Comparative localization of inositol 1,4,5-trisphosphate and ryanodine receptors in intestinal smooth muscle: an analytical subfractionation study

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[^{3}H]Ins(1,4,5)P_3- and [^{3}H]ryanodine-binding sites were characterized in membrane fractions from guinea-pig intestinal smooth muscle (longitudinal layer) and their subcellular localization was investigated by analytical cell-fractionation techniques. Fractions collected at low centrifugal fields (N and M fractions) contained predominantly low-affinity [^{3}H]Ins(1,4,5)P_3-binding sites (K_D 80 nM), whereas microsomal (P) fractions contained only high-affinity binding sites (K_D 5 nM). Total sedimentable high-affinity binding sites of [^{3}H]Ins(1,4,5)P_3 were 9–10-fold more numerous than those of [^{3}H]ryanodine. Both high-affinity binding sites were purified in microsomal fractions, and their sub-microsomal distribution patterns after isopycnic density-gradient centrifugation were similar to those of presumed endoplasmic reticulum (ER) constituents, indicating that Ins(1,4,5)P_3 and ryanodine receptors were localized primarily in ER and probably associated with rough as well as smooth ER. However, the stoichiometric ratio of Ins(1,4,5)P_3 to ryanodine receptors was distinctly higher in high-density RNA-rich subfractions than in low-density RNA-poor subfractions, suggesting that Ins(1,4,5)P_3 receptors were somewhat concentrated in the ribosome-coated portions of ER. The low overall stoichiometric ratio of ryanodine to Ins(1,4,5)P_3 receptors in intestinal smooth muscle (1:9–10) might explain, at least partly, the existence of a Ca^{2+}-storage compartment devoid of ryanodine-sensitive Ca^{2+} channels, but equipped with Ins(1,4,5)P_3-sensitive channels, in saponin-permeabilized smooth-muscle cells [Iino, Kobayashi and Endo (1988) Biochem. Biophys. Res. Commun. **152**, 417–422].

INTRODUCTION

Smooth-muscle contraction evoked by excitatory agonists is triggered by an elevation of cytoplasmic Ca^{2+}, and two separate Ca^{2+} sources, intra- and extra-cellular, are involved in this process, as evidenced in particular by the effects of drugs that inhibit Ca^{2+} entry (Godfraind and Kaba, 1969; Godfraind et al., 1986). Physiologically relevant internal stores are usually considered as associated with endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) in most cell types, including smooth muscle (Somlyo and Somlyo, 1992), although the existence of specific organelles (calciosomes) has been proposed (Meldolesi et al., 1990). Two Ca^{2+} channels may be responsible for physiological Ca^{2+} release from internal stores in smooth muscle, (1) a channel sensitive to Ins(1,4,5)P_3 and (2) a channel which can be activated by a rather pronounced elevation of cytosolic Ca^{2+} (Ca^{2+}-induced Ca^{2+} release) and is sensitive to caffeine and ryanodine (the so-called ryanodine receptor). Indeed, an Ins(1,4,5)P_3 receptor has been purified from bovine aorta microsomes (Chadwick et al., 1990) and from membranes of rat vas deferens smooth muscle (Mourey et al., 1990) and shown to form an Ins(1,4,5)P_3-gated Ca^{2+} channel after incorporation in planar lipid bilayers (Mayrleitner et al., 1991). In addition, a ryanodine-binding protein purified from pig and dog aortic microsomes and reconstituted into planar lipid bilayers has been shown to behave as a Ca^{2+}-gated ryanodine-sensitive Ca^{2+} channel with properties comparable with those of skeletal and cardiac ryanodine receptors (Herrmann-Frank et al., 1991).

