, using this procedure, the tritiated compound has recently become commercially available with a high specific activity of > 50 Ci/mmol (NEN Dupont, Boston, MA). The early availability of [^{3}H]ryanodine, distributed generously by John Sutko's laboratory, has facilitated the recent progress made by at least two groups of investigators toward identification and purification of the ryanodine receptor (Campbell *et al.*, 1987; Lai *et al.*, 1988*a*).

An initial report by Pessah *et al.* (1985) showed [^{3}H]ryanodine to bind with high affinity, and in a Ca^{2+}-dependent manner, to heavy SR membranes from skeletal and cardiac muscle. In this and subsequent studies, skeletal heavy SR preparations specifically bound 4–25 pmol [^{3}H]ryanodine/mg protein, and with a K_D of 4–200 nM (Pessah *et al.*, 1985; Fleischer *et al.*, 1985; Lattanzio *et al.*, 1987; Lai *et al.*, 1988*a*). The binding of [^{3}H]ryanodine to skeletal heavy SR was, in addition to the Ca^{2+} dependence, found to be affected by ionic strength, Mg^{2+}, caffeine, and adenine nucleotide with

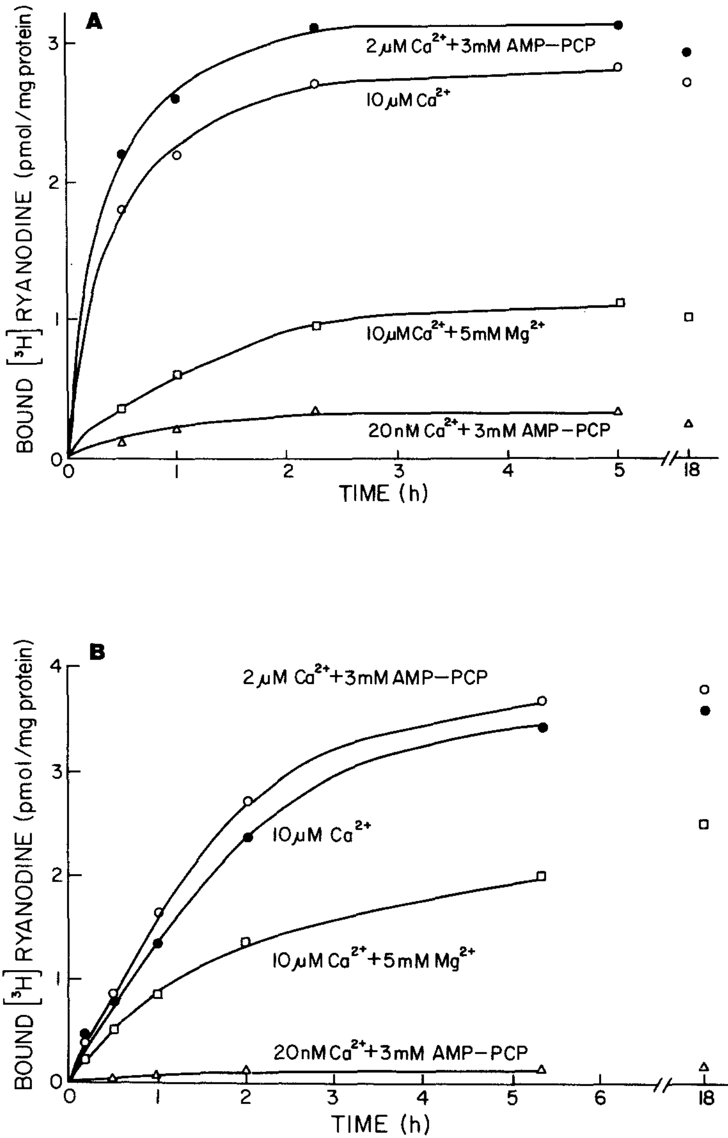

Fig. 2. Time course of [^{3}H]ryanodine binding to rabbit skeletal membranous and solubilized heavy SR preparations: (A) SR vesicles at 37°C and (B) Chaps-solubilized membranes (in the presence of 5 mg/ml phospholipid and 1.6% Chaps) at 23°C were incubated in media containing 10 nM [^{3}H]ryanodine, 1.0 M NaCl, and the indicated concentrations of free Ca^{2+}, Mg^{2+}, and adenine nucleotide. Total [^{3}H]ryanodine binding is shown.

