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A simple, fast, one-step method for the purification of the skeletal-muscle ryanodine receptor

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In this paper we describe a simple, fast, one-step method for the purification of the skeletal-muscle ryanodine receptor. The ryanodine receptor from CHAPS-solubilized junctional sarcoplasmic-reticulum membranes was adsorbed to a spermine–agarose column and eluted by 2 mM-spermine. The purified receptor, consisting predominantly of a 450 kDa polypeptide on SDS/PAGE, binds [^{3}H]ryanodine with a specific activity of $\sim$ 300 pmol/mg of protein and with a high affinity ($K_D = 4.7 \pm 2$ nM). The purified receptor appears to retain the pharmacological properties of the receptor in the original membranes. The purification resulted in over 80% recovery of the initial ryanodine-binding sites and about 30–96-fold purification. This simple and fast method is highly reproducible and suitable for purification of small as well as large quantities of ryanodine receptor.

INTRODUCTION

In skeletal muscle, contraction is initiated by a depolarization of the transverse tubular membrane, which in turn signals the release of Ca^{2+} from the sarcoplasmic reticulum (SR), an intracellular membrane surrounding each myofibre [1,2]. A key protein involved in the excitation–contraction coupling is the ryanodine receptor, a protein which binds the toxic alkaloid ryanodine with nanomolar affinity [3].

The ryanodine receptor from skeletal and cardiac muscle SR has been purified by several laboratories. Inui *et al.* [4,5] subjected a CHAPS-solubilized preparation to sequential column chromatography on heparin–agarose and hydroxyapatite. Campbell *et al.* [6] used immunoaffinity purification of a digitonin-solubilized preparation. Meissner's group [7,8] subjected their CHAPS-solubilized preparation to a single-step purification, using sucrose-gradient centrifugation. Hawkes *et al.* [9] later combined ion-exchange chromatography with density-gradient centrifugation, and Imagawa *et al.* [10] combined heparin–agarose column chromatography with sucrose-gradient centrifugation. These purification procedures resulted in purified protein which binds ryanodine with high affinity (4–30 nM) and very high activity (160–650 pmol/mg of protein). However, except for the density-gradient centrifugation method [7], the recovery of ryanodine-binding activity was very low ($\leq 25\%$).

The purified protein was found to comprise high-molecular-mass ($\sim$ 450 kDa) polypeptides, which are assembled into a tetrameric complex of apparent sedimentation coefficient 30 S [4–10]. Ryanodine-receptor cDNA from skeletal muscle has been cloned and sequenced, and a molecular mass of 565 kDa was determined [11,12]. When incorporated into planar lipid bilayers, the purified protein exhibited a Ca^{2+} conductance with pharmacological properties of the native SR Ca^{2+}-release channel [7,13]. The purified ryanodine receptor was found to be the major component of the 'foot' structures which connect the transverse tubular and SR membranes [2–4,7].

In this paper we describe a new simple, fast, one-step method with high recovery for the purification of ryanodine receptor, using spermine–agarose affinity chromatography.

EXPERIMENTAL

Materials

ATP, EGTA, Tricine, Mops, spermine, phosphatidylcholine (type IV-S) spermine–agarose, PEG 600, CHAPS, phenylmethanesulphonyl fluoride, polyethylenimine, leupeptin, benzamidine, aprotinin, pepstatin A and soybean trypsin inhibitor (type IIS) were obtained from Sigma Chemical Co. [^{3}H]Ryanodine (60 Ci/mmol) was purchased from New England Nuclear, and unlabelled ryanodine was obtained from Calbiochem.

Membrane preparations

Junctional SR membranes were prepared from rabbit fast-twitch skeletal muscle as described by Saito *et al.* [14], and the fractions R_4 or R_3 were used. The membranes were suspended to a final concentration of about 25 mg of protein/ml in a buffer containing 0.25 M-sucrose, 10 mM-Tricine, pH 8.0, and 1 mM-histidine and stored at -70 °C. Protein concentration was determined by the method of Lowry *et al.* [15].

