Ryanodine Receptor Purified from Crayfish Skeletal Muscle

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Abstract. The ryanodine receptor was isolated from the sarcoplasmic reticulum of crayfish skeletal muscle. Ryanodine binding to the native fraction was measured by Scatchard analysis and values of 60 nmol/l and 9 pmol/mg were obtained for K_D and B_{max} respectively. The identity of purified receptor was confirmed by electron microscopy, electrophoresis and incorporation into planar lipid bilayers. At least two conductance states (100 pS and 50 pS) were observed in 100 mmol/l NaCl both for native and purified receptor.

Key words: Ryanodine receptor — Purification — Planar lipid bilayer — Electron microscopy

Abbreviations: CHAPS — (3[(3Cholamidopropyl) dimethyl -ammonio] 1-propanesulfonate); DIFP — Diisopropylfluorophosphate; EGTA — Ethyleneglycol-bis- (-aminoethylether) N, N, N′, N′-tetraacetic acid; PIPES — Piperazine-N, N′,-bis [2-ethanesulfonic acid]; TRIS — Tris-(hydroxymethyl)-aminomethane.

Introduction

In skeletal and cardiac muscle, contraction is initiated by rapid release of Ca^{2+} ions from the sarcoplasmic reticulum (SR). Recent studies (Rousseau et al. 1986; Anderson et al. 1989; Smith et al. 1988; Lai et al. 1987; 1988) have suggested that the Ca^{2+} release from the SR is mediated by a high-conductance, ligand gated "Ca release" channel. This channel is significantly affected by the neutral plant alkaloid ryanodine, which specifically binds at nanomolar concentrations to the cardiac and skeletal Ca^{2+} release channels (Pessah et al. 1986; Alderson et al. 1987; Fleischer et al. 1985). Using [^{3}H]ryanodine as the Ca^{2+} release channel specific ligand, the detergent-solubilized ryanodine receptor has been identified in rabbit cardiac and skeletal muscle (Lai et al. 1988; Anderson et al. 1989; Imagawa et al. 1987) and frog skeletal muscle (Liu et al. 1989).

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The Ca^{2+}-release channel has been shown to be a polypeptide with a monomeric relative molecular mass (M_r) of ≈ 350,000—450,000 (Lai et al. 1988; Lai and Meissner 1989). The sequence of 5037 amino acids composing the ryanodine receptor from rabbit skeletal muscle sarcoplasmic reticulum has been deduced by cloning and sequencing the complementary DNA (Takeshima et al. 1989). The calculated M_r of the rabbit skeletal muscle ryanodine receptor is 565,223; this is in reasonable agreement with the values estimated by SDS-polyacrylamide gel electrophoresis. Electron microscopy revealed a fourfold symmetric structure (Lai et al. 1988; Saito et al. 1988) and three-dimensional architecture (Wagenknecht et al. 1989). When incorporated into planar lipid bilayers, the purified protein exhibited Ca^{2+} conductance with pharmacological properties of the native Ca^{2+} channel (Anderson et al. 1989; Imagawa et al. 1987; Hymel et al. 1988; Smith et al. 1988; Liu et al. 1989; Lai et al. 1988). The purified channel was permeable to monovalent ions, such as Na^+, K^+, with high conductances, and showed at least four distinct conductance levels for both Na^+ and Ca^{2+} conducting ions (Liu et al. 1989; Smith et al. 1988).

Herein, we report the properties of the ryanodine receptor from crayfish skeletal muscle, which is known to use external calcium ions for the activation of contraction (for a review see Zachar 1971).

Materials and Methods

Materials. [^{3}H]Ryanodine was obtained from New England Nuclear, unlabelled ryanodine was from Calbiochem. Asolectin was from Fluka, CHAPS, DIFP, EGTA, PIPES from Sigma, TRIS from Loba Feinchemie.

Isolation of SR membranes. Sarcoplasmic reticulum membrane fractions derived from crayfish tail muscle were prepared as follows: 50 g of tail muscle tissue was homogenized in 250 ml of 0.1 mol/l NaCl, 5 mmol/l TRIS maleate, pH = 6.8 at 4 °C for 60 s with a Polytron homogenizer. The homogenate was centrifuged for 40 min. at 2600 × *g*. The heavy SR was isolated as a pellet after centrifugation for 1 h at 36,000 × *g*. The pellet was resuspended in solution A (10 mmol/l PIPES, 0.5 mol/l NaCl, 70 μmol/l $CaCl_2$, 1 mmol/l DIFP, pH = 7.2), and stored frozen in liquid nitrogen.