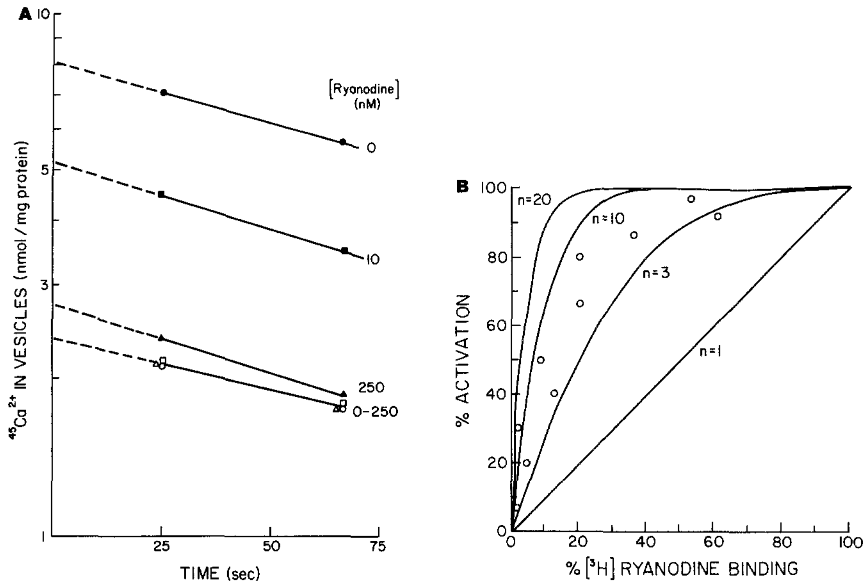

Fig. 3. Ryanodine binding and activation of Ca^{2+} release from skeletal SR vesicles. Skeletal muscle SR Ca^{2+} release vesicles were incubated for 60 min at 37°C with 0–10 μM ryanodine in a medium containing 0.1 M KCl, 20 mM K-PIPES, pH 7, 1 mM DIFP, 0.1 mM EGTA, 0.3 mM Ca^{2+}, and 5 mM AMP. "Activation" of Ca^{2+} release by ryanodine was determined by including $^{45}Ca^{2+}$ in the incubation medium and dilution of vesicles with a quench (Mg/ruthenium red, ●, ■, ▲) or release (5 μM free Ca^{2+}, ○, □, △) medium (Meissner, 1986*a*). Specific high-affinity ryanodine binding was determined using [^{3}H]ryanodine. (A) The $^{45}Ca^{2+}$ release behavior of vesicles incubated with 0 (●, ○), 10 (■, □) or 250 (▲, △) nM ryanodine. (B) Solid lines indicate expected ryanodine activation-binding behavior for vesicles assuming 1, 3, 10 and 20 Ca^{2+} release channels/vesicle.

respect to affinity, cooperativity, and/or receptor site density (Pessah *et al.*, 1987; Imagawa *et al.*, 1987; Michalak *et al.*, 1988). Our studies have indicated that [^{3}H]ryanodine binding to its membrane-bound receptor site was not appreciably affected by adenine nucleotide, but was decreased by mM Mg^{2+} and reduction of free Ca^{2+} from micromolar to nanomolar concentrations (Fig. 2A) (Meissner *et al.*, 1988*a*). A similar behavior was found for the Chaps-solubilized ryanodine receptor (Fig. 2B). Results of analysis of ryanodine binding and activation of Ca^{2+} release from skeletal heavy SR (Meissner 1986*a*) correlated with a majority of the vesicles containing 3–10 channels per vesicle (Fig. 3).

Studies of [^{3}H]ryanodine binding to cardiac SR vesicles have revealed that, in addition to a high-affinity site, with a K_D of 4–36 nM and a B_{max} of 0.5–14 pmol [^{3}H]ryanodine/mg protein (Pessah *et al.*, 1985; Alderson and

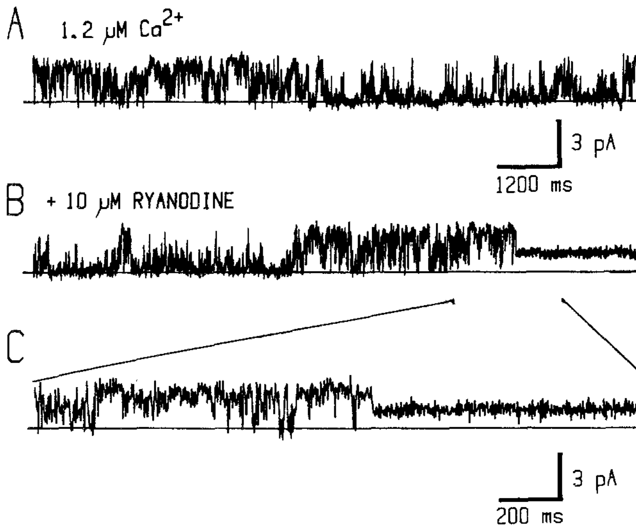

Fig. 4. Effect of ryanodine on single-channel recordings of the cardiac Ca^{2+} release channel. (A) Single-channel currents, shown as upward deflections, were recorded with 1.2 μM free Ca^{2+} (1 mM EGTA and 0.95 mM Ca^{2+}), 125 mM Tris/250 mM HEPES, pH 7.4 *cis*, and 50 mM $Ba(OH)_2$/250 mM HEPES, pH 7.4 *trans*. (B) At 1 min after addition of 10 μM ryanodine to the *cis* chamber (the cytoplasmic side of the channel). (C) Transition from normal into subconducting state on an extended time scale. Filter = 300 Hz. Sampling = 1 kHz. Holding potential = 0 mV (With permission from Rousseau *et al.* (1987).

