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High affinity ryanodine binding sites in rat liver endoplasmic reticulum

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The binding of [^{3}H]ryanodine to liver microsomal subfractions was investigated. The smooth microsomal membranes were enriched with ryanodine binding sites and also with a polypeptide of 360 kDa. Caffeine completely inhibited [^{3}H]ryanodine binding. Ryanodine also affected the membrane Ca^{2+} permeability. At low concentrations (< 10 μM) ryanodine stimulated Ca^{2+} efflux and at higher concentrations (> 50 μM) it blocked Ca^{2+} efflux. These results suggest that hepatic microsomes contain ryanodine binding sites which can modify the membrane permeability for Ca^{2+}.

Endoplasmic reticulum; Ryanodine; Caffeine; Ca^{2+} release

1. INTRODUCTION

Ryanodine is a highly toxic neutral alkaloid that causes an irreversible contracture of skeletal muscle and a decrease in the developed tension of cardiac muscle [1]. These effects are due to the interaction of ryanodine with the SR, thereby modulating the Ca^{2+} release activity of this membrane [2,3]. Recent studies identified morphologically the purified ryanodine receptor with the 'foot' structure [4] and with the junctional Ca^{2+} release channel [5–7]. The purified ryanodine receptor is a large, 30S complex comprised of a single polypeptide with apparent relative molecular mass (M_r) of 400000 (400 kDa). Recently, however, the ryanodine receptor from skeletal muscle has been cloned and a molecular weight of 565000 Da was determined [8,9].

Although it is now accepted that the principal site of ryanodine action is the SR, these previous studies did not rule out the possibility that specific ryanodine binding sites might be also present in non-excitable tissues. This possibility was, therefore, explored in the present study by using hepatic microsomal fractions.

2. MATERIALS AND METHODS

2.1. *Membrane preparations*

Liver microsomes were prepared from male Sprague-Dawley rats as described previously [10], except that the liver was homogenized and the 105000 × *g* pellet was resuspended in 0.25 M sucrose; 10 mM Mops, pH 7.1, and 1 mM DTT containing the following protease inhibitors: 0.8 mM benzamidine; 0.5 μg/ml aprotinin; 0.2 mM phenylmethylsulfonylfluoride. Microsomes were fractionated by Cs-containing sucrose-density-gradient centrifugation [11] into: rough ER (pellet); the membranes at 1.3 M sucrose phase; intermediate ER and the 1.3/0.75 M sucrose interface, smooth ER. The microsomes were quickly frozen in liquid nitrogen and stored at −70°C. The protein concentration was determined as described previously [12].

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Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum

2.2. *Ca^{2+} efflux*

This was assayed as described in the legend to Fig. 3.

2.3. *[^{3}H]Ryanodine binding*

Equilibrium binding to the different microsomal fractions was determined by incubation of the membranes (1 mg/ml), for 10 min at 37°C in 0.5 M NaCl; 20 mM Tris-HCl, pH 7.4; 0.5 mM EGTA, and 20 nM [^{3}H]ryanodine (60 Ci/mmol). The unbound ryanodine was separated from protein-bound ryanodine by the filtration method. Protein aliquots (80 μg) were filtered through 0.22 μm Millipore filters and washed two times with 5 ml of cold washing buffer containing 0.2 M NaCl, and 10 mM Hepes, pH 7.4. The counts retained on the filters were determined using liquid scintillation counting techniques.

2.4. *SDS-polyacrylamide gel electrophoresis*

This was performed as described by Laemmli [13].

3. RESULTS AND DISCUSSION

Microsomal membranes, prepared from rat liver, were subfractionated by sucrose-density gradient centrifugation to three subfractions: smooth membranes at the 0.75/1.3 M sucrose interface; intermediate membranes at the 1.3 M sucrose phase; and the rough membranes sedimenting as the pellet. The electrophoretic protein pattern of these fractions demonstrated the presence of a high molecular weight protein doublet of about 360 kDa, which is considerably enriched in the smooth membrane fraction (Fig. 1). The smooth ER fraction is also enriched with two other protein bands

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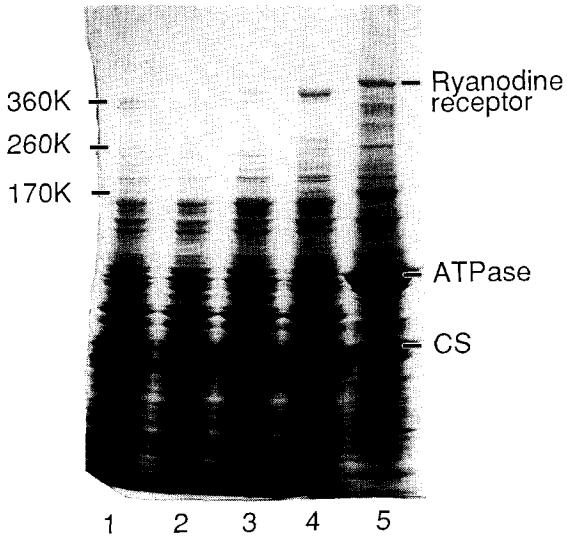

