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Characterization of high-affinity ryanodine-binding sites of rat liver endoplasmic reticulum

Differences between liver and skeletal muscle

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In this study, the binding of [^{3}H]ryanodine to liver microsomal subfractions was investigated. The specific binding of [^{3}H]ryanodine, as determined both by vacuum filtration and by ultracentrifugation, is to a single class of high-affinity binding sites with a K_d of 10 ± 2.5 nM and density of 500 ± 100 and 1200 ± 200 fmol/mg of protein by the filtration and centrifugation methods respectively. [^{3}H]Ryanodine binding reached equilibrium in about 1 min and 2 min at 36 °C and 24 °C respectively, and the half-time of dissociation at 37 °C was approx. 15 s. The binding of [^{3}H]ryanodine is Ca^{2+}-independent; it is slightly stimulated by NaCl, Mg^{2+}, ATP and InsP_3 but strongly inhibited by caffeine, diltiazem and sodium dantrolene. Thus the binding of ryanodine to endoplasmic reticulum membranes shares some of the characteristics of its binding to the sarcoplasmic reticulum but also differs from it in several important properties, such as its Ca^{2+}-independence, its rapid association and dissociation, and its inhibition by caffeine. The structural similarities between the skeletal muscle and liver binding sites were further explored by employing *in vitro* DNA amplification techniques, using the known sequence of the skeletal muscle receptor as reference point. The data obtained with this method indicate that the liver does not process mRNA for the skeletal muscle ryanodine receptor.

INTRODUCTION

The endoplasmic reticulum (ER) in the liver plays an essential role in the regulation of cytosolic Ca^{2+} levels. In contrast with the relatively well-characterized mechanisms of Ca^{2+} uptake, the mechanism by which Ca^{2+} is released from the hepatic ER is poorly understood. Several mechanisms for Ca^{2+} release from the ER have been suggested, including mediation by InsP_3 [1] or GTP [2,3], reversal of the Ca^{2+}-ATPase reaction [4] and oxidation of thiol groups [5]. However, no specific Ca^{2+}-efflux pathway or channel has been characterized.

In skeletal and cardiac muscle, the toxic alkaloid ryanodine has been successfully employed as an experimental tool to study Ca^{2+}-release channels [6,7]. At low concentrations ($< 1\ \mu$M) ryanodine was shown to stimulate the efflux of Ca^{2+} from the sarcoplasmic reticulum (SR), presumably by opening a Ca^{2+} channel [8,9], while at higher concentrations ($> 100\ \mu$M) it inhibited Ca^{2+} release [6,7]. Because of these findings it has been suggested that ryanodine is a Ca^{2+}-release-channel-specific marker. The binding site for this marker was localized in the junctional SR membranes [10].

The ryanodine receptor from skeletal and cardiac muscle SR has been purified and found to comprise a high-molecular-mass polypeptide [11–13]. Recently the molecular cloning of the cDNA encoding the ryanodine receptor of cardiac muscle SR has been reported [14]. The purified ryanodine receptor has been incorporated into planar lipid bilayers and found to form Ca^{2+}-conducting pathways [12,13,15]. Structural analysis of the purified receptor suggested that it is morphologically identical to the junctional feet structures which connect the SR junction face membranes to the transverse tubule [11,12].

We have recently reported that ryanodine-binding sites are present in liver microsomes [16,17[. In the present study, ryanodine binding to the liver smooth ER is characterized and its differences from the skeletal muscle binding site are explored.

EXPERIMENTAL

Materials

Tris, EGTA, ATP, caffeine, aprotinin, DL-dithiothreitol (DTT), benzamidine and phenylmethane sulphonyl fluoride were obtained from Sigma. [^{3}H]Ryanodine was from Du Pont/New England Nuclear. Unlabelled ryanodine was obtained from Agricultural System International (Windgap, PA, U.S.A.). Sodium dantrolene was obtained from Norwich Eaton Pharmaceuticals (Norwich, NY, U.S.A.), and was given to us by Dr. T. Nelson.