Feher, 1987; Meissner and Henderson, 1987; Inui *et al.*, 1988; Michalak *et al.*, 1988), a low-affinity site exists that has a K_D of 28 nM–1 μM (Pessah *et al.*, 1985; Inui *et al.*, 1988; Michalak *et al.*, 1988).

In studies of Ca^{2+} loading and Ca^{2+} release using isolated heavy SR preparations, the apparently paradoxical observed effects of ryanodine have been interpreted to be due either to activation or inhibition of a Ca^{2+} permeable pathway (Fairhurst and Hasselbach, 1970; Jones *et al.*, 1979; Chamberlain *et al.*, 1983; Seiler *et al.*, 1984; Feher and Lipford, 1985; Fleischer *et al.*, 1985; Sutko *et al.*, 1985). The apparent discrepancies of these above studies can be resolved by considering the different assay conditions and SR membrane preparation procedures, since at low concentrations ryanodine opens the channel, whereas at high concentrations ($> 10\,\mu$M) ryanodine closes the channel (Meissner, 1986*a*; Lattanzio *et al.*, 1987; Alderson and Feher, 1987).

The profound effect of ryanodine on skeletal and cardiac muscle has been most convincingly demonstrated in single-channel recordings, where the addition of ryanodine to the *cis* chamber, corresponding to the cytoplasmic side of SR, induced a long-lived open-channel subconductance state of the

skeletal (Rousseau *et al.*, 1987; Imagawa *et al.*, 1987; Nagasaki and Fleischer, 1988) and cardiac (Fig. 4) (Rousseau *et al.*, 1987) SR Ca^{2+} release channels. The ryanodine-modified channel was largely insensitive to subsequent addition of Ca^{2+}, ATP, or Mg^{2+}, physiological regulators that otherwise greatly affect the gating behavior of the channel.

Purification of the Ryanodine Receptor Complex

An important initial observation made by Casida and coworkers was that the ryanodine receptor could be solubilized from skeletal and cardiac SR membranes with retention of high-affinity [^{3}H]ryanodine-binding activity in the presence of the zwitterionic detergent Chaps and high salt concentrations (Pessah *et al.*, 1986; Seifert and Casida, 1986). Gel permeation chromatography suggested that the ryanodine receptor migrated as a large protein complex with $M_r > 1{,}200{,}000$. Subsequent reports have shown that the Chaps-solubilized skeletal ryanodine receptor could be purified to apparent homogeneity by sequential column chromatography (Inui *et al.*, 1987*b*) and, in essentially a single step, by immunoaffinity chromatography (Smith *et al.*, 1988) or density-gradient centrifugation (Lai *et al.*, 1988*a*). The receptor has also been immunoaffinity purified in the presence of digitonin (Campbell *et al.*, 1987). Using the isolation procedure described in Table I, we obtained a maximal value for specifically bound [^{3}H]ryanodine of 650 pmol/mg

Table I. Purification of Ryanodine Receptor[a]

		[^{3}H]Ryanodine binding	
Fraction	Protein (mg)	Specific activity (pmol/mg protein)	Purification
1. Crude homogenate	5000	0.1 ± 0.05	1
2. Heavy SR membranes	7.8	15.0	150
3. Chaps-solubilized heavy SR	7.5	14.4	144
4. Purified ryanodine receptor	0.15	650	6500

[a] Rabbit skeletal heavy SR, isolated as a 2600 to 35,000-g pellet in the presence of 2.5 mM EGTA and 1 mM DIFP from whole muscle homogenate, was solubilized and labeled (1.3 mg protein/ml) for 2 h at 23°C in buffer A (1 M NaCl, 150 μM $CaCl_2$, 100 μM EGTA, 25 mM Na-PIPES pH 7.1, and 1 mM DIFP) containing 1.6% Chaps, 3 mg/ml phosphatidylcholine, 2 mM AMP-PCP, and 300 nM [^{3}H]ryanodine (2 Ci/mmol). The 100,000-g supernatant was loaded onto six 5–20% linear sucrose gradients in buffer A containing 0.9% Chaps, 3 mM AMP, and 4 mg/ml phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (PC : PE : PS = 16 : 3 : 1) and then centrifuged for 16 h at 26,000 rpm and 2°C in a Beckman SW41 rotor. The purified ryanodine receptor was isolated as a 30 S complex which sedimented at a much faster rate than other solubilized SR proteins (Lai *et al.*, 1988*a*).