If two Ca^{2+}-release channels seem to coexist in most smooth-muscle types, little is known about their comparative subcellular localization. Are they distributed within all parts of the ER, or concentrated in a specialized subcompartment? Are the two types of channel co-localized in the same ER membranes? In contrast with many other cell types (Berridge, 1993), smooth-muscle cells seem to contain Ins(1,4,5)P_3-sensitive and ryanodine- (or caffeine-) sensitive stores that overlap largely (van Breemen and Saida, 1989). However, on the basis of studies performed mainly on saponin-permeabilized tissue, but also on isolated cells, Iino and co-workers (Iino et al., 1988; Yamazawa et al., 1992) proposed that smooth-muscle cells contained two compartments of mobilizable Ca^{2+}: one (Sα) that possessed both the caffeine-induced and the Ins(1,4,5)P_3-induced Ca^{2+}-release mechanism; and a second one (Sβ) that could be mobilized only by Ins(1,4,5)P_3. Similar data were obtained by Itoh et al. (1992) on β-escin-permeabilized smooth-muscle strips from the rabbit mesenteric artery. In order to detect a possible heterogeneous repartition of Ca^{2+}-release channels within intracellular membranes, we decided to investigate by analytical tissue-fractionation techniques the subcellular localization of ryanodine and Ins(1,4,5)P_3 receptors in intestinal smooth muscle. The distribution patterns of the two receptors indicated that they were both localized primarily in ER. However, their distributions were not totally superimposable, and Ins(1,4,5)P_3 receptors were somewhat more concentrated than were ryanodine receptors in the RNA-rich microsomal subfractions, that is, most probably, in vesicles derived from the ribosome-coated portions of ER. In addition, we found that the average surface density of ryanodine receptors in the ER membrane was very low, and much lower than that of Ins(1,4,5)P_3 receptors, which could favour the appearance of a Sβ compartment, devoid of ryanodine-sensitive Ca^{2+}-release channels, in detergent-permeabilized smooth-muscle cells.

Abbreviations used: $B_{max.}$, maximal number of binding sites; ER, endoplasmic reticulum; K_A, equilibrium association constant; K_D, equilibrium dissociation constant; SR, sarcoplasmic reticulum.

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MATERIALS AND METHODS

Subcellular fractionation

Homogenization and differential centrifugation

Ileal longitudinal smooth muscle (4 g wet wt.) was collected from five female guinea-pigs as previously described (Wibo et al., 1981). Tissue was either processed immediately or stored frozen overnight in 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole/HCl and supplemented with 0.1 mM phenylmethanesulphonyl fluoride (buffered sucrose). All subsequent operations were performed at 2–4 °C. Minced tissue was homogenized in buffered sucrose (7 ml/g of tissue) in an all-glass Potter–Elvehjem-type grinder (Braun, Melsungen, Germany). The homogenate (40–50 ml) was centrifuged in two 30 ml tubes at 1000 *g* for 10 min. The supernatant was collected, and the pellets were rehomogenized and re-centrifuged as above. This step was repeated twice. The final 1000 *g* pellets were resuspended in buffered sucrose and designated N fraction. The combined supernatants (100–120 ml) were centrifuged at 10000 *g* ($r_{av.}$ 8.3 cm) for 20 min in a Centrikon fixed-angle (TFT 50.38) rotor (Kontron, Zurich, Switzerland). The pellets were resuspended and re-centrifuged. The washed pellets were resuspended in buffered sucrose and designated M fraction. The combined supernatants were centrifuged at 200000 *g* for 30 min. The pellets were resuspended with buffered sucrose, combined in a single tube and re-centrifuged. The suspension obtained from the washed pellet was designated total microsomal fraction (P).

Separation of light and heavy microsomes

In some experiments, the microsomal fraction was subfractionated into light (P_l) and heavy (P_h) microsomes as follows. The total (unwashed) microsomal fraction (20 ml) was loaded over 12 ml of 31.5% (w/w) sucrose (buffered with 3 mM imidazole/HCl, pH 7.4; density 1.14 g/ml at 0 °C) and spun at 200000 *g* for 60 min in the 50.38 rotor. The light microsomes banding at the interface and the heavy microsomes in the pellet and the lower part of the 31.5%-sucrose layer were recovered separately, diluted with 3 mM imidazole, resuspended and centrifuged at 200000 *g* for 30 min; the corresponding pellets, resuspended in buffered sucrose, were designated light (P_l) and heavy (P_h) microsomal fraction respectively.