Membrane preparations

Liver microsomes were prepared from male Sprague–Dawley rats as described previously [18], except that the liver was homogenized and the 105000 *g* pellet was resuspended in 0.25 M-sucrose/10 mM-Mops (pH 7.1)/1 mM-DTT containing the following proteinase inhibitors: 0.8 mM-benzamidine, 0.5 μg of aprotinin/ml and 0.2 mM-phenylmethanesulphonyl fluoride. Microsomes were fractionated by sucrose-density-gradient centrifugation into rough ER (pellet), the membranes at the 1.3 M-sucrose phase (intermediate ER), and the 1.3/0.74 M- and 0.75/0.6 M-sucrose interfaces (smooth ER I and II respectively), as described previously [17,18]. Briefly, the post-mitochondrial supernatant was sedimented at 105000 *g* for 1 h, and the pellet was resuspended in the above buffer to a final concentration of 15–20 mg of protein/ml. A sample (2 ml) of the suspension was carefully layered on top of 2 ml of 0.6 M-sucrose, 3 ml of 0.75 M-sucrose and 5 ml of 1.3 M-sucrose (containing 15 mM-CsCl and 10 mM-Hepes, pH 7.2). After centrifugation for 2 h at 80000 *g* in

Abbreviations used: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; DDT, dithiothreitol.

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a Beckman SW41 swinging bucket rotor, the membranes at the 0.6/0.75 M-sucrose and 0.75/1.3 M-sucrose interfaces and at the 1.3 M-sucrose phase were collected, diluted 3-fold with 100 mM-KCl/20 mM-NaCl/5 mM-Hepes, pH 7.2, and centrifuged at 105000 *g* for 1 h. The pellets obtained were resuspended in the sucrose/Mops buffer, quickly frozen in liquid N_2 and stored at −70 °C. We added the 0.6 M-sucrose layer because the membranes at the 0.75/1.3 M-sucrose interface appeared as a fluffy double layer; the upper layer was a reddish colour and the lower layer was light brown. Some of the fluffy layer was separated on the 0.6/0.75 M-sucrose interface. In some experiments, the post-mitochondrial supernatant (5.5 ml per tube) was applied directly to the sucrose gradient. In this case the volume of the sucrose solutions applied in the gradient was half of that used above.

Hepatocytes, obtained by the collagenase liver perfusion method [19], were homogenized in sucrose/Mops buffer by six up-and-down strokes in a Teflon homogenizer. The 3000 *g* pellet was homogenized again, the two post-mitochondrial supernatants were combined, and microsomes were obtained and fractionated as described above for the whole liver.

[^{3}H]Ryanodine binding

Equilibrium binding to the different membrane 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/20 nM-[^{3}H]ryanodine (60 Ci/mmol). The unbound ryanodine was separated from protein-bound ryanodine by one of two methods: vacuum filtration and ultracentrifugation. In the filtration method, after the incubation protein portions (80 μg) were filtered through 0.22 μm-pore-size Millipore filters, and rapidly washed twice with 5 ml of ice-cold washing buffer containing 0.2 M-NaCl and 10 mM-Hepes, pH 7.4. The counts of radioactivity retained on the filters were determined using liquid scintillation counting techniques. The results are the averages of duplicate samples which differed by 1–5%. Specific binding represents the difference between total binding (with [^{3}H]ryanodine alone) and non-specific binding (with [^{3}H]ryanodine and 100 μM unlabelled ryanodine). The assay of ryanodine binding using other filters such as glass fibre (GF/C) or 0.45 μm-pore-size Millipore filters gave about 20–40% of the specific binding obtained with 0.22 μm-pore-size Millipore filters. In the ultracentrifugation technique, the membranes were incubated with ryanodine, as described above, in a total volume of 150 μl in the centrifugation tubes of the Beckman Airfuge. After the incubation time the samples were centrifuged for 7 min at 130000 $g_{max.}$. The supernatant was removed immediately and carefully, the pellet was resuspended in 20 μl of 10% SDS and the radioactivity was counted. The specific binding was determined as described above for the filtration method.

Amplification of ryanodine receptor sequences

Total RNA was isolated from rat quadriceps muscle and liver by homogenization in RNAsol (Biotech Laboratories, Houston, TX, U.S.A). Subsequent purification of the RNA was according to the manufacturer's recommendations. First-strand cDNAs were synthesized from 5 μg of total RNA using the cDNA cycle kit of Invitrogen (San Diego, CA, U.S.A.). One-tenth of the cDNA products were subjected to 40 cycles of polymerase chain reaction as described by Gibbs *et al.* [20], using oligonucleotide primer pairs designed to be specific for the ryanodine receptor of skeletal muscle. A cycle consisted of 15 s at 94 °C, 15 s at 40 °C and 60 s at 72 °C. The final 72 °C incubation was extended to 8 min. The amplified products were characterized by electrophoresis through a 1% agarose/1% Nusieve gel (FMC BioProducts, Rockland, ME, U.S.A.).