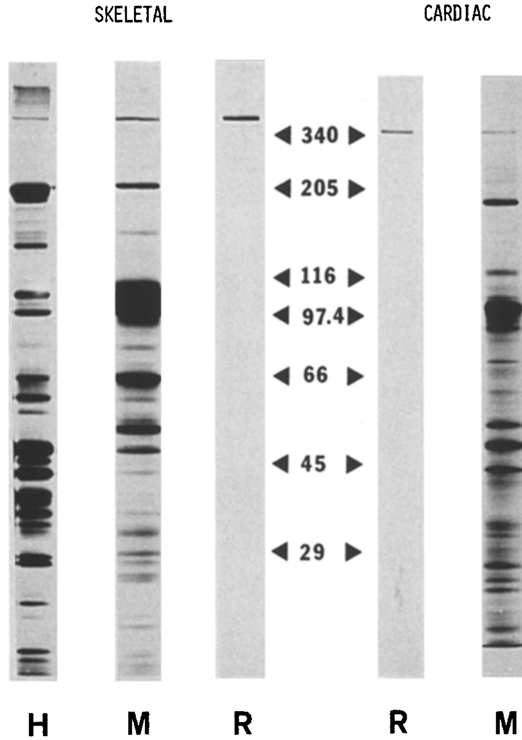

Fig. 5. Silver-stained gel of purified skeletal and cardiac ryanodine receptors. SDS–polyacrylamide gradient (5–12%) gel analysis of whole homogenate (H), heavy SR membranes (M), and purified 30 S ryanodine receptor complex (R) from rabbit skeletal and canine cardiac muscle. Sizes of molecular weight standards are shown ($\times 10^{-3}$). With permission from Meissner *et al.* (1988*b*).

protein, which corresponds to a purification of ~ 6500-fold versus the whole muscle homogenate (~ 0.1 pmol [^{3}H]ryanodine/mg protein) and 45-fold versus the heavy SR membranes (15 pmol [^{3}H]ryanodine/mg protein). The ryanodine receptor was demonstrated to exist as an oligomeric complex with an apparent sedimentation coefficient of 30 S (Lai *et al.*, 1987) and comprising a single high-molecular-weight protein band of M_r ~ 400,000 upon SDS–gel electrophoresis (Fig. 5) (Lai *et al.*, 1988*a*). A specific activity of 650 pmol/mg protein for [^{3}H]ryanodine binding to the purified 30 S complex is approximately one-quarter of the theoretical maximum value expected (2500 pmol/mg protein) for one high-affinity site per M_r ~ 400,000 polypeptide, suggesting that an oligomeric association of four M_r ~ 400,000 subunits may comprise a single ryanodine receptor complex (Lai *et al.*, 1988*a*). Similar identification of the ryanodine receptor as a high-molecular-weight protein of M_r 350,000–450,000 was made by other laboratories (Inui

et al., 1987*b*; Campbell *et al.*, 1987). The purification of the ryanodine receptor as a single high-molecular-weight band on SDS gels required the extensive use of protease inhibitors throughout the isolation procedure; otherwise additional components of $M_r < 380{,}000$, putative degradation products of the high-molecular-weight $M_r \sim 400{,}000$ protein, became apparent (Inui *et al.*, 1987*a*, b; Lai *et al.*, 1987, 1988*a*).

The cardiac SR ryanodine receptor has also been purified by column chromatography (Inui *et al.*, 1987*b*), density-gradient centrifugation (Lai *et al.*, 1988*b*), and a combination of the two methods (Rardon *et al.*, 1988). The receptor sedimented as a large complex of 30 S upon density-gradient centrifugation of Chaps-solubilized cardiac SR membranes (Lai *et al.*, 1988*b*), and comprised a single high-molecular-weight protein of $M_r \sim 400{,}000$ that was observed to migrate at a slightly faster rate on SDS gels than did the skeletal ryanodine receptor (Fig. 5) (Inui *et al.*, 1987*b*; Lai *et al.*, 1988*b*).

Ryanodine Binding to the Purified Ryanodine Receptor Complex

The three laboratories that have reported isolation of the skeletal ryanodine receptor observed retention of high-affinity [H^3]ryanodine-binding activity to their purified preparations, with B_{max} values of ~500 pmol [^3H]ryanodine/mg protein, although differences in binding affinity were obtained. A comparable K_D of 4 and 7 nM determined by Lai *et al.*, (1988*a*) and Smith *et al.* (1988), respectively, contrasted with a > 10-fold larger value reported by Inui *et al.* (1987*b*) of 79 nM. A lower K_D of 17 nM for the purified cardiac ryanodine receptor isolated under similar conditions (Inui *et al.*, 1987*b*) was interpreted by these authors to indicate a greater sensitivity of the cardiac SR Ca^{2+} release channel to modification by ryanodine.

Table II summarizes a study in which the dependence of [^3H]ryanodine binding to the purified skeletal ryanodine receptor, isolated and assayed in the presence or absence of Ca^{2+} and/or exogenous phospholipid, was examined. [^3H]Ryanodine at 20 nM was used for the binding study, a concentration at which a majority of the high-affinity sites ($K_D \sim 4$ nM) were occupied. When the 30 S complex was isolated and assayed for binding activity in the presence of Ca^{2+} and exogenous phospholipid, the purified protein bound 310 pmol [^3H]ryanodine/mg protein. The binding of [^3H]ryanodine appeared not to depend on the presence of exogenous phospholipid since its addition to the isolated receptor, purified in its absence, did not alter binding activity (110 vs. 110 pmol/mg protein), although a significant loss of binding activity had occurred during the isolation procedure (110 vs. 310 pmol/mg protein). A dramatic reduction in ryanodine binding was found, however, when the receptor was isolated and assayed in the presence of low (0.1 μM) Ca^{2+}