Isopycnic density-gradient centrifugation

The total microsomal fraction was also subfractionated by density equilibration in a linear sucrose gradient. In these experiments, we used a modified microsomal fraction, which was prepared as follows. The upper, gel-like, layer of the washed microsomal pellet was collected separately, and the lower, brownish, part was resuspended with buffered sucrose and designated P′ fraction. In two experiments, part of the P′ fraction was treated with digitonin before density-gradient centrifugation, as described previously (Wibo et al., 1980). We added 0.25 mg of digitonin/mg of protein (0.45 mg of digitonin/ml) in order to obtain a digitonin:cholesterol molar ratio of about 1 (Amar-Costesec et al., 1974; Wibo et al., 1981). The P′ fraction (6 ml) was loaded on the gradient (30 ml), prepared from 10% and 56% (w/w) sucrose solutions by means of a Beckman gradient former. After centrifugation for 2.5 h at 100000 *g* ($r_{av.}$ 7.3 cm), or 12 h at 50000 *g*, in a Centrikon vertical rotor (TV-850), 15 subfractions were recovered by puncturing the bottom of their tube. Their density was determined from their sucrose concentration, measured by refractometry. The volume of each subfraction was deduced from its weight and density. Average density/frequency histograms were constructed as described by Beaufay and Amar-Costesec (1976).

Binding and biochemical assays

[^{3}H]Ins(1,4,5)P_3 binding

Tissue fractions (0.1–0.5 mg of protein) were incubated with [^{3}H]Ins(1,4,5)P_3 on melting ice for 8–10 min in a medium (0.5 ml) containing (mM): KCl 100, NaH_2PO_4 25, Tris 25, EDTA 1, adjusted to pH 8.7 (2–4 °C) with NaOH. Saturation experiments were carried out either with increasing concentrations of [^{3}H]Ins(1,4,5)P_3 (1 Ci/mmol) or with a fixed amount of [^{3}H]Ins(1,4,5)P_3 (40 Ci/mmol) and increasing concentrations of unlabelled Ins(1,4,5)P_3. Non-specific binding was usually determined in the presence of 5 μM Ins(1,4,5)P_3. Bound and free radioligand were separated by centrifugation of Eppendorff tubes (1.5 ml) at 100000 *g* for 5 min (N and M fractions) or 15 min (microsomal fractions). Centrifugation could be performed safely in swinging buckets designed for 5 ml centrifuge tubes, provided that Eppendorff tubes were partially immersed in 2 ml of water. After careful removal of supernatant fluid by suction, pellets were dissolved in 0.1 ml of Soluene-100 at 40 °C (1–3 h). After transfer into scintillation vials and addition of 4 ml of Pico-Fluor 40, radioactivity was measured with an efficiency of 44%.

[^{3}H]Ryanodine and [^{3}H]PN200-110 binding

[^{3}H]PN200-110 binding was measured as reported previously (Wibo et al., 1991). [^{3}H]Ryanodine binding was determined in the medium (0.5 ml) described by McPherson and Campbell (1990), i.e. 1.5 M KCl, 10 mM Hepes, 10 mM ATP and 0.8 mM $CaCl_2$ (pH 7.4). Tissue fractions (0.04–0.2 mg of protein) were usually incubated with 1 nM [^{3}H]ryanodine for 2 h at 37 °C. Non-specific binding was determined in the presence of 10 μM ryanodine. Bound and free radioligand were separated by vacuum filtration on Whatman GF/F filters. Filters were washed with 2 × 10 ml of a chilled solution [150 mM KCl, 10 mM Tris, pH 7.4, poly(ethylene glycol) 6000 50 mg/ml] and transferred into scintillation vials. After addition of 10 ml of Pico-Fluor 40/toluene (1:3, v/v), radioactivity was measured with an efficiency of 44%.