Assays

Protein was determined according to [21], with BSA as a standard. SDS/PAGE was performed as described by Laemmli [22].

RESULTS

The distribution of ryanodine-binding sites in isolated microsomal fractions prepared from either intact liver or isolated hepatocytes is shown in Table 1. This comparison was necessary in order to ascertain that binding sites present in the microsomes prepared from whole liver are not due to the small amounts of vascular smooth muscle that are also part of the organ. The results demonstrate that the [^{3}H]ryanodine-binding capacities are similar in the two membrane preparations. The results also show that the smooth ER fraction is clearly enriched in [^{3}H]ryanodine-binding sites.

Very low binding to the mitochondrial fraction was also detected and amounted to less than 10% of that obtained with the smooth ER fraction (results not shown). This binding could be due to contamination with fragments of ER, as indicated by the presence of Ruthenium-Red-insensitive Ca^{2+} uptake and glucose 6-phosphatase activity in this fraction (results not shown).

Fig. 1 shows representative experiments in which the binding of [^{3}H]ryanodine to the smooth ER fraction as a function of its concentration was determined by the vacuum filtration and centrifugation techniques. With the filtration method (Figs. 1*a* and 1*b*), the specific binding of [^{3}H]ryanodine reached a saturation at concentrations below 100 nM, whereas the total binding was increased with increasing [^{3}H]ryanodine concentration (Fig. 1*a*). The non-specific binding (see the Experimental section) at low concentrations of ryanodine (1–20 nM) is relatively low (18–25%); however, it increased with increasing ryanodine concentration. Scatchard analysis of these data revealed the presence of a single type of binding site for specific binding (Fig. 1*b*). The apparent K_d, calculated from the slope, for the specific binding in four different membrane preparations was 10 ± 2.5 M, and the $B_{max.}$ was 400–600 fmol/mg of protein. The apparent concentration of the binding sites determined by the filtration method was a minimal value, since it did not take into account loss of microsomal protein through the filters and the loss of bound ryanodine during the filtration and washing time (about 20 s), which is expected because of the fast dissociation of ryanodine from its binding site (see Fig. 3). Therefore we also employed the centrifugation technique for measuring ryanodine binding (Figs. 1*c* and 1*d*). As expected, the K_d obtained with this

Table 1. [^{3}H]Ryanodine binding to microsomal subfractions isolated from whole liver or hepatocytes

Specific binding of [^{3}H]ryanodine to different fractions was assayed as described in the Experimental section. The results do not take into account the loss of membranes through the 0.22 μm pore-size Millipore filters, which was found to be between 30 and 40% of the total protein applied to the filters.

	[^{3}H]Ryanodine bound (fmol/mg of protein)	
Membrane fraction	Liver	Hepatocytes
Total microsomes	147	137
Rough ER	101	112
Intermediate ER	180	161
Smooth ER	280	190