Purification of the ryanodine receptor

Frozen SR membranes (5–20 mg) were thawed and suspended (to a final concentration of 2 mg/ml) in 1.0 M-NaCl/25 mM-Mops (pH 7.4)/100 μM-$CaCl_2$. For prelabelling with [^{3}H]ryanodine, the membranes were incubated with 40 nM-[^{3}H]ryanodine (diluted 1:4 with unlabelled ryanodine) for 2 h at 37 °C before membrane solubilization. The following proteinase inhibitors were added to the indicated final concentrations before the solubilization: 0.3 mM-phenylmethanesulphonyl fluoride, 0.8 mM-benzamidine, 0.5 μg of aprotinin/ml, 0.5 μg of pepstatin A/ml, 1 μg of leupeptin/ml, 10 μg of soybean trypsin inhibitor/ml and 0.5 mM-EGTA, and also 1 mM-dithiothreitol. Solubilization of the membranes was initiated by adding 10% (w/v) CHAPS to a final concentration of 1.2% or 1.3%. The sample was incubated at 4 °C for 20 min and centrifuged at 40000 *g* for 30 min. The CHAPS extract was diluted about 5-fold with 10 mM-Mops, pH 7.4, and CHAPS (to bring the NaCl and CHAPS concentrations to 0.2 M and 0.4% respectively), and loaded on to a small spermine–agarose column (5 cm × 0.8 cm) pre-equilibrated with 10 mM-Mops (pH 7.4)/0.2 M-NaCl/0.4%

Abbreviations used: SR, sarcoplasmic reticulum; PEG, poly(ethylene glycol) 600.

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CHAPS, containing 0.3 mM-phenylmethanesulphonyl fluoride and 0.8 mM-benzamidine (buffer A). The column was washed with cold (~ 10 °C) buffer A (20–30 ml) until no protein could be detected in the eluate. The rate of loading and washing was about 1 ml/3 min. Ryanodine receptor was eluted with buffer A (10 ml) containing 2.0 mM-spermine. Other proteins, such as calsequestrin, were eluted with higher concentrations of spermine (5–20 mM). Fractions (0.6–0.9 ml) were collected at the rate of 1 ml/3 min, and 30 μl samples were assayed for protein content by two methods [15,16] and for [^{3}H]ryanodine radioactivity. The purified ryanodine receptor was stored at −70 °C in a solution containing 10 mM-Mops, pH 7.4, 1 mM-dithiothreitol, 0.5 mg of soybean lecithin/ml, 0.5 M-NaCl and 15% (w/v) sucrose. Under these conditions its activity did not change for at least 2 months. In some purifications, the partially purified ryanodine receptor (contaminated with Ca^{2+}-ATPase) was further purified on a second spermine–agarose column (1 ml syringe) after dilution with 0.2 M-NaCl, 10 mM-MOPS, pH 7.4, and 0.4% CHAPS to decrease spermine to 0.3 mM. Finally the spermine–agarose column was washed with 10 ml of 1 M-NaCl, followed by 10 ml of 0.5 M-NaCl, and then stored at 4 °C.

[^{3}H]Ryanodine binding

Unless otherwise specified, junctional SR membranes (0.5–2 mg/ml) were incubated with 20 nM-[^{3}H]ryanodine in a standard binding solution containing 1 M-NaCl, 25 mM-Mops, pH 7.4, and 100 μM-$CaCl_2$ for 2 h at 37 °C. The unbound ryanodine was separated from the protein-bound ryanodine by filtration of samples (40 μg of protein) through Whatman GF/B filters, followed by washing with 3 × 5 ml of ice-cold buffer containing 0.2 M-NaCl, 5 mM-Mops, pH 7.4, and 50 μM-$CaCl_2$. The filters were dried and the retained radioactivity was determined by liquid-scintillation-counting techniques. [^{3}H]Ryanodine binding to the soluble and the purified ryanodine receptor was assayed essentially as described by Hawkes *et al.* [9]. [^{3}H]Ryanodine-binding medium was identical with that used for the membranes. The solubilized purified proteins (4–12 μg) were incubated for 5 min at 24 °C with 0.5 mg of soybean lecithin (type IV-S) before their inclusion in the assay medium. After incubation for 2 h at 30 °C, BSA was added to a final concentration of 1.4 mg/ml, followed by 10-fold dilution with an ice-cold solution containing 10% PEG in 0.3 M-NaCl and 10 mM-Mops, pH 7.4 (buffer B). After 15 min on ice, the samples were filtered on Whatman GF/B filters (presoaked in 0.5% polyethylenimine), followed by washing with 3 × 4 ml of an ice-cold 10% PEG solution (buffer B). Specific binding of [^{3}H]ryanodine is defined as the difference between the binding in the absence and in the presence of 20 μM unlabelled ryanodine. Filters were counted for radioactivity as above.

Gel electrophoresis

Analysis of the protein profile by SDS/polyacrylamide-slab-gel electrophoresis was performed with the discontinuous buffer system of Laemmli [17] in 1.5 mm-thick slab gels with 4–13% acrylamide. Gels were stained with Coomassie Brilliant Blue. Molecular-mass standards (Bio-Rad) were: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 42.7 kDa.

