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Distinct ryanodine- and inositol 1,4,5-trisphosphate-binding sites in hepatic microsomes

Varda SHOSHAN-BARMATZ,* Guo H. ZHANG,† Leon GARRETSON† and Naomi KRAUS-FRIEDMANN†‡
*Department of Biology, Ben-Gurion University of Negev, Beer-Sheva, Israel, and †Department of Physiology and Cell Biology, The University of Texas Medical School, Health Science Center at Houston, P.O. Box 20708, Houston, TX 77225, U.S.A.

A light hepatic microsomal preparation was fractionated by sucrose-density centrifugation into one rough, one intermediate and two smooth fractions. The four fractions were characterized with respect to parameters relevant to Ca^{2+} sequestration. Ca^{2+}-ATPase activity was similar in the rough, intermediate and smooth I fractions, but lower in the smooth II fraction. Ca^{2+} accumulation was the highest in the smooth I and intermediate fractions. On the other hand, Ca^{2+} efflux from the rough fraction was several-fold faster than from the smooth I fraction. All four subfractions exhibited specific binding sites for inositol 1,4,5-trisphosphate (IP_3) and ryanodine; however, the receptors were especially enriched in the smooth I fraction. The total binding sites for ryanodine in that fraction exceeded the number of binding sites for IP_3 by about 10-fold. The two receptors responded differently to pharmacological agents; caffeine and dantrolene strongly inhibited ryanodine binding but not IP_3 binding, whereas heparin inhibited IP_3 binding only. Thus the two receptors are distinct entities. The four fractions also showed distinct gel electrophoretic patterns. The use of two different SDS/polyacrylamide-gel gradients and two protein-staining methods revealed major differences in the distribution of the bands corresponding to M_r values of ($\times 10^{-3}$) 380, 320, 260, 170, 90, 29 and 21. These proteins were enriched in the smooth fraction. The results indicate that the smooth I fraction might have special importance in stimulus-evoked Ca^{2+}-release processes.

INTRODUCTION

A membrane network, the endoplasmic reticulum (ER), is ubiquitously present in the cytoplasm of higher animals and plants. A subcellular fraction, the so-called microsomal fraction, corresponding to the ER, can be obtained by differential centrifugation. In the liver, depending on the methods of preparation, microsomal fractions with somewhat different characteristics have been described (Fleischer & Kerrina, 1974; De Pierre & Dallner, 1975). The most obvious difference relates to the presence or absence of bound ribosomes, resulting in rough and smooth microsomes respectively. Smooth microsomes isolated from the liver derive almost exclusively from the smooth ER (Dallner & Ernster, 1968). Subfractions of both the rough and the smooth microsomal fractions have been prepared and shown to exhibit structural and functional differences (Lewis & Tada, 1973; Beaufay *et al.*, 1974; Pryme, 1986; Gierow & Jergil, 1989). It has also been shown that the characteristics of the ER are different in the different zones of the liver (Jungermann & Katz, 1982; Lindros & Penttila, 1985).

Characterization of the different microsomal fractions has been carried out in studies by determination of chemical composition and enzyme activities, and by the use of molecular biology and of electron microscopic imagery methods. These studies have led to an in-depth understanding of many activities carried out by the ER. However, more recently it has become evident that, as is the case with the SR in the muscle, the ER in the liver plays a major role in Ca^{2+} sequestration and in the regulation of cytosolic free Ca^{2+} levels. Indeed, the ER seems to be the origin, to a large extent, of the Ca^{2+} released from intracellular stores upon hormonal stimulation (Streb *et al.*, 1983; Taylor & Putney, 1985; Bond *et al.*, 1987; Berridge, 1987). Because of this, characterization of the microsomal subfractions with respect to Ca^{2+} sequestration, Ca^{2+} release and the presence of specific receptors likely to be involved in the hormonal responses seemed necessary. This was the focus of the present study.

EXPERIMENTAL

Materials

Tris, EGTA, ATP, IP_3, glucose 6-phosphate, mannose 6-phosphate, aprotinin, DL-dithiothreitol, benzamidine and phenylmethanesulphonyl fluoride were obtained from Sigma. [^{32}P]IP_3 and [^{3}H]ryanodine were obtained from Du Pont/New England Nuclear. Unlabelled ryanodine was obtained from Progressive Agri System, Inc. $^{45}CaCl_2$ was obtained from Amersham.

Membrane preparations

Liver microsomes were prepared from male Sprague–Dawley rats as described previously (Fleschner & Kraus-Friedmann, 1986), except that the liver was homogenized and the 105000 *g* pellet was resuspended in a buffer containing 0.25 M-sucrose, 10 mM-Mops, pH 7.1, 1 mM-dithiothreitol and the following proteinase inhibitors: 0.8 mM-benzamidine, 0.5 μg of aprotinin/ml and 0.2 mM-phenylmethanesulphonyl fluoride. Microsomes were fractionated by Cs-containing sucrose-density-gradient centrifugation into the following fractions: rough ER (pellet), the membranes at the 1.3 M-sucrose phase (intermediate ER) and the 1.3/0.75 M- and 0.75/0.6 M-sucrose interfaces (smooth ER I and II respectively), essentially as described previously (Benedetti *et al.*, 1988). 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 portion (2 ml) of the suspension was carefully layered on top of 2 ml of 0.6 M-sucrose, 3 ml of 0.75 M-

Abbreviations used: IP_3, D-*myo*-inositol 1,4,5-trisphosphate: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum.