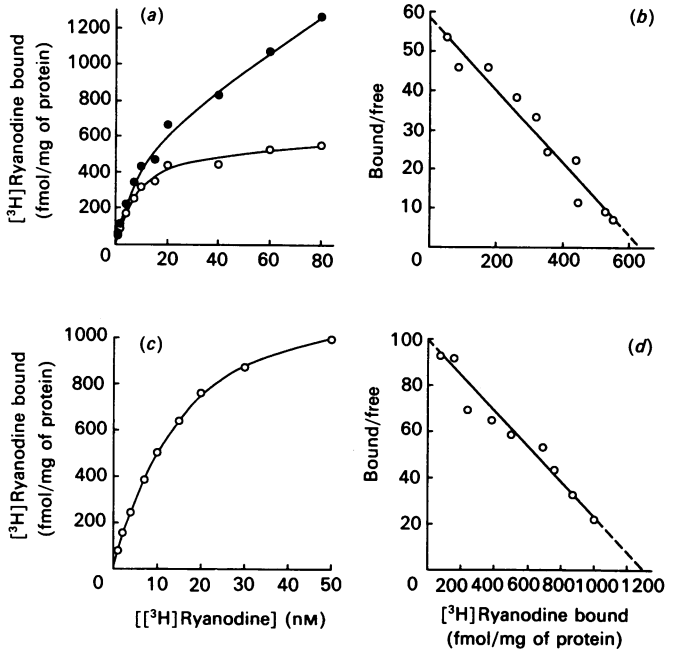

Fig. 1. [³H]Ryanodine binding to liver smooth ER membranes

Saturation isotherms and Scatchard plots are shown for total binding (●) and specific binding (○). [³H]Ryanodine binding was carried out for 20 min as described in the Experimental section, except that the [³H]ryanodine concentration was varied. Specific [³H]ryanodine binding was determined as described in the Experimental section. The binding was determined by Millipore filtration (*a* and *b*) or ultracentrifugation (*c* and *d*). (*b*) $B_{max.}$ = 620 fmol/mg of protein; K_d = 10.5 nM. (*d*) $B_{max.}$ = 1280 fmol/mg of protein; K_d = 12.8 nM.

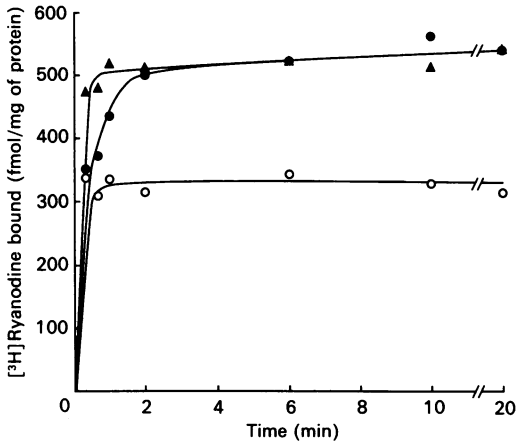

Fig. 2. Time courses of [³H]ryanodine binding to microsomes and smooth ER membranes

Microsomes (○) or smooth ER (●, ▲) (1 mg/ml) were incubated with 20 nM-[³H]ryanodine at 37 °C (▲, ○) or 24 °C (●). After the indicated time, samples were assayed for ryanodine binding by the filtration method as described in the Experimental section.

method was similar to the K_d obtained by filtration; the $B_{max.}$, however, was about 2-fold higher.

The time course of [³H]ryanodine binding to unfractionated microsomes and to the smooth ER subfraction is shown in Fig. 2. In the standard assay buffer at 37 °C, [³H]ryanodine (20 nM) rapidly associated with its binding sites, and this association appeared to be complete by the first time point for both membrane fractions. Decreasing the assay temperature from 37 °C to 24 °C increased the time for half-maximal saturation by about 5-fold.

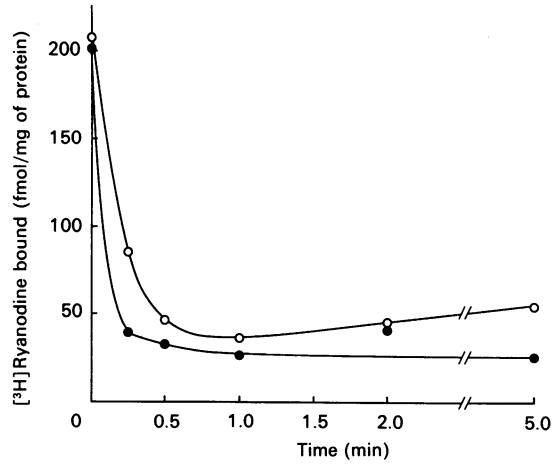

Fig. 3. Displacement of [³H]ryanodine by unlabelled ryanodine

Smooth ER membranes were incubated with 20 nM-[³H]ryanodine at 37 °C. After 15 min, samples were assayed for bound [³H]ryanodine. Dissociation of specifically bound [³H]ryanodine at equilibrium was initiated by addition of 2 μM (○) or 20 μM (●) unlabelled ryanodine and determination of the residual radioligand bound at the indicated times.