RESULTS AND DISCUSSION

Spermine is a polyamine, which are polycationic metabolites in prokaryotic and eukaryotic cells which influence a variety of cell functions such as growth, differentiation and regulation of enzyme activities [18]. We have found recently [19] that spermine interacts with the ryanodine receptor and modulates its properties. The effect of spermine on ryanodine-receptor properties suggests that this receptor has spermine-binding site(s). On the basis of this suggestion, we have developed a procedure for purification of the ryanodine receptor from skeletal muscle. A representative purification of [^{3}H]ryanodine-prelabelled receptor is shown in Fig. 1. The membranes were prelabelled, as described in the Experimental section, with 40 nM-[^{3}H]ryanodine, a concentration at which most of the high-affinity sites (K_D = 4 nM;

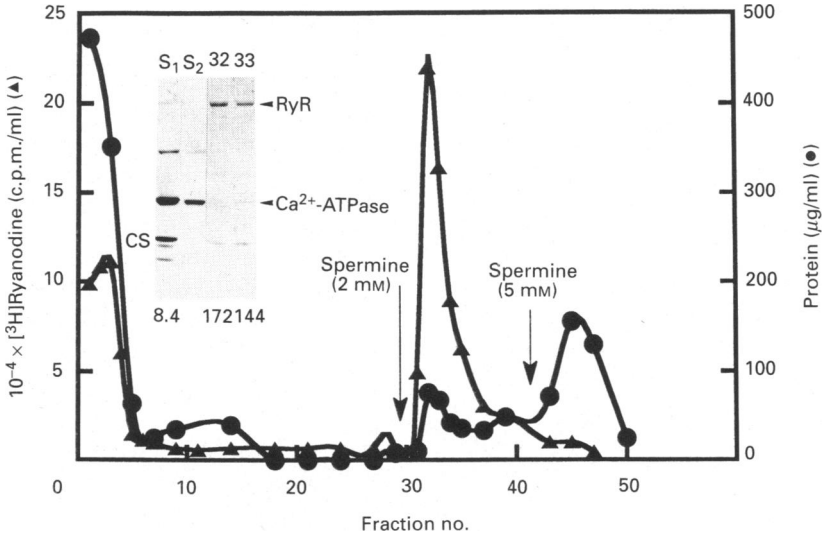

Fig. 1. Purification of ryanodine receptor by spermine–agarose column

Junctional SR membranes (R_4 fraction) (8.8 mg) were labelled with [^{3}H]ryanodine (40 nM), solubilized by CHAPS, and ryanodine receptor was purified as described in the Experimental section. The [^{3}H]ryanodine radioactivity (▲) and the protein content (●) of the various fractions (30 μl) are shown. The SDS/PAGE pattern of CHAPS extract before (S_1, lane 1) and after its passage through the column (S_2, lane 2) and of fractions 32 and 33 is shown in the inset. The calculated specific activity (in pmol of [^{3}H]ryanodine bound/mg of protein) of the indicated fractions is also presented. Abbreviations: RyR, ryanodine receptor; CS, calsequestrin.

see Fig. 3) were occupied. To decrease the dissociation of ryanodine from its binding site, unlabelled ryanodine, to a final concentration of 10 μM, was added 20 min before membrane solubilization. Ryanodine receptor has low-affinity binding sites (K_D = 1–5 μM) which are allosterically coupled to the high-affinity site, their occupation leading to a decrease in the off-rate of ryanodine from the high-affinity site [20,21]. The solubilization of the membranes and the column-chromatography procedures are described in the Experimental section. On the spermine–agarose column, the unbound or loosely bound proteins as well as unbound [^{3}H]ryanodine were effectively washed off with 0.2 M-NaCl. The protein-bound [^{3}H]ryanodine adsorbed to the spermine–agarose is eluted as a single peak by 2.0 mM-spermine. Further increase in spermine concentration to 5 mM or higher elutes a second protein peak with no [^{3}H]ryanodine radioactivity. The SDS/PAGE profile and the values for bound ryanodine of the CHAPS extract applied to the column (S_1), and of two fractions (nos. 32 and 33) eluted from the column by spermine, are also presented in Fig. 1 (inset).