Table II. [^{3}H]Ryanodine Binding to the Purified Skeletal 30 S Protein Complex[a]

Conditions of isolation	[^{3}H]Ryanodine binding: Under conditions of isolation	[^{3}H]Ryanodine binding: In presence of phospholipid and 10 μM Ca^{2+}
Phospholipid + 10 μM Ca	310	310
Phospholipid + 0.1 μM Ca	28	265
10 μM Ca	110	110
0.1 μM Ca	35	120

[a]The ryanodine receptor–Ca^{2+} release channel complex was isolated by solubilization of heavy SR vesicles (Meissner *et al.*, 1986) in 1.6% Chaps, followed by sucrose density-gradient centrifugation in the presence of 1% Chaps, the presence and absence of 5 mg/ml soybean phosphatidylcholine, 1.0 M NaCl, 3 mM AMP, 0.1 or 10 μM free Ca^{2+}, 0.1 mM DIFP, and 20 mM Na-PIPES, pH 7.0, as previously described (Lai *et al.*, 1988*a*). After centrifugation, high-affinity [^{3}H]ryanodine binding was determined by a filter assay (Pessah *et al.*, 1986) after incubation of the 30 S complex containing gradient fractions for 10 h at 23°C in the presence of 20 nM [^{3}H]ryanodine (1) under conditions of isolation, and (2) in the presence of 5 mg/ml phospholipid and 10 μM free Ca^{2+}. Values were not extrapolated back to zero time to account for loss of [^{3}H]ryanodine binding during the 10-h incubation period.

concentrations (28 and 35 pmol/mg protein in the presence and absence of exogenous phospholipid, respectively), which in the complete assay medium (plus Ca^{2+} and lipid) could be restored to levels close (265 and 120 pmol/mg protein) to those found for the receptor isolated and assayed in the presence of exogenous phospholipid (310 and 110 pmol/mg protein). The data in Table II suggest that high-affinity ryanodine binding to the purified receptor depends on μM Ca^{2+}, as observed for the membrane-bound, and Chaps-solubilized (Fig. 2) (Pessah *et al.*, 1986) and digitonin-solubilized, immunoaffinity purified (Imagawa *et al.*, 1987) receptor. However, Table II further shows that ryanodine binding does not require exogenous phospholipid, although receptorstability during isolation is enhanced by addition of phospholipid.

Structure of the Ryanodine Receptor Complex

Rotary-shadowed images of junctional SR vesicles have revealed a four-leaf-clover appearance (quatrefoil) for the feet structure that spans the T–SR junctional gap (Ferguson *et al.*, 1984). The side of the square that circumscribed the shadowed feet on the surface of the SR membrane was ~ 25 nm. Similarly, squarelike structures of slightly smaller size were observed in thin sections of SR vesicles (Franzini-Armstrong, 1970; Saito *et al.*, 1984). The ryanodine receptor purified by sequential column chromatography or density-gradient centrifugation, when viewed by negative staining, exhibited a shape with dimensions that correlated well with that of membrane-bound

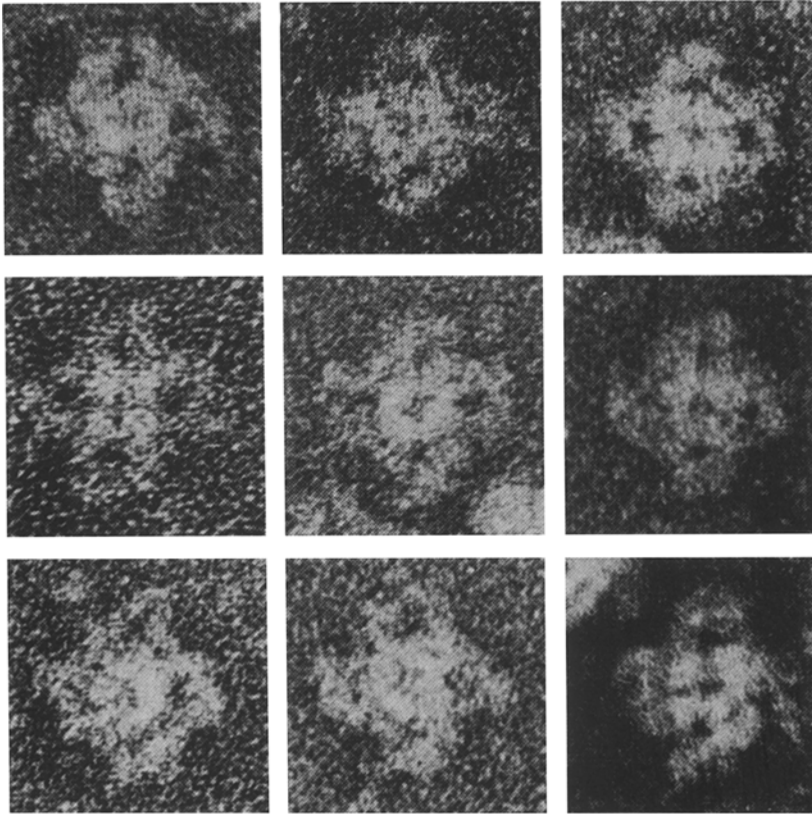

Fig. 6. Negative-stain electron microscopy of the 30 S ryanodine receptor complex. A selected panel of particles with the characteristic cloverleaf shape of the feet structures (Ferguson *et al.*, 1984) stained by uranyl acetate. Dimensions of the quatrefoil are 34 nm from tip of one leaf to the tip of the opposite one, with each leaf 14 nm wide. The central electron-dense region is ~ 14 nm in diameter with central hole 1–2 nm in diameter. With permission from Lai *et al.* (1988*a*).