Ryanodine binding assay. Ryanodine binding to heavy SR vesicles was studied as described by Michalak (1988) with some modifications. Briefly, the heavy SR of crayfish tail muscle (at a protein concentration 300 μg/ml) was incubated with [^{3}H]ryanodine (spec. act. = 60 Ci/mmol) in a standard solution A for 2 h at room temperature. The unbound ryanodine was separated from the protein-bound ryanodine by filtration through Whatman GF/B filters, followed by washing with unlabelled icecold solution containing 0.2 mol/l NaCl, 10 mmol/l PIPES, 70 μmol/l $CaCl_2$ pH = 7.0. The activity retained on the filters was measured using standard liquid scintillation counting techniques. For the purpose of this study, specific binding of ryanodine has been defined as the difference between total binding (with [^{3}H]ryanodine alone) and nonspecific binding (with [^{3}H]ryanodine and 10 μmol/l unlabelled ryanodine). Nonspecific binding made up approx. 10—15 % of total binding.

Purification of the ryanodine receptor. The procedure of Lai et al. (1988) with some modification was used. Crayfish tail muscle heavy SR (1.5 mg/ml) was solubilized in solution A, containing 17 nmol/l [^{3}H]ryanodine (spec. act. = 60 Ci/mmol). The 100,000 × *g* supernatant was loaded onto a 5—20 % linear sucrose gradient in buffer A containing 0.9 % CHAPS, 4 mg/ml asolectin and centrifuged for 16 h at 130,000 × *g*. The sucrose gradient was fractionated (0.35 ml/fraction) and taken for the analyse of radioactivity and protein composition on 7.5 % SDS-PAGE.

Planar lipid bilayer measurements were performed as described by Liu et al. (1989) with some modifications. Lipid bilayers from asolectin (40 mg/ml in *n*-decane) were formed on a circular aperture (600 μm in diameter) in the wall of Teflon separating two chambers. The purified sample was added to one side; the two chambers contained the symmetrical solutions (100 mmol/l NaCl, 20 mmol/l PIPES, 200 μmol/l $CaCl_2$, 100 μmol/l EGTA, pH = 7.0). Data were digitized at 500 Hz and stored in a SM 4/20 computer.

Electron microscopy. One drop of sample in solution A containing 0.9 % CHAPS and 4 mg/ml asolectin was transferred onto a carbonated formvar covered copper grid. After 5 minutes it was drained with filter paper. Double staining: the sample was contrasted with 1 % uranyl acetate in distilled water for 3 minutes. The grid was then washed with several drops of distilled water. Then, 1 % lead citrate (pH = 12.0) was dropped on the grid and after additional 3 min the grid was washed with distilled water. The samples were drained again with filter paper, carbonated and studied under a JEM 1200 EX electron microscope at 120,000x magnification.

Results and Discussion

Fig. 1 *A*. illustrates ryanodine binding to the heavy fraction of crayfish skeletal muscle SR membrane. Our preparation of crayfish skeletal muscle bound [^{3}H]ryanodine in a saturable manner. The Scatchard analysis of specific ryanodine binding to skeletal muscle SR revealed a single class of high affinity binding sites with K_D and B_{max} values of 60 nmol/l and 8.95 pmol/mg protein (Fig. 1*B*). These values are close to those reported by Michalak et al. (1988) and Pessah et al. (1985) for rabbit skeletal muscle SR and for canine cardiac SR membranes by Anderson et al. (1989). All experiments were performed at 70 μmol/l concentration of Ca^{2+} in solutions due to a maximum of [^{3}H]ryanodine binding at this concentration (Fig. 2.).

CHAPS-solubilized ryanodine receptor was isolated by centrifugation through a linear sucrose gradient (Fig. 3). A peak of radioactivity appeared in approximately the middle of the gradient. The peak did not appear when the solubilized membranes were incubated in the presence of 10 μmol/l unlabelled ryanodine. The large amount of radioactivity on the top of the gradient was due to unbound [^{3}H]ryanodine. The activity in the peak was 1 % of that on the top of the gradient, i. e. less than reported by other authors.