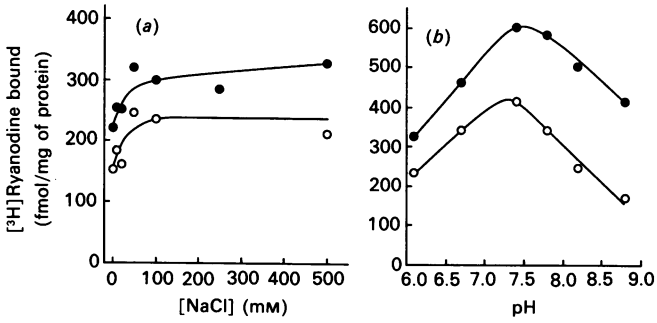

Fig. 4. Effects of NaCl and pH on [³H]ryanodine binding to smooth ER membranes

In (*a*), the binding of [³H]ryanodine (20 nM) was assayed in 0.1 M-sucrose/20 mM-Tris/HCl (pH 7.4)/0.5 mM-EGTA containing the indicated concentrations of NaCl. Total (●) and specific (○) [³H]ryanodine binding was determined as described in the Experimental section. In (*b*) [³H]ryanodine binding was assayed by Millipore filtration as described in the Experimental section, except that the pH was varied as indicated. The buffers used were Mops for pH values 6.1, 6.4 and 7.1, and Tris/HCl for pH values 7.4, 7.8, 8.2, and 8.8.

Dissociation experiments were performed by equilibrium binding of 20 nM-[³H]ryanodine with the membranes for 15 min at 37 °C, and then dissociation of the labelled ryanodine was induced by the addition of excess unlabelled ryanodine and residual specific binding was determined after various periods of incubation at 37 °C (Fig. 3). Addition of 20 μM unlabelled ryanodine resulted in displacement of [³H]ryanodine which was complete by the first data point. Addition of 2 μM-ryanodine caused a displacement of 70% of the radioactivity by that time point.

The binding of [³H]ryanodine to skeletal muscle SR is Ca^{2+}-dependent, stimulated by ionic strength and ATP, and inhibited by Mg^{2+} and Ruthenium Red [6,7,10,15,23–26]. Fig. 4 presents the effects of NaCl and pH on the binding of [³H]ryanodine to the liver smooth ER membranes. Fig. 4(*a*) shows that both total and specific binding were increased as the NaCl concentration

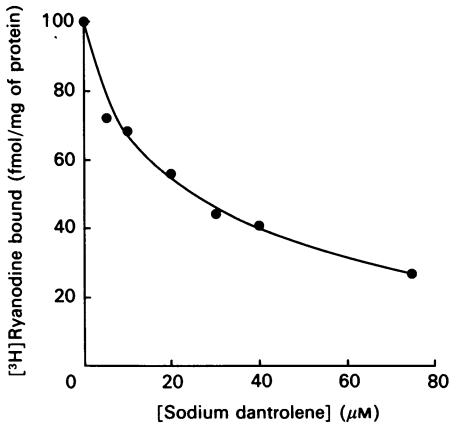

Fig. 5. Inhibition by sodium dantrolene of [^{3}H]ryanodine binding to smooth ER membranes

[^{3}H]Ryanodine binding was assayed in the absence and in the presence of sodium dantrolene as described in the Experimental section. The data, derived from two separate experiments, are expressed as percentages of the specific binding obtained in the absence of sodium dantrolene (250 ± 4 fmol/mg of protein).

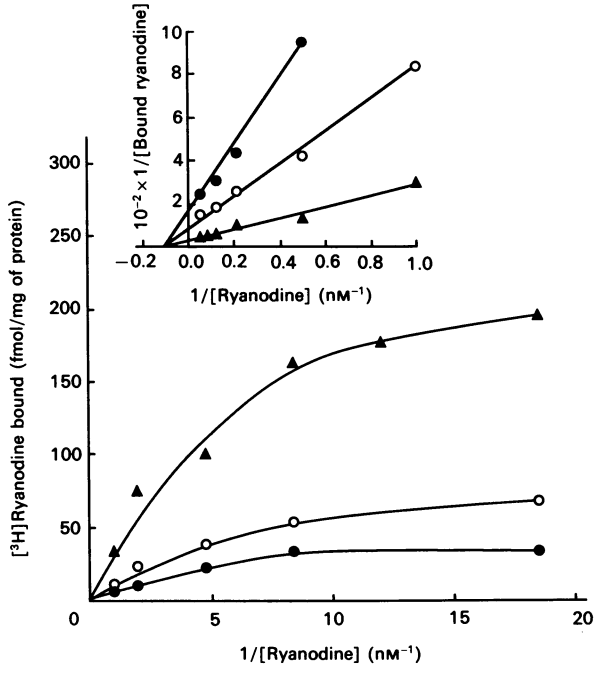

Fig. 6. Inhibition by caffeine and dantrolene of [^{3}H]ryanodine binding to smooth ER membranes