feet structures (Inui *et al.*, 1987*a*, b; Lai *et al.*, 1987). Recently, Lai *et al.* (1988*a*) reported a more detailed description of the substructure of the ryanodine receptor complex purified by density-gradient centrifugation. In negative-stained images of the purified receptor complex using uranyl acetate, a striking resemblance to the four-leaf clover feet structures, previously described by Ferguson *et al.* (1984), was clearly evident (Fig. 6). At the centre of the quatrefoil was an electron-dense region of ~ 14 nm in diameter surrounding a central hole 1–2 nm in diameter. Four "loops" of lower electron density extended from the central region delineating four peripheral holes or depressions within each leaf of the quatrefoil. Subse-

quently, Saito *et al.* (1988) have reported similar substructural details of the ryanodine receptor purified by sequential column chromatography, including views that appear to show images of the transmembrane stalk of the complex.

The ryanodine receptor has been proposed to exist as an oligomer comprising either four (Lai *et al.*, 1988*a*) or 12 (Inui *et al.*, 1987*a*, *b*) subunits of the high-molecular-weight protein (M_r 350,000–450,000). The estimate of 12 subunits was based on the assumption that the whole foot structure was completely occupied with protein, giving an overall M_r totaling 4,400,000 (Inui *et al.*, 1987*a*, *b*). However, the images shown in Fig. 6 clearly demonstrate that many holes and/or depressions are present in the quatrefoil, indicating that the higher value may be an overestimate. The alternative, tetrameric structure with M_r 1,600,000 proposed by Lai *et al.* (1988*a*) agrees well with the apparent sedimentation coefficient value of 30 S, the ryanodine-binding stoichiometry of one high-affinity site per four subunits, and the fourfold symmetry of the negatively stained ryanodine receptor complex (Fig. 6).

Reconstitution of the Ryanodine Receptor Channel

The ryanodine receptor purified in Chaps detergent has been reconstituted at the single-channel level into Mueller–Rudin-type planar lipid bilayers (Lai *et al.*, 1988*a*; Smith *et al.*, 1988), and shown to comprise a Ca^{2+}-conducting channel with conductance and gating behavior similar to that previously described for the native Ca^{2+} release channel from heavy SR vesicles. Dilution of the purified skeletal ryanodine receptor resulted in the spontaneous appearance of a cation conductance suggesting that the protein had been incorporated into the planar lipid bilayer. In symmetric 500 mM NaCl buffer, a large conductance of ~600 pS was obtained that displayed a pharmacological profile characteristic of the native Ca^{2+} release channel (Fig. 7). Na^+, and not Cl^-, was the major conducting ion under these conditions since a similar conductance resulted when the NaCl buffer was replaced by Na-PIPES buffer, and conductance was reduced to background levels when replaced by choline chloride (or choline Cl^-). Unit conductances of 500, 461, 406, and 205 pS in 250 mM of the test cation were reported for Cs^+, Na^+, K^+, and Li^+, respectively (Smith *et al.*, 1988*a*). When monovalent cations were replaced with 50 mM Ca^{2+} in the *trans* chamber, a single-channel conductance of 91 pS, similar to that of ~100 pS for the native Ca^{2+} release channel, was obtained (Fig. 7). Under bi-ionic conditions with 60 mM K^+ *cis* and 60 mM Ca^{2+} *trans*, the channel was selectively permeable to Ca^{2+}, with a permeability ratio (P_{ca}/P_K) of ~6 (Smith *et al.*, 1988).