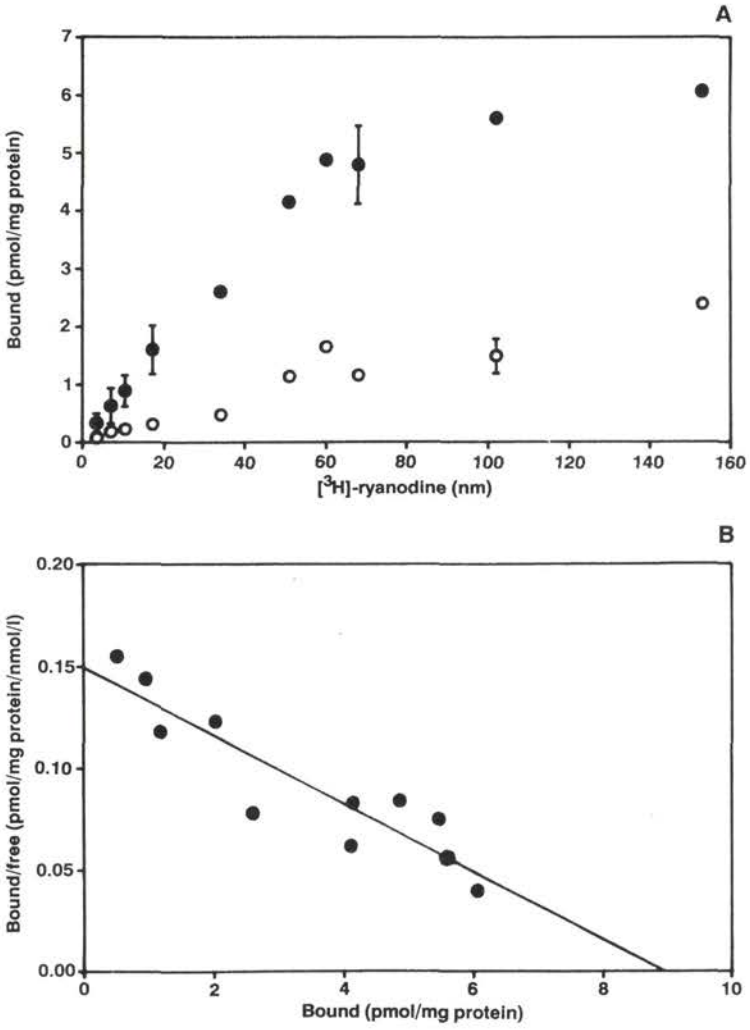

Fig. 1. (*A*) Specific (●) and nospecific (○) binding of [^{3}H]ryanodine to crayfish skeletal muscle SR vesicles. (*B*) Scatchard plot of [^{3}H]ryanodine binding.

SDS-polyacrylamide gel electrophoresis of the ryanodine receptor peak fraction showed specific comigration of a single high molecular weight polypeptide of M_r 380—400K (Fig. 4), which was also present in the neighbouring fractions.

The solubilized ryanodine receptor was incorporated into a bilayer membrane in symmetrical NaCl solution, because of its large conductance under these conditions (Liu et al. 1989; Lai et al. 1988; Smith et al. 1988). Na^+ current

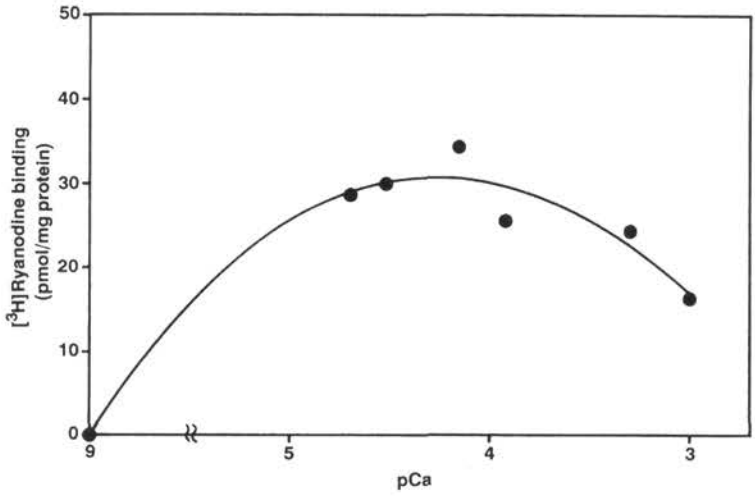

Fig. 2. The Ca^{2+} dependence of [^{3}H]ryanodine binding to heavy SR vesicles. For details see Materials and Methods.