‡ To whom correspondence should be addressed.

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 80 000 *g* in a Beckman SW41 swing-out rotor, the membranes at the 0.6/0.75 M- 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 suspended 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. Similar results were obtained when the post-mitochondrial supernatant was applied directly on to the sucrose gradient.

The relative amounts of the various fractions were found to be dependent on the extent of homogenization of the 105000 *g* pellet. In the present experiments, the pellets were suspended in sucrose/Mops buffer by five strokes in a tight-fitting Teflon homogenizer. Under these conditions the yield was about 38, 43, 15 and 4% for the rough, intermediate, smooth I and smooth II fractions respectively.

Electron microscopy

Microsomal fractions prepared as above were diluted 1:10 in sucrose buffer and pelleted for fixation in a variant of 50% strength Karnovsky's fixative in 0.1 M-sodium cacodylate, pH 7.4. Following rinses with buffer, post-fixation proceeded using osmium tetroxide (1%) reduced with potassium ferrocyanide (1.5%). Samples were washed in water, dehydrated through a graduated series of ethanol and embedded in Spurr's epoxy medium. All exchanges of solutions were achieved by first centrifuging using a bench-top Microfuge and then resuspending the pellet in the subsequent mixture. Thin sections were contrasted with uranyl and lead salts and examined with a JEOL 100-CX electron microscope operated at 60 keV.

[^{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 the filtration method. Protein samples (80 μg) were filtered through 0.22 μm 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 radioactivity retained on the filters was determined using liquid scintillation counting techniques. 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).

[^{32}P]IP_3 binding

Binding of IP_3 to the different fractions was assayed as described previously (Guillemette *et al.*, 1988). Membranes (1 mg/ml) were incubated at 0 °C in a medium containing 25 mM-Na_2HPO_4, pH 7.4, 100 mM-KCl, 20 mM-NaCl, 1 mM-EDTA, 0.1% BSA and 1 nM-[^{32}P]IP_3 (110 Ci/mmol). After 30 min, protein samples (100 μg) were filtered through phosphate-buffered presoaked 0.22 μm Millipore filters and rapidly washed with 5 ml of ice-cold incubation medium. The receptor-bound radioactivity was analysed by liquid scintillation counting techniques. Non-specific binding was determined in the presence of 5 μM-IP_3.

Assays

Glucose-6-phosphatase was measured as described previously (Harper, 1965) and mannose-6-phosphatase was measured in the absence and the presence of 0.2% CHAPS by the method of Vanstapel *et al.* (1986). ($Na^+ + K^+$)-ATPase was determined as described by Scharschmidt *et al.* (1979) and Ca^{2+}-ATPase activity was assayed as described previously (Fleschner & Kraus-Friedmann, 1986). All enzyme activities were measured at 37 °C, except for mannose-6-phosphatase (20 °C). Ca^{2+} uptake by the microsomal fractions was determined by Millipore filtration using $^{45}CaCl_2$. The basic reaction mixture contained 20 mM-Mops, pH 6.8, 100 mM-KCl, 2 mM-$MgCl_2$, 0.5 mM-EGTA, 0.5 mM-$CaCl_2$ (containing ^{45}Ca, 3×10^6 c.p.m./μmol) and 1 mM-ATP. The uptake was started by the addition of microsomes to a final concentration of 0.25 mg/ml. After incubation for 1–20 min at 37 °C, 0.18 ml samples were filtered through 0.22 μm Millipore filters and washed with 5 ml of 0.1 M-KCl/5 mM-Mops, pH 6.8. Radioactivity remaining on the filters was measured in a liquid scintillation counter. Ca^{2+} efflux from passively loaded vesicles was carried out by incubation of microsomes (5 mg/ml) in a medium containing 100 mM-KCl, 20 mM-Mops, pH 6.8, and 5 mM-$CaCl_2$ [containing $^{45}Ca^{2+}$, $(3–4) \times 10^3$ c.p.m./nmol] for 2 h at 24 °C. For the 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/10 mM-Mops, pH 6.8, or with the same solution containing 1 mM-EGTA, for the indicated times. The flow rate was 1 ml/s.

Other analytical procedures

Protein was determined according to Smith *et al.* (1985); SDS/PAGE was performed as described by Laemmli (1970). Gels were stained either with 0.1% Coomassie Brilliant Blue R or with Stains-All, according to King & Morrison (1976).