Fig. 1. Gel-electrophoresis pattern of liver microsome subfractions; an enrichment in 360 kDa protein band in the smooth ER fraction. Liver microsomes (lane 1) were fractionated by Cs-containing sucrose-density-gradient centrifugation into: rough ER (lane 2); intermediate ER (lane 3); and smooth ER (lane 4) as described in section 2. Samples (100 μg) were solubilized in SDS and proteins were separated on 4–13% gradient SDS-polyacrylamide gels using a 3% stacking gel. In the heavy fraction of skeletal muscle SR (lane 5), the ryanodine receptor, the (Ca^{2+} + Mg^{2+})ATPase and calsequestrin (CS) are indicated. Molecular weight standards were myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin, with their respective molecular weights at 200000, 116000, 92500, 66200 and 45000. (Bio-Rad).

of 260 and 170 kDa. We have found (results not shown) that four proteins cross-reacted with the affinity purified polyclonal antibodies against the 360 kDa protein (ryanodine receptor) of the rabbit sarcoplasmic reticulum [14]. These proteins were 360 and 260 kDa, and a protein doublet of 200–170 kDa. Although several protease inhibitors were used during the microsomal preparation, we cannot rule out the possibility that the 260 and 170 kDa polypeptides are degradation products of the 360 kDa protein.

To test whether the protein(s) which cross-reacted with the antibodies against the SR ryanodine receptor represent ryanodine binding protein, we measured the binding of [^{3}H]ryanodine to the different membranes fractions. [^{3}H]Ryanodine binds to high affinity sites enriched in the smooth membrane fraction (Table I). The binding of [^{3}H]ryanodine to the smooth membranes is about 1.6- and 2-fold higher than the binding to the unfractionated microsomes or rough ER, respectively. A single class of binding sites with K_d of 8 nM and total binding sites (B_{max}) of 600 fmol/mg protein were obtained by Scatchard analysis of saturation isotherms of ryanodine binding results (data not shown). Quantitative correlation between the 360 kDa polypeptide, present in the different fractions (Fig. 1), and the specific binding of ryanodine (Table I) could not be carried out. This is because the binding values of the different fractions do not represent the maximal binding due to their different filtering efficiency with regard to loss of membranes through the 0.22/μm Millipore filters (data not shown).

The liver is supplied with blood through vessels which contain some smooth muscle. Though their amount in comparison to the liver mass is negligible, it seemed important to verify that ryanodine binds to microsomes derived from parenchymal cells. The possibility that ryanodine binding sites originate from smooth muscle was ruled out by fractionation of microsomes prepared from isolated hepatocytes. Both ryanodine binding to these subfractions and the enrichment in the 360 kDa protein in the smooth fraction were similar to those obtained with microsomes prepared from whole liver. Moreover, further purification of the smooth ER fraction on Mg^{2+} sucrose gradient [15] resulted in an increase in [^{3}H]ryanodine binding capacity of the membranes.

Caffeine, at concentrations which promote Ca^{2+} release from SR [16], inhibited the binding of [^{3}H]ryanodine to the liver smooth ER membranes (Fig. 2). It should be mentioned that caffeine-sensitive Ca^{2+} stores have been demonstrated in non-muscle tissue, the rat sympathetic neuron [17]. Also, it has been proposed that a 170 kDa protein of the SR is the caffeine receptor of the Ca^{2+} release channel [18].

If as suggested, ryanodine is a Ca^{2+} release channel-specific marker [3,6,19,20] then it should affect the Ca^{2+} permeability of the liver microsomes. This was tested by determining the Ca^{2+} permeability of vesicles passively loaded with $^{45}Ca^{2+}$ in the presence and absence of ryanodine. We have found that at concentrations below 10 μM ryanodine stimulated Ca^{2+} efflux, while at higher concentrations it blocked Ca^{2+}-permeable pathway(s) activated by low concen-

Table I

Ryanodine binding by different subfractions of liver microsomes

Fraction	[^{3}H]Ryanodine binding (fmol/mg protein)	
	Total	Specific
Microsomes	520	344
Rough ER	383	244
Intermediate ER	629	404
Smooth ER	770	544

The different membrane fractions were isolated and [^{3}H]ryanodine binding was measured as described in section 2. This is a representative experiment of 12 different membrane preparations. The results are the averages of duplicate samples which differed by 1–5%. The nonspecific binding was determined in the presence of 100 μM of unlabeled ryanodine. It should be mentioned that we used a high concentration of the unlabeled ryanodine, because of the presence of low affinity binding sites for ryanodine (Shoshan-Barmatz, V. and Kraus-Friedmann, N., unpublished results).