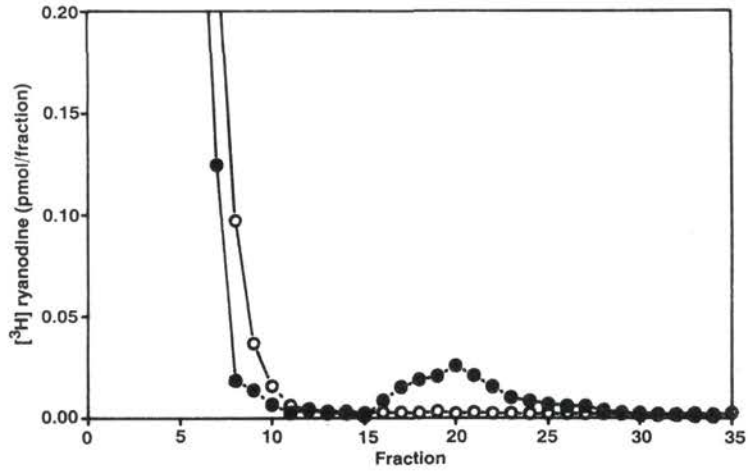

Fig. 3. Sedimentation profile of CHAPS-solubilized [^{3}H]ryanodine receptor from crayfish skeletal muscle SR in a 5—20 % linear sucrose gradient. Samples were labelled with [^{3}H]ryanodine in the presence (○) or absence (●) of 10 μmol/l unlabelled ryanodine.

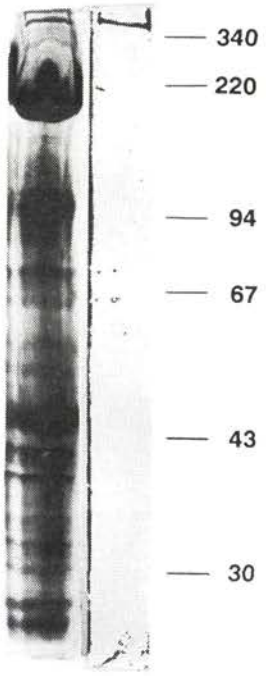

Fig. 4. Silver stained SDS-PAGE 7.5 % gel of heavy SR membranes (H) and purified ryanodine receptor (R) under non-reducing conditions. Molecular weight standards (Pharmacia): ferritin (220,000), phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000).

specifically appeared in the fractions containing receptor peak; no single channel activity could be detected in any other gradient fractions. All single-channel recordings in NaCl buffer displayed multiple levels of conductance, with the additional sublevel of approximately half of the amplitude of the maximal conductance. Fig. 5 shows the records obtained from purified and native ryanodine receptor; both showed the same unitary conductance of 100 pS, sublevel conductances of 50 pS and similar current-voltage relationships (Fig. 6). There were no differences observable between the records from the native and the purified ryanodine receptor. Similar subconduction states were also reported by Liu et al. (1989), although with a twofold higher amplitude in 100 mmol/l NaCl than in our experiments. In addition to the two conductance levels, two other conductance sublevels were occasionally observed. These conductance states may be related to the tetrameric structure of the receptor complex (Fig. 7). The