RESULTS

A total hepatic light microsomal preparation was fractionated into four subfractions by the use of a discontinuous Cs-containing sucrose gradient. Although we have used a well-established method (De Pierre & Dallner, 1975; Benedetti *et al.*, 1988) for the preparation of rough and smooth ER membranes, we verified the identity of the different microsomal subfractions by electron microscopy (Fig. 1) and marker enzyme distributions (Table 1). Examination by electron microscopy showed that the rough microsomal fraction consists mostly of various large-sized clear vesicles studded with ribosomes and surrounded by glycogen particles (Fig. 1*b*). In the intermediate fraction (Fig. 1*c*), both small and large vesicles are present. The smooth microsomal fraction I consists of small, more uniformly sized vesicles of varying density (Fig. 1*d*). This fraction also contains profiles of tubular ER and a few smaller vesicles with adherent ribosomes. All fractions appear to be free of contamination with other cellular organelles. The difference in the vesicular size of the various fractions is in agreement with other reports (De Pierre & Dallner, 1975; Benedetti *et al.*, 1988) and with measurements of intramicrosomal water space (Nilsson *et al.*, 1973).

In order to evaluate the leakiness of the vesicles, the activity of an intravesicular enzyme, mannose-6-phosphatase, was measured as an index of microsomal permeability (Arion *et al.*, 1976) in the presence and absence of a detergent. With the exception of the rough fraction, which showed high mannose-6-phosphatase activity in the absence of the detergent, the other vesicular preparations were sealed (Table 1). The relatively higher leakiness of the rough vesicles could be due to mechanical destruction caused by the high content of glycogen particles present in these

fractions (see Fig. 1*b*). The differences in the membrane stability of rough and smooth ER is also reflected by the observation that GTP increased the permeabilities to mannose-6-phosphate mainly in the rough microsomes (Godelaine *et al.*, 1983; Paiement *et al.*, 1987). The activity of mannose-6-phosphatase in the fully disrupted smooth I and intermediate microsomal fractions is about 2-fold that in the other fractions (Table 1).

The different microsomal subfractions also differed in the activity of glucose-6-phosphatase, an ER marker enzyme (Table 1). The smooth I and intermediate fractions contained the most activity. The Ca^{2+}-dependent ATPase activity in the various membrane fractions, measured in the presence of the Ca^{2+} ionophore A23187, is shown in Table 1. Except for smooth II, the activity in the various fractions was similar. Table 1 also shows that the activity of the $(Na^+ + K^+)$-ATPase, a plasma membrane marker enzyme, in the total microsomes and in the two smooth ER fractions is very similar, and represents about 2% of the activity present in purified plasma membranes (Codina *et al.*, 1988).

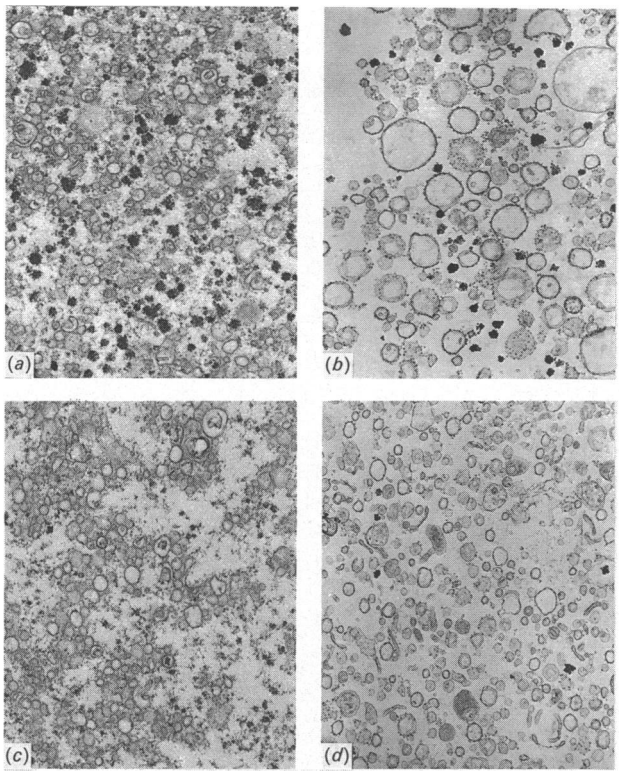

Fig. 1. Electron micrographs of total microsomes and of three submicrosomal fractions

The various membrane fractions were obtained and prepared for electron microscopy as described in the Experimental section. (*a*)–(*d*) are representative micrographs of the total, rough, intermediate and smooth I fractions respectively. Magnification × 12420.

The time courses of ATP-dependent Ca^{2+} accumulation by the different microsomal subfractions are presented in Fig. 2. In the absence of oxalate, the accumulation of Ca^{2+} by the various fractions, except rough ER, reaches a steady-state level in less than 10 min (Fig. 2*a*). When oxalate was added, as a permanent anion to support high levels of Ca^{2+} accumulation, the uptake of Ca^{2+} was linear over the 20 min test period, in which the accumulated Ca^{2+} was 10–18-fold higher than in the absence of oxalate. Under both conditions the rate and the extent of Ca^{2+} accumulation were highest in smooth I and intermediate microsomal fractions, and lowest in the rough microsomes. These results are in agreement with previous reports (Moore *et al.*, 1975; Benedetti *et al.*, 1988).