An uncertainty inherent in single-channel reconstitution studies with

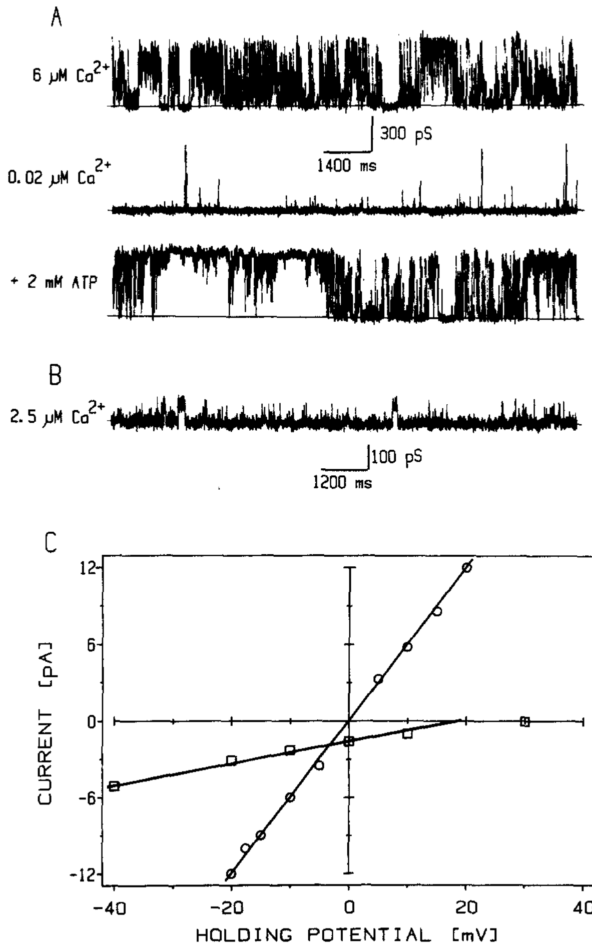

Fig. 7. Reconstitution of the 30 S ryanodine receptor complex into planar lipid bilayers. (A) Single-channel Na^+ currents, shown as upward deflections, were recorded in symmetric 0.5 M NaCl, 20 mM Na-PIPES, pH 7, with 6 μM free Ca^{2+} *cis* (100 μM EGTA and 100 μM $CaCl_2$) (top trace), or with 0.02 μM free Ca^{2+} *cis* (2.1 mM EGTA and 100 μM $CaCl_2$) (middle trace), or with 0.02 μM free Ca^{2+} plus 2 mM ATP in the *cis* chamber (bottom trace). Holding potential (HP) = −15 mV.) (B) Single-channel current recorded after perfusion with 50 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4, 10% glycerol *trans*, and 125 mM Tris/250 mM HEPES, pH 7.4, 10% glycerol, plus 2.5 μM free Ca^{2+} (100 μM EGTA and 100 μM $CaCl_2$) *cis*. HP = 0 mV. (C) Current–voltage relationship for recordings A (top trace) and B. Values of unit conductance: γ_{Na}, 595 pS with 0.5 M Na^+ (○); and γ_{Ca}, 91 pS with 50 mM Ca^{2+} (□) as the conducting ion. With permission from Lai *et al.* (1988*a*).

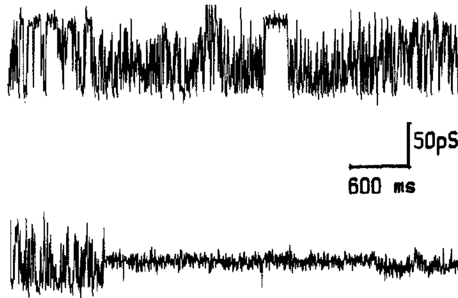

Fig. 8. Effect of ryanodine on single channel activity of the reconstituted purified skeletal 30 S ryanodine receptor. Single-channel Ca^{2+} currents, shown as upward deflections, were recorded in 50 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4 trans, and 125 mM Tris/250 mM HEPES, pH 7.4, 2.5 μM free Ca^{2+} cis buffer before (upper trace) and 1 min after the addition of 10 μM ryanodine cis (lower trace). HP = 0 mV. (Q.Y. Liu, unpublished studies).

purified ion channel proteins is the possibility that a miniscule contaminant in the purified preparation, undetectable by SDS-gel analysis followed by silver staining, may give rise to the observed channel activity. It was therefore important to show that the assumed ryanodine receptor channels recorded in planar lipid bilayers were sensitive to ryanodine modification. Lai *et al.* (1988*a*) and Smith *et al.* (1988) reported that addition of ryanodine to the *cis* chamber did indeed induce the typical response reported for native Ca^{2+} release channels (Rousseau *et al.*, 1987; Imagawa *et al.*, 1987; Nagasaki and Fleischer, 1988). A long-lived open-channel state is formed with a reduced conductance of $\sim$50% that of the native channel (Fig. 8). Interestingly, a long-lived open subconducting and pharmacologically insensitive Ca^{2+} channel was also recorded upon incorporation of the digitonin-solubilized receptor purified by immunoaffinity chromatography (Imagawa *et al.*, 1987).

A novel feature not readily observed in lipid bilayer recordings with native channels was the appearance of subconductance states with the reconstituted Chaps-purified ryanodine receptor (Lai *et al.*, 1988*a*; Smith *et al.*, 1988; Liu *et al.*, 1988, 1989). The presence of subconducting states was also indicated in patch-clamp recordings of Ca^{2+} currents in native frog muscle "sarcoball" membranes that showed two predominant conductance levels of $\sim$90 pS and $\sim$140 pS (Stein and Palade, 1988).

The purified skeletal ryanodine receptor has also been functionally reconstituted into a lipid bilayer using the monolayer spreading method (Hymel *et al.*, 1988*a*). Using this technique, Ca^{2+} conductances in multiples of 3.8 pS up to 120 pS or larger were recorded. Although a clear structural assignment of the 3.8 pS conductance was not possible, the presence of multiple conductance levels that increased with time was interpreted to indicate the continuous association of 12–16 monomers of the high-molecular-weight

protein subunits (M_r 360,000) into oligomers of increasing conductance (Hymel *et al.*, 1988*a*).