To demonstrate the reproducibility of this method, we show another representative purification (Fig. 2) in which we have started with the less-enriched ryanodine-receptor fraction (R_3). Fig. 2 shows the SDS/PAGE profile of the CHAPS extract before and after its passage through the column, indicating that the ryanodine receptor (450 kDa), its 350 kDa fragment and the 63 kDa species (calsequestrin) are the major proteins adsorbed by the column (Fig. 2, lanes S_1 and S_2). The SDS/PAGE profiles of various fractions from the spermine–agarose column indicate that the fractions obtained by column washing with 0.2 M-NaCl contained most of the SR proteins (except the ryanodine receptor and calsequestrin). In the fractions (nos. 25–29) of bound ryanodine receptor eluted with 2.0 mM-spermine, those of 450 and 350 kDa are the major polypeptides, with minor contamination with Ca^{2+}-ATPase. In the following fractions (nos. 31–35) some calsequestrin was eluted with the ryanodine receptor. The values for specifically bound [^{3}H]ryanodine are also indicated. Fractions 25 and 27 have the maximal specific activity of 318 and 304 pmol of [^{3}H]ryanodine bound/mg of protein respectively, corresponding to a purification of about 96-fold over the original SR membranes. The specific radioactivity of bound ryanodine in the following fractions is decreased, presumably because of increased contamination with calsequestrin. The protein peak eluted with 5 mM spermine contained the negatively charged protein calsequestrin (fractions 45 and 47). It should be mentioned that the 150 kDa protein [22] is also co-purified with the ryanodine receptor.

Table 1. Summary of ryanodine-receptor purification

Summary of ryanodine-receptor purification as detailed in Fig. 1. The values indicated here are from a typical experiment.

Fraction	Protein (mg)	[^{3}H]Ryanodine bound: Specific activity (pmol/mg)	[^{3}H]Ryanodine bound: Total activity (pmol)	Purification (fold)	Yield (%)
Junctional SR membranes	8.8	8.1	71	–	
CHAPS-solubilized junctional SR membrane	8.2	8.3	68	–	96
Spermine-eluted protein (fractions 31–36)	0.34	166.5	56.6	20.6	80

Table 1 summarizes the purification of ryanodine receptor using this simple and fast one-step procedure. Using 8.8 mg of membrane protein, we obtained 0.34 mg of purified ryanodine receptor, with specific [^{3}H]ryanodine-binding activity of 166 pmol/mg of protein, corresponding to 20-fold purification, and overall recovery of ryanodine-binding activity of about 80 % of the initial membrane activity. It should be mentioned that in our calculations the loss of bound [^{3}H]ryanodine during the purification (for about 4 h at 10 °C) was not taken into account. This is because the dissociation of bound ryanodine from its

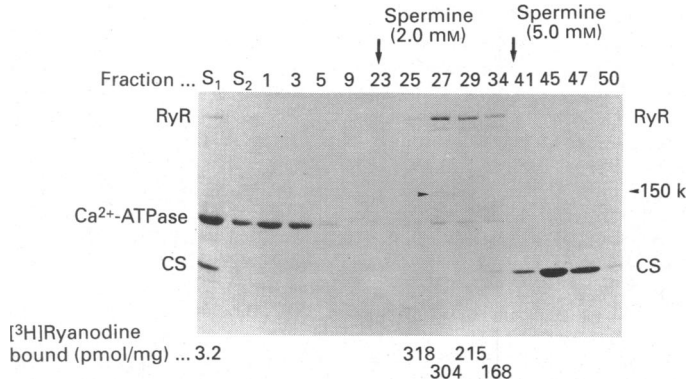

Fig. 2. SDS/PAGE profile of various fractions during purification of ryanodine receptor

Junctional SR membranes (R_3 fraction, 5 mg of protein) were labelled with 20 nM-[^{3}H]ryanodine (diluted 1:4 with unlabelled ryanodine), solubilized by CHAPS, and purified as described in the Experimental section. The SDS/PAGE pattern of the CHAPS extract before (S_1) and after its passage through the column (S_2) and of the indicated fractions obtained during the purification is shown. Fractions 1–22 were obtained by column washing with buffer A, and the other fractions were eluted with buffer A containing 2 mM-spermine (fractions 23–35) or 5 mM-spermine (fractions 36–50). The calculated specific activity, in pmol of ryanodine bound/mg of protein, is also indicated. Abbreviations: RyR, ryanodine receptor; 150 k, 150 kDa protein; CS, calsequestrin.