[^{3}H]Ryanodine binding, in the absence (▲) and the presence of caffeine (7 mM) (●) or sodium dantrolene (40 μM) (○), was measured as in the legend to Fig. 1. The inset represents a double-reciprocal plot of the data. The K_m for [^{3}H]ryanodine is 7 ± 1 nM. The results are averages of two experiments with two different membrane preparations.

was increased (by 1.5-fold at 0.5 M-NaCl). However, this stimulation was not as great as that obtained for the binding of ryanodine to skeletal muscle SR (over 10-fold) [11,26].

The results given in Fig. 4(*b*) show that the binding of ryanodine is pH-dependent, being optimal at pH 7.4 followed by a progressive decrease. This is in contrast with the SR, where the

Table 2. Effects of caffeine, dantrolene and diltiazem on [^{3}H]ryanodine binding to skeletal muscle SR and hepatic smooth microsomes

[^{3}H]Ryanodine binding was assayed in the absence and the presence of the indicated compounds by Millipore filtration as described in the Experimental section, except that for the SR, EGTA was omitted from the reaction mixture and 50 μM-$CaCl_2$ was added to it, and the non-specific binding was measured in the presence of 20 μM unlabelled ryanodine. Control activity (100%) was 2.83 and 0.22 pmol/mg of protein for SR and smooth ER respectively. Heavy SR from the 45/38% sucrose interface were obtained from rat skeletal muscle as described previously [36].

Compound	[^{3}H]Ryanodine bound (% of control)	
	SR	Smooth ER
Caffeine (5 mM)	117.5	35.8
Caffeine (10 mM)	131.7	27.5
Caffeine (20 mM)	151.2	11.0
Dantrolene (20 μM)	81.8	75.0
Dantrolene (50 μM)	70.4	55.3
Dantrolene (100 μM)	54.2	16.6
Diltiazem (20 μM)	94.5	55.2
Diltiazem (50 μM)	84.6	33.7
Diltiazem (100 μM)	77.9	18.0

binding of ryanodine is increased with increasing pH up to 8.6 [27]. Also in contrast with the SR, [^{3}H]ryanodine binding was evident in liver microsomes in the presence of EGTA. Addition of Ca^{2+} (5–500 μM free Ca^{2+}) had no effect on the binding. [^{3}H]Ryanodine binding was slightly stimulated by ATP, $InsP_3$ and Mg^{2+}, but was not affected by Ruthenium Red.

The pharmacological characteristics of the liver ryanodine-binding sites are presented in Figs. 5 and 6 and in Table 2. Dantrolene, which is an inhibitor of Ca^{2+} release by the SR of skeletal muscle [28–30], inhibited [^{3}H]ryanodine binding to the liver smooth ER membranes (Fig. 5). Half-maximal inhibition was obtained at 25 μM-dantrolene, a similar concentration to that reported to result in 50% inhibition of ryanodine binding to the CHAPS-solubilized ryanodine receptor of the SR [23]. We have shown previously [16] that caffeine inhibited the binding of ryanodine to the hepatic smooth ER membranes. The inhibitory effects of caffeine and dantrolene on ryanodine binding were obtained regardless of whether the binding was assayed by vacuum filtration or by ultracentrifugation.

Caffeine and dantrolene were found to be non-competitive inhibitors of [^{3}H]ryanodine binding (Fig. 6).

Table 2 compares the effects of caffeine, dantrolene and diltiazem on the binding of [^{3}H]ryanodine to skeletal muscle SR and liver smooth ER membranes. As shown previously [24], caffeine stimulated the binding of ryanodine to the SR; however, it strongly inhibited the binding of ryanodine to the liver smooth ER (see also Fig. 6 and ref [16]). Table 2 also shows that, although the Ca^{2+}-channel blocker diltiazem strongly inhibited the binding of ryanodine to the liver ER, it only slightly affected the binding to the SR (see also [23]). Similarly, dantrolene almost completely inhibited binding to the liver ER, but it only partially inhibited binding to the SR (Fig. 5).