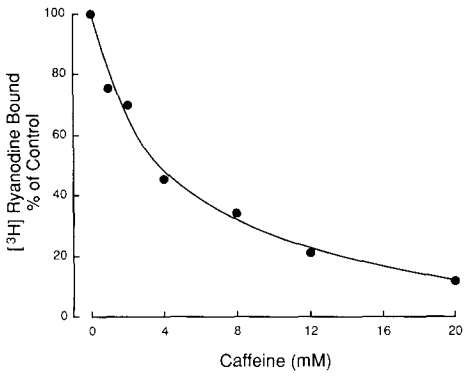

Fig. 2. Caffeine inhibition of [^{3}H]ryanodine binding to liver smooth ER. [^{3}H]Ryanodine binding was assayed in the absence and the presence of the indicated concentrations of caffeine, as described in section 2. Specific binding in the absence of caffeine (100%) = 363 fmol/mg protein. Specific binding represents the difference between total binding (with [^{3}H]ryanodine alone) and nonspecific binding (with [^{3}H]ryanodine and 100 μM unlabeled ryanodine).

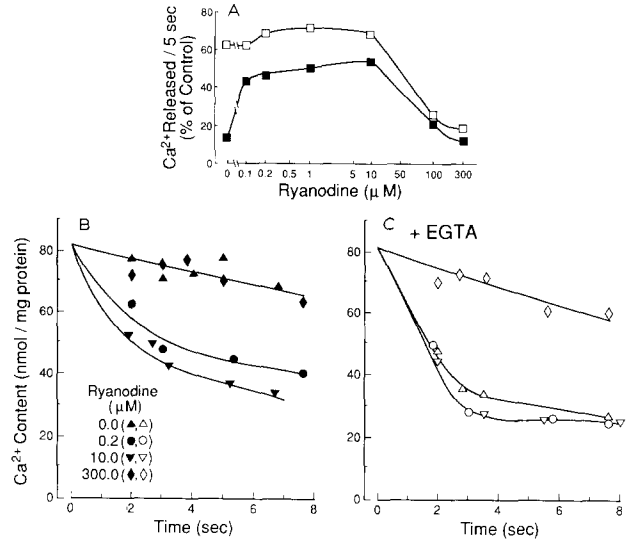

Fig. 3. Effect of ryanodine on the Ca^{2+} permeability of isolated liver microsomes. Microsomes (5 mg/ml) were loaded with 5 mM $CaCl_2$ (containing ^{45}Ca, 3.4×10^3 cpm/nmol) by incubation for 1 h at 37°C in a medium containing 100 mM KCl and 20 mM Mops, pH 6.8 in the absence or the presence of the indicated concentrations of ryanodine. For Ca^{2+} efflux assay, the loaded vesicles (20 μl) were placed on 0.22 μm Millipore filters and rinsed with different volumes of 0.1 M KCl and 10 mM Mops, pH 6.8 (B) or with the same solution containing 1 mM EGTA (C), for the indicated time. The flow rate was 1 ml/s. The dependence of $^{45}Ca^{2+}$ efflux on ryanodine concentration is shown in the upper part of the figure. Closed symbols indicate $^{45}Ca^{2+}$ efflux assayed in the absence, and open symbols in the presence of 1 mM EGTA.

tration of ryanodine or by the presence of EGTA (Fig. 3).

Thus, our results are similar to those reported for the skeletal and cardiac muscle sarcoplasmic reticulum [19–21], and suggest that smooth ER membranes contain a Ca^{2+} release channel which can be activated or inhibited by ryanodine, depending on the experimental conditions. On the other hand, the liver ryanodine receptor differs from the cardiac and skeletal muscle receptors by its fast association and dissociation (both reached steady state at less than 5 min at 24°C), by its Ca^{2+}-independent binding, and by its insensitivity to Ruthenium red and Mg^{2+} (Shoshan-Barmatz and Kraus-Friedmann, unpublished results). These observations suggest that the ryanodine binding sites in the liver microsomes are exposed, in contrast to the cardiac and skeletal SR membranes where ryanodine binds to a site which is accessible only in the open state of the Ca^{2+} release channel/ryanodine receptor [19]. The observed differences between the *liver* and the *muscle* ryanodine binding sites are not surprising in light of the noticeable differences between the cardiac and the skeletal muscle ryanodine receptors, such as their molecular weight and their sensitivity to Mg^{2+}, Ca^{2+} and Ruthenium red [22–24].

The presented data demonstrates the existence of a ryanodine binding protein/Ca^{2+} release system in the liver. This is the first demonstration of such activities in non-excitable tissue. However, ryanodine binding to brain microsomal preparation with similar K_d and B_{max} as we have obtained for the liver was reported recently [25]. These findings raise the possibility that the ryanodine-receptor/Ca^{2+} release system has a wider distribution and role in Ca^{2+}-regulated processes than previously considered.

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