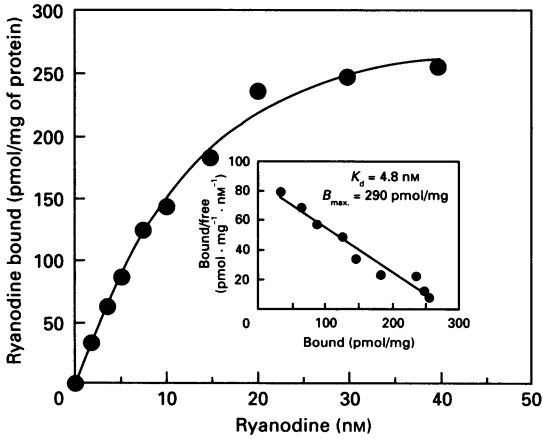

Fig. 3. [^{3}H]Ryanodine binding to the purified receptor

The binding of [^{3}H]ryanodine to the purified receptor (40 μg of protein/ml) was assayed as described in the Experimental section. The [^{3}H]ryanodine was diluted 1:1 or 1:4 with unlabelled ryanodine and used at concentrations from 1.7 to 40 nM. The non-specific binding is 2–5 % of the radioactivity obtained in the absence of unlabelled ryanodine. The inset shows a Scatchard analysis of the data. The lines are the best fit to all data points.

Table 2. Pharmacological profile of the membrane-bound and the spermine–agarose purified ryanodine receptor

The binding of [^{3}H]ryanodine (20 nM) to the junctional SR membranes (0.5 mg/ml) and to purified receptor (35 μg/ml) was determined as described in the Experimental section, except that NaCl concentration was 1 M (Expt. I) or 0.2 M (Expt. II) and the indicated compounds were added to the assay medium. This is a representative experiment.

Assay conditions	[^{3}H]Ryanodine bound (pmol/mg of protein)	
	Membrane-bound	Purified
Expt. I (1 M-NaCl)		
Control	8.10	216.7
Ruthenium Red (25 μM)	3.25	108.9
Ruthenium Red (50 μM)	1.10	79.2
Expt. II (0.2 M-NaCl)		
Control	2.28	94.9
Spermine (5 mM)	3.54	151.6
Spermine (10 mM)	4.16	159.5

receptor is very slow ($t_{\frac{1}{2}} \geqslant 10$ days at 4 °C [7]). Thus minimal loss of bound ryanodine can be expected.

A similar purification protocol was performed with unlabelled membranes. By Scatchard analysis of ryanodine binding to this purified receptor (Fig. 3) a $B_{max.}$ of 280 ± 10 ($n = 2$) pmol/mg of protein and a K_D of 4.8 ± 2 nM ($n = 3$) were obtained. This K_D is similar to that of the original junctional SR membranes ($K_D = 6 \pm 2$ nM), indicating that the ryanodine receptor was purified unchanged with respect to ryanodine binding. The $B_{max.}$ values in different purifications were between 140 and 318 pmol/mg of protein.

The effects of different reagents on ryanodine binding to the membrane-bound and the purified ryanodine receptor is shown in Table 2. Ruthenium Red, known to inhibit ryanodine binding [2,3], inhibits the binding to both preparations. Spermine, as we have found for the membrane-bound receptor [19], also stimulates the binding of ryanodine to the purified receptor. Thus the pharmacological specificity of the membrane-associated high-affinity [^{3}H]ryanodine-binding site is preserved in the purified receptor.

The procedure described here for the purification of ryanodine receptor is very rapid (4–5 h), relatively simple and highly reproducible. This method yields very high recovery of over 80%. The reported recovery of ryanodine-binding activity in previous methods has been reported as 18–21% [4–6,9,10], except for the purification of ryanodine receptor by sedimentation in sucrose gradient [7]. The latter method yields high recovery and high specific binding activity, but this method was designed for small quantities (starting from 1.3–2 mg of SR protein).

The maximal specific activity ($B_{max.}$) of [^{3}H]ryanodine binding to the purified receptor is about 300 pmol/mg of protein. In agreement with previous reports [5,7,9], these data suggest that there is only one ryanodine-binding site per tetrameric complex.

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This method should greatly facilitate studies with the purified receptor aimed at elucidating its multiple functions as a key protein involved in excitation–contraction coupling and as a high-conductance Ca^{2+}-release channel [2,3]. Purified receptor will be particularly useful in studies related to the multiple recognition sites for several regulators such as the activators Ca^{2+}, ATP, ryanodine and spermine [2,3,19] and the inhibitors Mg^{2+}, high concentrations of Ca^{2+}, Ruthenium Red and calmodulin [2,3]. These studies may provide an insight into the control mechanism(s) of opening and closing of this Ca^{2+}-release channel.

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