Enzyme assays

Sulphatase C and NADPH:cytochrome *c* reductase were assayed as reported previously (Morel et al., 1981; Wibo et al., 1981). Ca^{2+}-ATPase activities were determined essentially as described by Simonides and van Hardeveld (1990). Tissue fractions (0.05 mg of protein) were first preincubated for 10 min at room temperature with Triton X-100 (0.035%) in a final volume of 0.1 ml, to inactivate a large part of the basal Mg^{2+}-ATPase activity and to remove membrane permeability barriers. The suspension was then diluted to a final volume of 0.7 ml with a solution, prewarmed at 37 °C, containing (final concns. mM): ATP 4, $MgCl_2$ 4, NaN_3 10, phosphoenolpyruvate 5, pyruvate kinase and lactase dehydrogenase (14 units/ml), $CaCl_2$ 1.35, EGTA 1, KCl 200, Hepes 20, pH 7.5. NADH (0.2 mM) was then added and its oxidation was monitored spectrophotometrically at 37 °C for 12 min. To determine the Ca^{2+}-dependent activity, samples were incubated in parallel without $CaCl_2$. The thapsigargin-sensitive and -resistant Ca^{2+}-ATPase activities were distinguished by using the drug at a final concentration of 50 nM. Activities were expressed in nmol of ATP hydrolysed/min, by using a molar absorption coefficient for NADH (340 nm) of 6.22×10^3 $M^{-1} \cdot cm^{-1}$.

Protein and RNA

Protein was measured as reported by Lowry et al. (1951). RNA was determined using ethidium bromide as a fluorescent indicator, essentially as described by Bentle et al. (1981). Fluorescence (excitation 360 nm; emission 595 nm) was measured in an Aminco spectrofluorometer (SPF-500) using thermostated cuvettes (30 °C). The decrease in fluorescence 15 min after addition of ribonuclease A was taken as reflecting the amount of RNA. *E. coli* transfer RNA was used as standard.

Materials

Female Dunkin–Hartley guinea-pigs (400–600 g) were obtained from Bantin & Kingman (Hull, U.K.). [^{3}H]Ins(1,4,5)P_3 (1 and 40 Ci/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.); [^{3}H]PN200-110 and [^{3}H]ryanodine (87 Ci/mmol) were from Du Pont–NEN Research Products (Boston, MA, U.S.A.). Ins(1,4,5)P_3, cytochrome *c*, ATP, NADH and NADPH were obtained from Boehringer (Mannheim, Germany); ryanodine was from AgriSystems International (Wind Gap, PA, U.S.A.); heparin (low-molecular-mass), phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, thapsigargin, cyclopiazonic acid, *Escherichia coli* RNA and RNAase A were from Sigma (St. Louis, MO, U.S.A.); methylumbelliferyl sulphate was from Koch–Light (Haverhill, Suffolk, U.K.); and Soluene-100 and Pico-Fluor 40 were from Packard (Groningen, The Netherlands).

RESULTS

Characterization of [^{3}H]Ins(1,4,5)P_3 binding to subcellular fractions from intestinal smooth muscle

In preliminary experiments (results not shown), we found that maximal binding of [^{3}H]Ins(1,4,5)P_3 to microsomes was attained within 5 min at 2–4 °C and that specific binding was completely reversible in a few minutes. (Rate constants could not be accurately determined, in view of the centrifugation technique used to separate bound from free [^{3}H]Ins(1,4,5)P_3; see the Materials and methods section). Specifically bound [^{3}H]Ins(1,4,5)P_3 was proportional to the amount of microsomes used. Sucrose did not inhibit binding at concentrations less than 0.5 M. No hydrolysis of [^{3}H]Ins(1,4,5)P_3 could be detected by anion-exchange chromatography (Downes and Michell, 1981) at the end of the incubation, whatever the subcellular fraction used.

With light (P_l) and heavy (P_h) microsomal fractions, only one class of specific binding site for [^{3}H]Ins(1,4,5)P_3 could be detected, with an equilibrium dissociation constant (K_D) of about 5 nM (Figure 1 and Table 1). Scatchard plots were obviously very different for N and M fractions, where binding was predominantly of lower affinity. However, Scatchard plots were distinctly concave upwards, and Ligand analysis (McPherson, 1985) showed that binding data were better fitted by a two-sites model ($P < 0.05$), with K_{D1} and K_{D2} values of 6–8 nM and 70–90 nM respectively. To improve the reliability of the estimated maximal number of sites ($B_{max.}$) for the high-affinity site in M and N fractions, we also performed Ligand analysis with a fixed value of K_{D1}, that is the K_D value obtained with the P_h fraction (see Table 1). The proportion of high-affinity sites was only 3–6 % in N and M fractions. High- and low-affinity binding could not be distinguished by their sensitivity to heparin (Figure 2). In contrast, they differed by their response to pH changes: binding of 0.5 nM [^{3}H]Ins(1,4,5)P_3 to microsomes (high-affinity) increased almost linearly from pH 7.2 to 9.5, whereas binding of 5.5 nM [^{3}H]Ins(1,4,5)P_3 to the M fraction (80 % low-affinity at pH 8.7) reached a plateau between pH 7.9 and 9.5 (results not shown; two experiments).