The results presented above (Table 2 and Figs. 4–6) illustrate the significant differences between the ryanodine-binding sites present in the SR and those in liver ER membranes.

Rather than representing additional forms of ryanodine-binding protein, the differences in the kinetics and pharmacological properties of ryanodine binding in liver and muscle

Table 3. Oligonucleotide primers directed against the sequence encoding the ryanodine receptor of skeletal muscle

Primers were derived from regions of the rabbit receptor sequence [25] with minimal codon redundancy. RR-C and RR-D were mixtures of oligonucleotides with 16-fold redundancy. In the sequences, Y represents any pyrimidine and N represents any nucleotide.

Name	Orientation	Sequence
RR-A	Sense	CCCAAGACGTATATGATG
RR-B	Anti-sense	CTCAAACTCGAAGTACCA
RR-C	Sense	TTYGCNCAYATGATGATG
RR-D	Anti-sense	ACYTGNGGCATYTCCCA

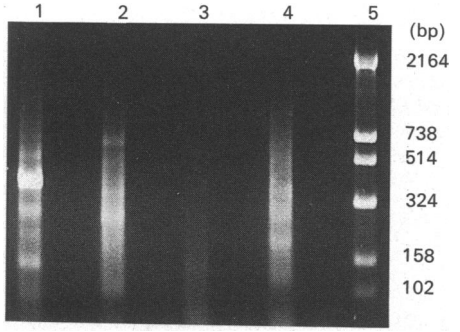

Fig. 7. *In vitro* amplification of skeletal muscle ryanodine receptor cDNA

The products of the amplification reaction were electrophoresed through 1% agarose/1% NuSieve, and then stained with ethidium bromide. Lanes 1 and 2 contain the products derived from skeletal muscle with primer pairs RR-A/RR-B and RR-C/RR-D respectively. Lanes 3 and 4 contain the products derived from liver under the same conditions with RR-A/RR-B and RR-C/RR-D respectively. Lane 5 contains size markers with the indicated numbers of nucleotide pairs.

might be explained by liver-specific modification of the muscle ryanodine receptor. To explore this possibility, we examined liver and skeletal muscle for the presence of the mRNA encoding the skeletal muscle ryanodine receptor using *in vitro* amplification techniques. Oligonucleotide primers were derived from regions of the rabbit receptor nucleotide sequence [25], with minimal codon redundancy to maximize the likelihood that the primers would also recognize a sequence of rat origin (Table 3). The first primer pair, RR-A and RR-B, were unique sequences and flanked the 414-nucleotide region between the codons for Pro-956 and Glu-1093 of the rabbit sequence. The second pair, RR-C and RR-D, consisted of two mixtures of oligonucleotides with 16-fold redundancy and flanked the 644-nucleotide region between the codons for Phe-3996 and Val-4210. Using RNA isolated from rat skeletal muscle, amplification with primers RR-A and RR-B yielded a major product with a mobility consistent with that of the expected 414 bp (Fig. 7). In contrast, RNA obtained from rat liver failed to yield a product of the expected mobility with RR-A and RR-B. As expected for multiple redundancy primers, amplification with RR-C and RR-D produced a smear of products in both skeletal muscle and liver. However, this primer pair generated a distinct product consistent with the expected 644 bp only in the skeletal muscle sample. Subsequent sequencing of the skeletal-muscle-derived product from the RR-A/RR-B primer pair confirmed that it encodes the appropriate region of the receptor (results not shown). These results strongly suggest that the mRNA encoding the skeletal muscle form of the receptor is not expressed in liver and that the binding of ryanodine observed in liver cannot result from expression of a modified form of the skeletal muscle receptor.

DISCUSSION

The characterization of the hepatic microsomal ryanodine-binding process reveals striking differences between ryanodine binding in the liver and in skeletal muscle. These differences are evident and manifested in two ways: (1) in the characteristics of the binding itself; and (2) in the effects of pharmacological agents on the binding.