The Ca^{2+} permeability of the different microsomal subfractions was tested by determining the Ca^{2+} efflux from vesicles passively loaded with $^{45}Ca^{2+}$. Fig. 3 shows that both the extent and the rate of Ca^{2+} release from rough microsomes are higher than those from total and smooth microsomes. Similar results were obtained when EGTA, which stimulates Ca^{2+} efflux was included in the efflux media (Fig. 3*b*). In the absence of EGTA, the rate of Ca^{2+} efflux from rough microsomes is about 3- and 12-fold faster respectively than that from total and smooth microsomes. The maximal amount of Ca^{2+} released from the rough fraction is about 2- and 3-fold higher respectively than that released from total and smooth microsomes (Fig. 3). These differences in Ca^{2+} efflux from rough and smooth microsomes could be due to the differences in membrane integrity, as reflected by the different degree of latency of mannose-6-phosphatase activity (Table 1).

Recently (Shoshan-Barmatz & Kraus-Friedmann, 1990) we have found that ryanodine, an SR Ca^{2+}-release-channel marker (for review, see Lai & Meissner, 1989), binds to hepatic microsomes. In the following experiments we compared the distribution of IP_3-binding sites in the various submicrosomal fractions with that of ryanodine binding sites in order to evaluate the relationship between these two receptors.

Table 2 shows the distribution of IP_3-binding sites in the microsomal subfractions. The results indicate the presence of IP_3-binding sites in all the microsomal subfractions. However, the data clearly demonstrate that the smooth I microsomal fraction is highly enriched in IP_3 receptors (concentration about 3.3-fold that of unfractionated microsomes). For comparison,

Table 1. Comparison of enzyme activities between microsomal subfractions

The membrane fractions were prepared and the enzyme activities were assayed as described in the Experimental section. Results are expressed as means ± S.E.M. of 3 experiments.

	Enzyme activity (nmol of P_i released/min per mg of protein)				
				Mannose-6-phosphatase	
Microsomal fraction	Glucose-6-phosphatase	$(Na^+ + K^+)$-ATPase	Ca^{2+}-ATPase	−CHAPS	+CHAPS
Total	386.7 ± 13.3	24.8 ± 3.3	69.4 ± 0.5	18.0 ± 2.3	83.6 ± 7.5
Rough	433.3 ± 88.6	12.1 ± 3.8	71.5 ± 1.9	35.7 ± 10.9	92.3 ± 4.3
Intermediate	626.3 ± 87.4	13.5 ± 6.5	75.2 ± 1.9	16.2 ± 4.8	166.9 ± 6.8
Smooth I	563.0 ± 24.0	26.6 ± 2.8	64.3 ± 1.4	10.8 ± 2.1	147.9 ± 17.5
Smooth II	380.0 ± 43.2	22.1 ± 3.4	49.9 ± 0.5	7.0 ± 3.5	89.2 ± 5.7

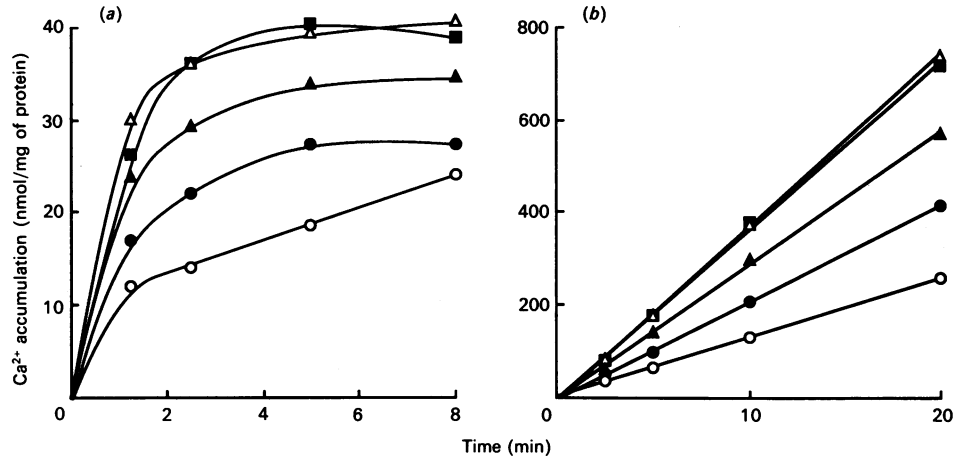

Fig. 2. Time course of Ca^{2+} accumulation in the absence (*a*) and the presence of (*b*) oxalate by different microsomal fractions

Ca^{2+} accumulation by total (●), rough (○), intermediate (■), smooth I (△) and smooth II (▲) microsomal fractions was assayed as described in the Experimental section, except that in (*b*), oxalate (20 mM) was also added to the assay medium.

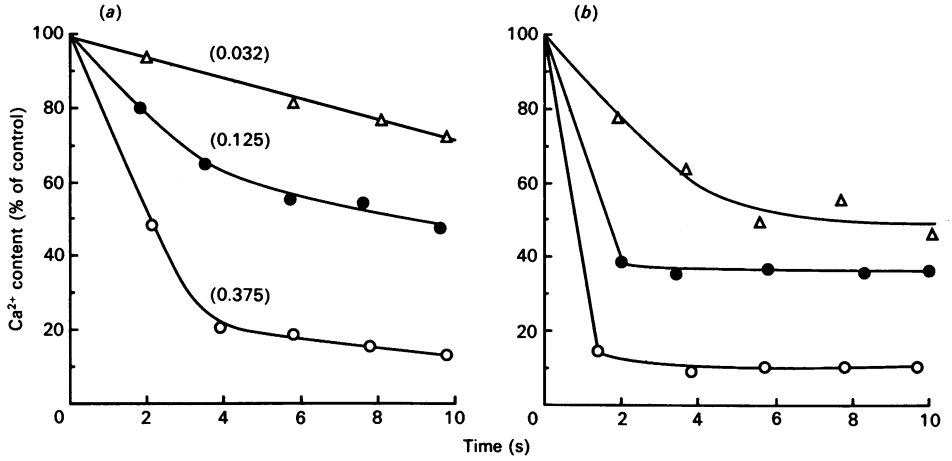

Fig. 3. Ca^{2+} efflux from total, rough and smooth microsomes passively loaded with $^{45}Ca^{2+}$