N and M fractions contain a large amount of protein material that sediments as a loosely packed gel-like layer in the upper part of the corresponding pellets obtained by differential centrifugation of the homogenate. (This gel-like material was also present, but much less prominent, in P fractions.) In one experiment, this gel-like layer (M_g) was recovered separately from the brownish, more densely packed, lower part of the M pellet (M_b), which was enriched with mitochondria (results not shown). Saturation binding experiments of M_g and M_b subfractions showed that the ratio $B_{max.2}/B_{max.1}$ was 21.1 and 2.5 respectively, indicating that

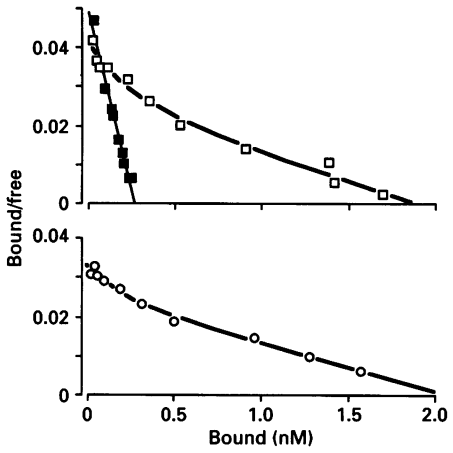

Figure 1 Scatchard analysis of [^{3}H]Ins(1,4,5)P_3 specific binding to subcellular fractions from intestinal smooth muscle

N (□) and M (○) fractions were incubated with 0.5 nM [^{3}H]Ins(1,4,5)P_3 (40 Ci/mmol) and various concentrations (1–600 nM) of non-radioactive Ins(1,4,5)P_3. P_h (■) fraction was incubated with various concentrations (0.5–40 nM) of [^{3}H]Ins(1,4,5)P_3 (1 or 40 Ci/mmol). Protein concentrations (mg/ml) were: N, 0.93; M, 0.89; P_h, 0.25. Parameters obtained by Ligand analysis are listed in Table 1.

Table 1 Specific binding parameters of [^{3}H]Ins(1,4,5)P_3 in various subcellular fractions

Saturation binding experiments (illustrated in Figure 1) were carried out with four fractions obtained from the same tissue preparation, as described in the Materials and methods section. Parameters estimated by the Ligand program (McPherson, 1985) are given with approximate standard errors. K_{A1} and K_{A2} are equilibrium association constants, $1/K_{D1}$ and $1/K_{D2}$ respectively. $B_{max.}$ values are expressed in fmol/mg of protein. For N and M fractions, we also performed Ligand analysis with a fixed value of K_{A1}, i.e. the K_{A1} value obtained with the P_h fraction (see the text).

	High affinity		Low-affinity	
Fraction	K_{A1} (nM^{-1})	$B_{max.1}$ (fmol/mg)	K_{A2} (nM^{-1})	$B_{max.2}$ (fmol/mg)
P_h	0.179 ± 0.028	1022 ± 105	–	–
P_l	0.187 ± 0.011	417 ± 22	–	–
M	0.127 ± 0.125	92 ± 127	0.011 ± 0.003	2290 ± 125
	0.179 (fixed)	59 ± 13	0.012 ± 0.001	2282 ± 109
N	0.165 ± 0.196	110 ± 164	0.014 ± 0.004	1929 ± 135
	0.179 (fixed)	100 ± 25	0.014 ± 0.002	1934 ± 97