The presented data demonstrate that the binding sites originated from parenchymal-cell-derived microsomes and are not due to contamination with vascular smooth muscle SR. This is confirmed by the observation that microsomes and their subfractions prepared from isolated hepatocytes exhibited similar protein patterns and [^{3}H]ryanodine binding to those of membrane fractions prepared from intact liver (Table 1). The smooth ER fraction, which was obtained by a specific purification procedure on a Mg^{2+}/sucrose gradient [17,31], showed an increase in [^{3}H]ryanodine-binding sites compared with that of total microsomes. However, in these studies the contribution of ribosomal protein to the protein content of the fractions was not taken into consideration. If it was taken into consideration the differences between the rough and smooth microsomal fraction might be less pronounced.

The binding of ryanodine to the ER fractions shares some of the characteristics of binding to the SR, but also differs from it in several important ways. In the SR, ryanodine binds to high-affinity binding sites; recently, low-affinity binding sites have also been demonstrated [32]. In the liver, ryanodine also seems to bind to high-affinity sites, but low-affinity binding sites might be also present (results not shown). In contrast with that in skeletal muscle, the binding in liver is Ca^{2+}-independent, it is not inhibited by either Mg^{2+} or Ruthenium Red, and it decreases at pH values above 7.5. According to the data presented here, the values for the total ryanodine binding sites ($B_{max.}$) in the liver are lower than the reported values for skeletal muscle SR [6,7,23,24]. Binding affinities and $B_{max.}$ values similar to those reported here for the liver were described in a recent study on a brain microsomal preparation [33].

An additional important difference relates to the association/dissociation of ryanodine to and from its binding sites. In the liver at 37 °C, the half-times of association and dissociation were 100–1000-fold lower than values reported for muscle (23.1 min and 14.4 h for association and dissociation respectively [24]).

A comparison between the pharmacological profiles of the skeletal muscle SR and the liver ER ryanodine-binding sites also indicates significant differences. Ryanodine binding to the liver ER was strongly inhibited by caffeine, while at the same concentrations caffeine stimulated ryanodine binding to the SR (Table 2, Fig. 6, [24]). The muscle relaxant sodium dantrolene almost completely inhibited the binding of ryanodine to the liver ER, but it only partially inhibited binding to SR membranes. The inhibition of ryanodine binding by caffeine and dantrolene (Figs. 5 and 6) may suggest that these compounds bind to the same ryanodine-binding protein or to a closely associated polypeptide(s). The Ca^{2+}-channel antagonist diltiazem also strongly inhibited the binding of ryanodine to the hepatic ER, but only slightly affected the binding to SR membranes (see Table 2 and [23]). It should be mentioned, however, that diltiazem was found to be as effective as dantrolene in preventing the abnormal contraction of skeletal muscle induced by halothane

and caffeine in malignant hyperpyrexia-susceptible muscle (34,35). Also, as mentioned above, Ruthenium Red has no significant effect on binding of ryanodine to the hepatic ER, but it is a potent inhibitor of ryanodine binding to the SR [6,23].

Further structural differences are indicated by the observation that, by using different antibodies, and in contrast with a previous report [16], no interaction was found between the hepatic microsomal fractions and antibodies raised against the purified skeletal muscle ryanodine receptor in two laboratories (J. R. Dedman & A. Lai, personal communication).

The most probable explanation for the differences in the binding of ryanodine to liver and skeletal muscle is the existence of multiple proteins capable of binding ryanodine. Indeed, the existence of multiple forms of the ryanodine receptor has been confirmed by the recent isolation of the cDNA encoding the cardiac form [14]. Binding studies alone, however, cannot exclude the possibility that the observed differences between liver and skeletal muscle might result from the expression of a modified form of the skeletal muscle receptor in liver. This seems unlikely, as oligonucleotide primer pairs directed to two different sites on the skeletal muscle sequence failed to amplify the mRNA encoding the muscle form of the receptor in liver (Fig. 7). The observed absence of the muscle receptor mRNA in liver on using amplification techniques is consistent with recent studies in which the tissue specificity of the skeletal muscle and cardiac forms of the receptor were examined. Otsu *et al.* [14] found that neither the skeletal muscle cDNA nor the cardiac cDNA probes hybridized to any mRNA species in liver. These findings raise the possibility that the ryanodine-binding protein in the liver represents an additional form of the ryanodine receptor.

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