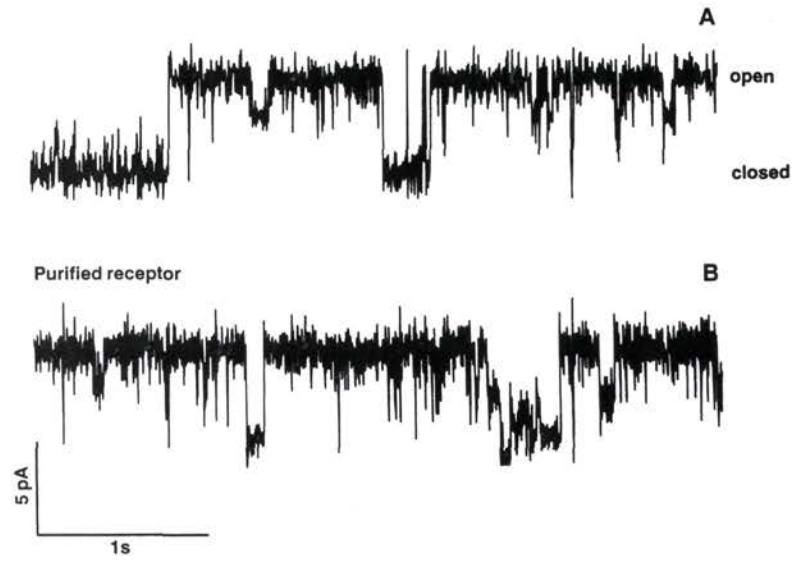

Fig. 5. Single channel recordings of Na^+ current in native (upper trace) and purified (lower trace) ryanodine receptor incorporated into a planar lipid bilayer in symmetrical 100 mmol/l NaCl; holding potential HP = −60 mV.

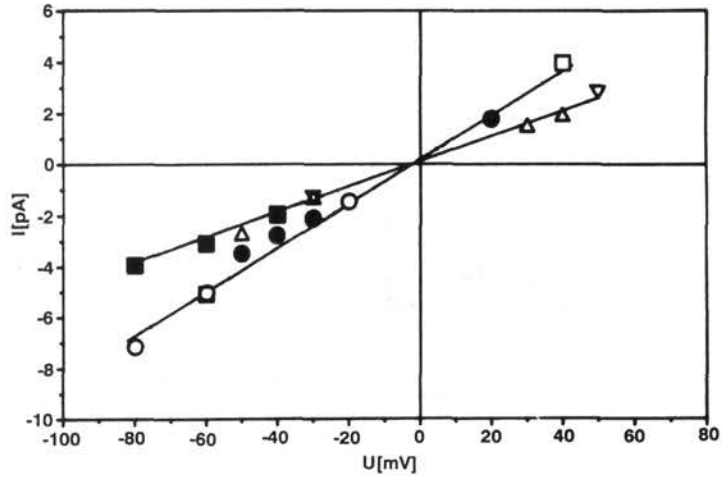

Fig. 6. The current — voltage relationships for the purified (○) and native (other symbols) ryanodine receptor, giving two major conductance levels of 100 and 50 pS.

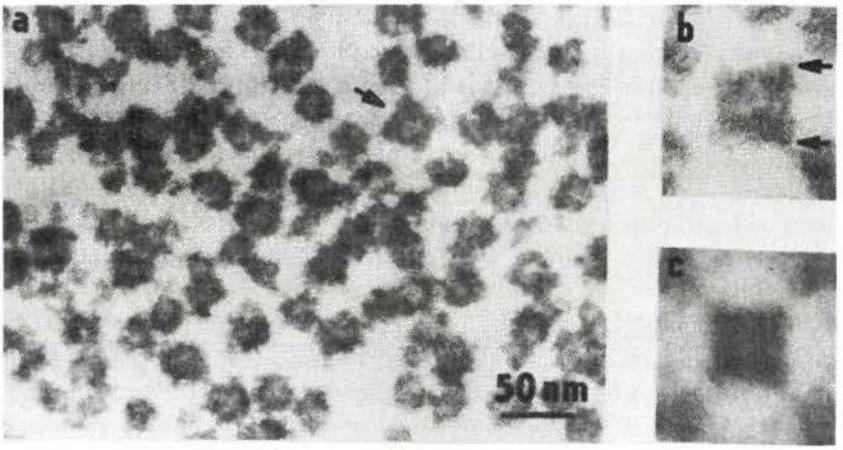

Fig. 7. Electron micrograph of crayfish ryanodine receptor. Double staining (see Materials and Methods) shows the receptor structure which ressembles a square with a side length of 25 nm. Magnif. 120,000x.

receptor conductance in Ca^{2+} solution was not measured due to technical problems concerning solution exchange on both sides of the membrane after receptor incorporation into the membrane.

In conclusion, our data indicate that the rabbit and the crayfish skeletal muscle ryanodine receptor do not differ from each other significantly, though they operate on different excitation-contraction coupling mechanisms, at least as far as the role of the external Ca^{2+} ions is concerned (Zacharová and Zachar 1967; Valko et al. 1967).

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