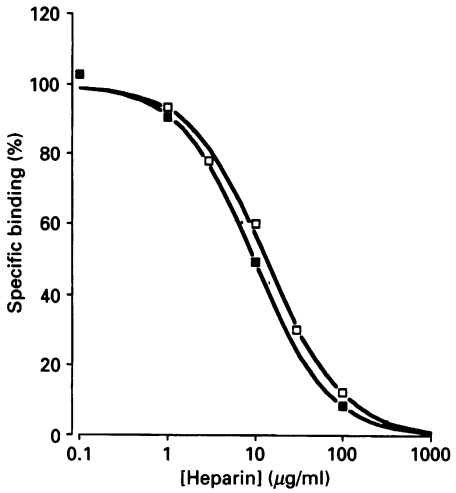

Figure 2 Effect of heparin on high- and low-affinity binding of [^{3}H]Ins(1,4,5)P_3

P_h (□) and M (■) fractions were incubated with [^{3}H]Ins(1,4,5)P_3 at concentrations of 0.4 nM and 9.5 nM respectively, in the presence of various concentrations of low-molecular-mass heparin. At the specified concentrations, [^{3}H]Ins(1,4,5)P_3 bound exclusively to high-affinity sites in the P_h fraction, and predominantly (81%) to low-affinity sites in the M fraction. Heparin concentrations that inhibited 50% of specific binding, calculated by a non-linear least-squares curve-fitting technique (EBDA; McPherson, 1985), were 13.5±2.3 and 9.8±2.1 μg/ml for high- and low-affinity binding respectively.

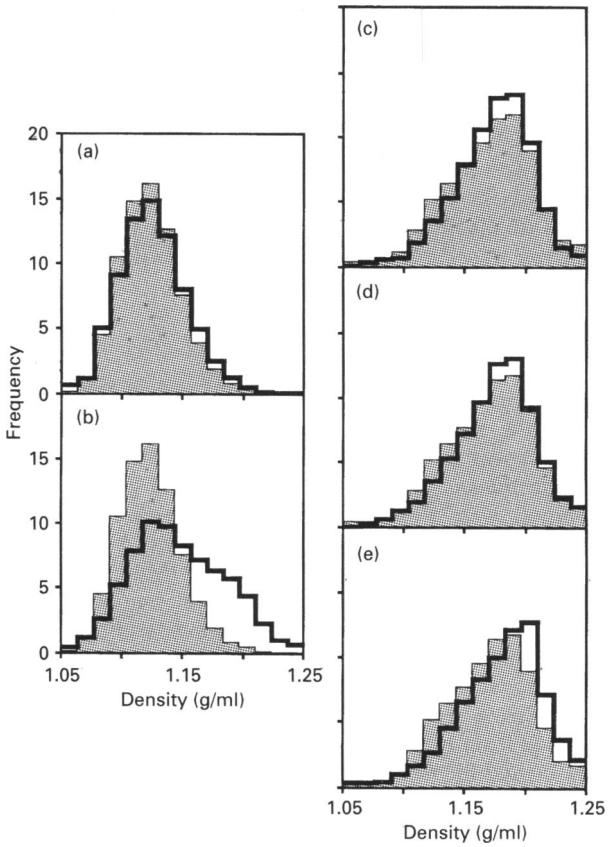

Figure 3 Subfractionation of microsomal fractions by density-gradient centrifugation

Average density distributions were obtained from two (protein, RNA, Ca^{2+}-ATPases) or three (binding sites) P′ fractions, as described in the Materials and methods section. Gradients were centrifuged for 2.5 h at 100000 *g*. Left panels: [^{3}H]PN200-110 binding (shaded), thapsigargin-resistant Ca^{2+}-ATPase (**a**) and protein (**b**). Right panels: [^{3}H]ryanodine binding (shaded), [^{3}H]Ins(1,4,5)P_3 binding (**c**), thapsigargin-sensitive Ca^{2+}-ATPase (**d**), RNA (**e**). The frequency is the fractional amount recovered between two density boundaries ($Q/\Sigma Q$, where ΣQ is the total amount recovered in all subfractions) divided by the corresponding density increment. Average recoveries with respect to total microsomes ranged from 88 to 98%, except for [^{3}H]Ins(1,4,5)P_3 binding (74%). Median densities are listed in Table 4.

Table 2 Specific binding parameters in microsomal (P) fractions

Values are means±S.E.M. from 8 preparations. K_D and $B_{max.}$ values were estimated by the Ligand program, and Hill coefficients by the EBDA program (McPherson, 1985).