Total (●), rough (○) and smooth (△) microsomes were passively loaded with $^{45}Ca^{2+}$ and Ca^{2+} efflux was assayed in the absence (*a*) or in the presence (*b*) of 1 mM-EGTA as described in the Experimental section. The numbers in parentheses indicate the rates of Ca^{2+} efflux in nmol/s per mg of protein.

Table 2. [^{3}H]Ryanodine and [^{32}P]IP_3 binding to different hepatic submicrosomal fractions

[^{3}H]Ryanodine and [^{32}P]IP_3 binding were assayed as described in the Experimental section. The values shown represent the averages of results derived from two different experiments with two separate membrane preparations, each done in duplicate. The results from the two sets of experiments differ by 1–26%. The numbers in parentheses indicate % of control.

Microsomal fraction	Binding (fmol bound/mg of protein)	
	[^{3}H]Ryanodine	[^{32}P]IP_3
Total	142.1 (100)	5.1 (100)
Rough	91.7 (64.5)	9.0 (176.5)
Intermediate	178.7 (125.8)	11.4 (223.5)
Smooth I	265.3 (186.7)	17.3 (339.2)
Smooth II	190.9 (134.3)	8.9 (174.5)

Table 2 also shows the distribution of [^{3}H]ryanodine-binding sites in the various microsomal subfractions. A comparison between the binding of [^{32}P]IP_3 and [^{3}H]ryanodine to the different fractions indicates that both binding sites are enriched in the smooth I fraction. However, whereas the unfractionated microsomes possess higher ryanodine-binding activity than the rough microsomes, their [^{32}P]IP_3 binding is much lower than that of the rough microsomes. These results show that the distribution of the two receptors in various subfractions is not identical.

In Fig. 4, the binding of [^{32}P]IP_3 to the total and smooth microsomes as a function of [^{32}P]IP_3 concentration is presented. Scatchard analysis of these data is consistent with a single class of high-affinity binding sites with K_d values of 2.7 and 2.8 nM and maximal binding capacities of 24 and 78 fmol/mg of protein for total and smooth microsomes respectively. Thus the total binding sites (rather than change in affinity) were enriched by about 3.4-fold in the smooth microsomal fraction. A single class of high-affinity sites with K_d of 1.7 ± 1.0 nM was previously obtained for

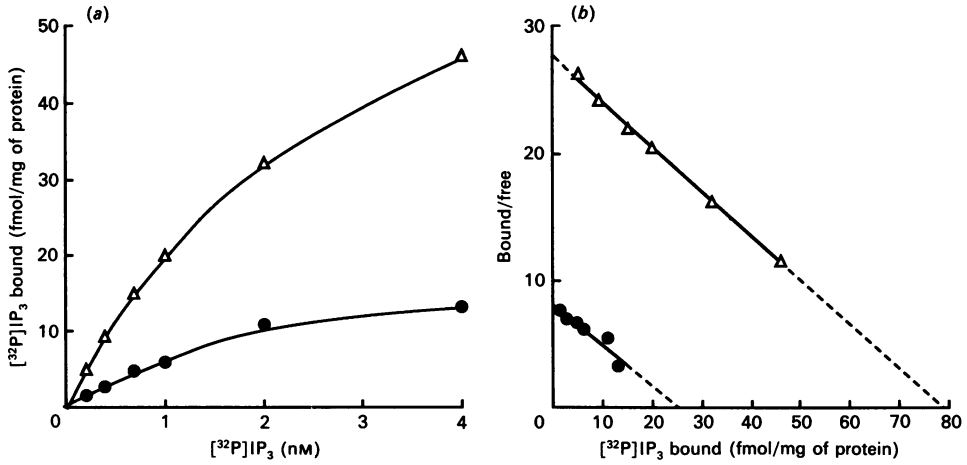

Fig. 4. [^{32}P]IP_3 binding to total and smooth microsomes

Saturation isotherms and Scatchard plots are shown for [^{32}P]IP_3 binding to total (●) and smooth (△) microsomes. [^{32}P]IP_3 binding was carried out as in Table 2, except that [^{32}P]IP_3 concentration was varied. In (*b*), $B_{max.}$ = 78 mol/mg of protein; K_d = 2.8 nM for smooth microsomes (△), and $B_{max.}$ = 24 fmol/mg; K_d = 2.9 nM for total microsomes (●).