The reconstitution of the purified cardiac ryanodine receptor into planar lipid bilayers has also been reported. Lai *et al.* (1988*b*), Anderson *et al.* (1988, 1989), and Rardon *et al.* (1988) described the cardiac ryanodine receptor channel as comprising a large conductance Ca^{2+} channel (72–80 pS in 50 mM Ca^{2+}) with a conductance and kinetic behavior similar to that of the native cardiac Ca^{2+} release channel (Rousseau *et al.*, 1986). In contrast, and as observed for the skeletal ryanodine receptor channel, Hymel *et al.* (1988*b*) reported that the cardiac ryanodine receptor comprised Ca^{2+} channels ranging from 4 to 60 pS and more.

Conclusion

The identification of [^{3}H]ryanodine as a specific ligand has enabled the rapid isolation of the ryanodine receptor–Ca^{2+} release channel complex and has thus opened the way to a systematic study of the molecular properties of a key component in E–C coupling. The ryanodine receptor has been purified by a number of laboratories (Campbell *et al.*, 1987; Imagawa *et al.*, 1987, 1989; Inui *et al.*, 1987*a*, *b*; Lai *et al.*, 1987, 1988*a*, *b*; Anderson *et al.*, 1988; Rardon *et al.*, 1988), shown to contain intrinsic Ca^{2+} channel activity (Lai *et al.*, 1988*a*, *b*; Smith *et al.*, 1988*a*; Hymel *et al.*, 1988*a*, *b*; Anderson *et al.*, 1988, 1989; Rardon *et al.*, 1988; Liu *et al.*, 1989), and identified to be the feet structures that span the junctional gap between T-tubule and SR (Inui *et al.*, 1987*a*, *b*; Lai *et al.*, 1987, 1988*a*; Anderson *et al.*, 1988, 1989; Saito *et al.*, 1988). Table III summarizes the presently known properties of the cardiac and skeletal SR ryanodine receptor–Ca^{2+} release channel complex as determined in this laboratory. The ryanodine receptor–Ca^{2+} release channel exists as a 30 S complex with a four-leaf-clover morphology, possesses a

Table III. Properties of SR Ryanodine Receptor–Ca^{2+} Release Channel Complex

1. Composition: 30 S complex with four polypeptides of $M_r \sim 400{,}000$
2. Structure: Four-leaf clover (quatrefoil)
3. Ryanodine binding: One high-affinity [^{3}H]ryanodine-binding site per 30 S complex
4. Conductance:

50 mM Ca^{2+} *trans*	100 pS (skeletal) 80 pS (cardiac)
500 mM Na^{+} *trans*	600 pS (skeletal) 550 pS (cardiac)

5. Regulation: Activation by μM Ca^{2+} and mM ATP
Inhibition by mM Mg^{2+} and μM calmodulin

single high-affinity site for ryanodine, and comprises high-molecular-weight proteins of $M_r \sim 400{,}000$ in an apparent tetrameric stoichiometry. The intrinsic cation channel within the purified ryanodine receptor complex has a large monovalent and divalent cation conductance, the activity of which is regulated by the endogenous ligands Ca^{2+}, Mg^{2+}, ATP, and calmodulin.

Although recent progress has enabled a much improved understanding of the mechanism of SR Ca^{2+} release, several major questions of immediate relevance regarding the structure and function of the Ca^{2+} release channel remain to be resolved:

1. Is the 30 S complex composed of four identical subunits (α_4) or different subunits of similar size ($\alpha_3\beta$ or $\alpha_2\beta_2$)?
2. Does the channel complex comprise a single ion-conducting pore or are there several highly cooperatively interacting ion pathways within the 30 S complex?
3. Does disruption of the T–SR junction during isolation of heavy SR vesicles result in an "uncoupled" channel with a loss of function and/or an altered sensitivity to regulation by physiological effectors such as IP_3 (Volpe *et al.*, 1985; Somlyo, 1985) and calmodulin (Meissner, 1986*b*; Kim and Ikemoto, 1986; Planck *et al.*, 1988; Smith *et al.*, 1989)?
4. What is the identity and function of the junctional proteins present in the T-tubule membrane and cytosol that are thought to specifically associate with the feet (Corbett *et al.*, 1985; Kawamoto *et al.*, 1986; Chadwick *et al.*, 1988)?
5. Do other SR proteins (smaller than $\sim 400{,}000$) have a modulatory role in Ca^{2+} release (Kim and Ikemoto, 1986; Zorzato *et al.*, 1986), or are they related to the acute lability of the channel to proteolysis (Seiler *et al.*, 1984; Chu *et al.*, 1988; Trimm *et al.*, 1988; Rousseau *et al.*, 1988; Meissner *et al.*, 1989)?
6. Is the Ca^{2+} release channel the sole contributor to physiological SR Ca^{2+} release during E–C coupling, or are there additional Ca^{2+} release pathways in SR (Smith *et al.*, 1986*b*; Suarez-Isla *et al.*, 1986)?

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