Table 3. Effects of different compounds on IP_3 and ryanodine binding to the smooth ER fraction

[^{32}P]IP_3 and [^{3}H]ryanodine binding were assayed as described in the Experimental section, except that the indicated compounds were added to the assay medium. Control activities (100%) were 19 and 260 fmol/mg of protein for bound [^{32}P]IP_3 and [^{3}H]ryanodine respectively. N.D., not determined.

Additions	Binding (% of control)	
	IP_3	Ryanodine
Caffeine, 5 mM	95	41
Caffeine, 10 mM	97	20
Ryanodine, 5 μM	101	N.D.
Ryanodine, 50 μM	104	9
Sodium dantrolene, 50 μM	96	36
Sodium dantrolene, 100 μM	109	14
IP_3, 5 μM	0	103
IP_3, 25 μM	N.D.	119
Heparin, 50 μg/ml	47	105
Heparin, 100 μg/ml	24	98

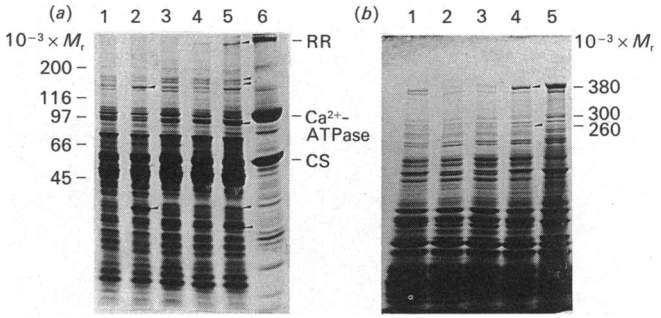

Fig. 5. Gel electrophoresis pattern of liver microsomal subfractions

Microsomal subfractions were obtained as described in the Experimental section. Membrane samples [50 μg (*a*) and 100 μg (*b*)] were solubilized in SDS and proteins were separated on 4–10% (*a*) or 7–15% (*b*) gradient SDS/polyacrylamide gel (Laemmli, 1970) using a 3% stacking gel. In (*a*), the protein pattern of the heavy fraction of skeletal muscle SR is also shown and the ryanodine receptor (RR), the (Ca^{2+} + Mg^{2+})-ATPase and calsequestrin (CS) are indicated. Lanes: 1, total microsomes; 2, rough ER; 3, intermediate ER; 4, smooth ER I; 5, smooth ER II; 6, SR. The arrowheads indicate the protein bands enriched in either the rough or the smooth I microsomes. M_r standards were myosin (200000), β-galactosidase (116000), phosphorylase *b* (92500), bovine serum albumin (66200) and ovalbumin (45000) (Bio-Rad).

hepatic IP_3-binding sites, co-purified with plasma membranes (Guillemette *et al.*, 1988). However, two classes of binding sites for IP_3 with K_d values of 7.9 nM and 0.16 μM have been also observed in liver microsomes (Spat *et al.*, 1986).

The pharmacological profile of [^{3}H]ryanodine and [^{32}P]IP_3 binding to the smooth microsomal fraction is shown in Table 3. Although caffeine and dantrolene were potent inhibitors of [^{3}H]ryanodine binding, they did not interfere with or promote binding of IP_3. Heparin, a compound known to inhibit IP_3 binding to brain microsomes (Worley *et al.*, 1987) had no effect on ryanodine binding but, as expected, inhibited IP_3 binding. Also, the two ligands did not influence the binding of each other. These results also suggest that the two compounds bind at distinct sites.

The different microsomal subfractions also showed distinct protein patterns when analysed by electrophoresis in two different SDS/polyacrylamide-gel gradients (Fig. 5). The major differences are in the distribution of the polypeptides with M_r values of ($\times 10^{-3}$) 380, 260, 170, 90, 29 and 21, which were enriched in the intermediate and smooth ER fractions. The rough microsomes are enriched with two polypeptides with M_r values of 27000 and 150000, which were also present in the smooth II fraction.

To characterize further the protein profile of the different microsomal subfractions, we subjected them to gel electrophoresis and then to staining with the carbocyanine dye Stains-All (King & Morrison, 1976). Several prominent stained bands with apparent M_r values of ($\times 10^{-3}$) 380, 320, 260, 170 and 150 were observed (Fig. 6). These bands stained violet (colour not shown). The 170000-M_r protein band was present exclusively in the smooth I fraction, whereas the 380000- 320000- and 260000-M_r bands were more enriched in the smooth I fraction. On the other hand, the 150000-M_r protein band seems to be present equally in all three fractions. Some other protein bands

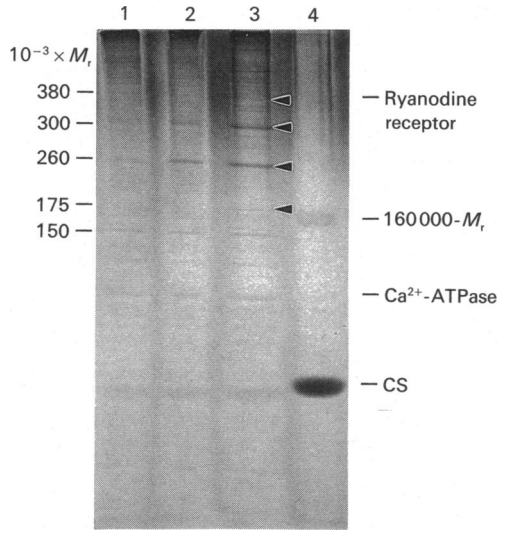

Fig. 6. Stains-All staining patterns of total, rough and smooth microsomes

Total (lane 1), rough (lane 2) and smooth (lane 3) microsomes were prepared and protein samples (100 μg) were separated by SDS/PAGE (4–10% gels) as described in the legend to Fig. 5, except that the gel was stained with Stains-All (King & Morrison, 1976). The proteins labelled in the SR (lane 4) are as in Fig. 5, except for the 160000-M_r protein (Cala & Jones, 1983). The arrowheads indicate the major stained protein bands enriched in the smooth microsomal fraction.

of M_r between 100000 and 30000 were weakly stained with Stains-All and were present in all subfractions.

DISCUSSION

Four subfractions of hepatic light microsomes have been characterized with respect to Ca^{2+} sequestration and the presence of IP_3- and ryanodine-binding sites.

The various submicrosomal fractions showed different levels of Ca^{2+} accumulation in either the absence or the presence of oxalate (Fig. 2). The smooth I and intermediate fractions were the most active, whereas the rough microsomes had the lowest Ca^{2+} accumulation capacity. Since the steady-state Ca^{2+} accumulation observed in the absence of oxalate (Fig. 2*a*) is likely to be determined by the balance between the uptake and efflux processes, these results suggest that the rough and smooth microsomes differ with respect to these activities. The Ca^{2+}-efflux data (Fig. 3), as well as the degree of mannose-6-phosphatase latency, as an index of membrane integrity, suggest that the rough microsomes are more leaky. This is a possible reason for the lower Ca^{2+} accumulation obtained in this fraction. It is likely that the leakiness of the rough fraction is due to the mechanical disruption of the ER during homogenization; therefore, Ca^{2+} accumulation is likely to occur *in situ* both in the rough and smooth I ER to about the same degree. One fraction, the smooth II fraction, shows considerably less Ca^{2+}-ATPase activity than the other fractions, and also less glucose-6-phosphatase activity.

In contrast with the distribution of Ca^{2+}-ATPase activity, which with the exception of the smooth II fraction is probably quite uniform, the distribution of the ryanodine- and IP_3-binding sites is not uniform: both receptors are enriched in the smooth I fraction. In light of recent findings that the amino acid sequence, deduced from cDNA, of the IP_3 receptor shows partial sequence similarity to the skeletal muscle ryanodine receptor (Furuchi *et al.*, 1989; Mignery *et al.*, 1989), it was important to verify the relationship between these two binding sites.

The results presented here suggest that ryanodine and IP_3 bind to two distinct sites. This conclusion arises from comparison of a number of characteristics. (1) The total number of binding sites for ryanodine is about 10-fold higher than for IP_3. (2) The distributions of the ryanodine- and IP_3-binding sites between the various submicrosomal fractions are not identical, and these differences are especially pronounced in the rough microsomal fraction. (3) The two binding sites can be distinguished also by their sensitivity to different inhibitors. Caffeine and dantrolene both inhibit ryanodine binding, but they have no effect on IP_3 binding. Heparin, a known inhibitor of IP_3 binding (Worley *et al.*, 1987), decreases IP_3 binding but has no influence on ryanodine binding. Unlabelled ryanodine displaces labelled ryanodine, but not labelled IP_3, and vice versa (Table 3). Taken together, these data strongly indicate that the two receptors are distinct and different entities.

The ryanodine-binding protein has been purified from skeletal muscle and found to be an oligomer of a single polypeptide estimated by SDS/PAGE to have an M_r of 350000–450000 (Inui *et al.*, 1987; Lai & Meissner 1989). Recently, however, the ryanodine receptor from skeletal muscle has been cloned and an M_r of 565000 was determined (Takeshima *et al.*, 1989; Marks *et al.*, 1989). The IP_3 receptor from cerebellum has been purified and characterized as a glycoprotein with an M_r of 260000 (Supattapone *et al.*, 1988; Mignery *et al.*, 1989).

Examination of the protein bands present in the submicrosomal fractions revealed the presence of proteins with M_r values of 380000 and 260000 which are enriched in the smooth I fraction (Figs. 5 and 6). Analysis of the protein profiles of the various membrane fractions using two different polyacrylamide gradient gels (4–10% and 7–15%) and two different staining methods allowed better resolution and characterization of these proteins. The 260000-M_r protein band stained in a pronounced fashion with Stains-All but stained much less with Coomassie (compare Figs. 5*b* and 6). If, as shown previously (King & Morrison, 1976; Cala & Jones, 1983) proteins stained blue with Stains-All represent glycoproteins, then the 260000 protein enriched in the smooth microsomal fraction might also be a glycoprotein. The 260000-M_r IP_3 receptor is a glycoprotein which has also been demonstrated to be present in the liver (Mignery *et al.*, 1989). Thus the enrichment of the smooth I fraction in a protein band of 380000-M_r and a glycoprotein band of 260000-M_r indicate that these proteins might correspond to the ryanodine and IP_3 receptors respectively. This suggestion requires further studies.

Analysis of the protein profiles of the various fractions reveals additional differences in their protein compositions. The function of these heterogeneously distributed proteins has not been identified. However, it is possible that the different protein compositions of the various subfractions are indicative of functional differences that are likely to exist in the different regions of the ER (De Pierre & Dallner, 1975). If this is also true for IP_3- and ryanodine-binding proteins, and if, as suggested (Berridge, 1987; Lai & Meissner, 1989), these receptors represent Ca^{2+}-release systems, then our findings suggest that the smooth ER might have a major role in the release of Ca^{2+} following stimulation. Further studies of the IP_3 and ryanodine receptors might lead to a better understanding of the control mechanism and structure of the Ca^{2+} release system(s) in liver cells.

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REFERENCES

Arion, W. J., Ballas, L. M., Lange, A. J. & Wallin, B. K. (1976) J. Biol. Chem. **251**, 4901–4907

Beaufay, H., Amar-Costesec, A., Thines-Sempoux, D., Wibo, M., Robbi, M. & Berthet, J. (1974) J. Cell Biol. **61**, 213–231

Benedetti, A., Fulceri, R., Romani, A. & Comporti, M. (1988) J. Biol. Chem. **263**, 3466–3472

Berridge, M. J. (1987) Annu. Rev. Biochem. **56**, 154–193

Bond, M., Vadasz, G., Somlyo, A. V. & Somlyo, A. P. (1987) J. Biol. Chem. **262**, 15630–15636

Cala, S. E. & Jones, L. R. (1983) J. Biol. Chem. **258**, 11932–11936

Codina, S., Kimura, S. & Kraus-Friedmann, N. (1988) Biochem. Biophys. Res. Commun. **150**, 848–852

Dallner, G. & Ernster, L. (1968) J. Histochem. Cytochem. **16**, 611–632

De Pierre, J. W. & Dallner, G. (1975) Biochim. Biophys. Acta. **415**, 411–471

Fleischer, S. & Kerrina, M. (1974) Methods Enzymol. **31**, 6–41

Fleschner, C. R. & Kraus-Friedmann, N. (1986) Eur. J. Biochem. **154**, 313–320

Furuchi, T., Yoshihawa, S., Miyawaki, A., Wada, K., Mead, N. & Mikoshiba, K. (1989) Nature (London) **342**, 32–38

Gierow, P. & Jergil, B. (1989) Biochem. J. **262**, 55–61

Godelaine, D., Beaufay, H., Wibo, M. & Ravoet, A. M. (1983) J. Cell Biol. **97**, 340–350

Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1988) J. Biol. Chem. **263**, 4541–4548

Harper, A. E. (1965) Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 788–792, Academic Press, New York

Inui, M., Saito, A. & Fleischer, S. (1987) J. Biol. Chem. **262**, 15637–15642

Jungermann, K. & Katz, N. (1982) Hepatology **2**, 385–395

King, L. E. & Morrison, M. (1976) Anal. Biochem. **71**, 223–230

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lai, F. A. & Meissner, G. (1989) J. Bioenerg. Biomembr. **21**, 227–245

Lewis, J. A. & Tada, J. R. (1973) J. Cell Sci. **13**, 447–459

Lindros, K. O. & Penttila, K. E. (1985) Biochem. J. **228**, 757–760

Marks, A. R., Tempt, P., Hwang, K. S., Taubman, M. B., Inui, M., Chadwick, C., Fleischer, S. & Nadel-Ginard, B. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 8683–8687

Mignery, G. A., Sudhof, T. C., Takei, K. & De Camilli, P. (1989) Nature (London) **342**, 192–195

Moore, L., Chen, T., Knapp, R. H., Jr. & London, E. J. (1975) J. Biol. Chem. **250**, 4562–4568

Nilsson, R., Peterson, E. & Dallner, G. (1973) J. Cell Biol. **56**, 762–776

Paiement, J., Rindress, D., Smith, C. E., Poliquin, L. & Bergeron, J. J. M. (1987) Biochim. Biophys. Acta **898**, 6–22

Pryme, I. F. (1986) Mol. Cell. Biochem. **71**, 3–18

Scharschmidt, B. F., Keefe, E. B., Blankenship, N. M. & Ochnes, R. K. (1979) J. Lab. Clin. Med. **93**, 790–799

Shoshan-Barmatz, V. & Kraus-Friedmann, N. (1990) Biophys. J. **57**, in the press

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Anal. Chem. **150**, 76–85

Spat, A., Fabiato, A. & Rubin, R. P. (1986) Biochem. J. **233**, 929–932

Streb, H., Irvine, R. F., Berridge, M. M. & Schultz, I. (1983) Nature (London) **306**, 67–69

Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988) J. Biol. Chem. **263**, 1530–1534

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Veda, M., Hanaoka, M., Hirose, T. & Numa, S. (1989) Nature (London) **339**, 439–445

Taylor, C. W. & Putney, J. W., Jr. (1985) Biochem. J. **232**, 435–438

Vanstapel, F., Pua, K. & Blankaert, N. B. (1986) Eur. J. Biochem. **156**, 73–77

Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S. & Snyder, S. H. (1987) J. Biol. Chem. **262**, 12132–12136

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