	K_D (nM)	$B_{max.}$ (fmol/mg of protein)	Hill coefficient (*h*)
[^{3}H]PN200-110	0.063±0.007	1084±88	0.98±0.03
[^{3}H]Ryanodine	0.807±0.060	82.0±7.8	0.99±0.01
[^{3}H]Ins(1,4,5)P_3	5.37±0.77	743±77	0.98±0.01

Table 3 Distribution of specific binding sites, protein and RNA between particulate fractions obtained by differential centrifugation

Results are means±S.E.M. from *n* experiments (in parentheses). (N+M+P) refers to the sum of the numbers of receptors ($B_{max.}$) or amounts of protein or RNA found in the three particulate fractions.

		Distribution (%)		
	(N+M+P)	N	M	P
	(pmol/g of tissue)			
[^{3}H]PN200-110 (6)	14.9±1.8	32.1±3.0	8.9±0.7	59.0±2.5
[^{3}H]Ryanodine (8)	1.54±0.09	42.5±2.7	11.7±1.3	45.8±2.2
[^{3}H]Ins(1,4,5)P_3 (4)	15.8±2.2	39.9±2.4	13.6±2.2	46.5±3.5
	(mg/g of tissue)			
Protein (10)	60.9±2.9	61.9±3.5	24.4±3.1	13.7±0.5
RNA (3)	0.562±0.017	50.9±2.6	14.1±3.5	35.0±3.6

low-affinity binding was associated with the gel-like material, rather than with the brownish pellet.

Distribution of binding sites and biochemical constituents between the fractions obtained by differential centrifugation

As for [^{3}H]PN200-110 and [^{3}H]ryanodine binding, only one class of binding sites was detected by Scatchard analysis, whatever the fraction used. Average parameters obtained from 8 microsomal fractions are listed in Table 2. Also included are average parameters obtained for high-affinity [^{3}H]Ins(1,4,5)P_3 binding. The $B_{max.}$ of [^{3}H]ryanodine binding to microsomes (82 fmol/mg of protein) was about one-ninth of the $B_{max.}$ of high-affinity [^{3}H]Ins(1,4,5)P_3 binding (743 fmol/mg of protein). As shown in Table 3, the P fraction contained about 46% of the [^{3}H]ryanodine- and [^{3}H]Ins(1,4,5)P_3-binding sites recovered in the three particulate fractions (N, M and P), compared with 14% of the protein. The distributions of these two binding sites were not significantly different, whereas [^{3}H]PN200-110-binding sites were recovered in somewhat greater proportion (59%) in the P fraction. The sum of N, M and P accounted for about 50%

Table 4 Median equilibrium densities in untreated and digitonin-treated microsomal fractions

Median densities (means ± S.E.M. from three experiments, or individual values from two experiments) were computed from the distributions shown in Figure 3 (2.5 h; 100000 *g*) and in Figures 4 and 5 (12 h; 50000 *g*). Digitonin-shift is the difference between median density in digitonin-treated and untreated preparations. Abbreviations: (tha.-res.), thapsigargin-resistant; (tha.-sens.), thapsigargin-sensitive; ND, not determined; cyt., cytochrome.

	Density (g/ml)			
	(2.5 h; 100000 *g*)	(12 h; 50000 *g*)		
	Untreated	Untreated	Digitonin-treated	Digitonin-shift
[^{3}H]PN200-110	1.122 ± 0.003	1.118–1.124	1.152–1.160	0.034–0.036
Ca^{2+}-ATPase (tha.-res.)	1.120–1.125	ND–1.125	ND	
Protein	1.138–1.146	ND	ND	
NADPH:cyt. *c.* reductase	ND	1.176–1.181	1.184–1.186	0.008–0.005
[^{3}H]Ryanodine	1.174 ± 0.002	1.178–1.180	1.187–1.191	0.009–0.011
Sulphatase C	ND	1.180–1.183	1.194–1.194	0.014–0.011
Ca^{2+}-ATPase (tha.-sens.)	1.174–1.180	ND–1.191	ND	
[^{3}H]Ins(1,4,5)P_3	1.176 ± 0.002	1.184–1.192	1.197–1.200	0.013–0.008
RNA	1.180–1.188	ND	ND	

of the protein, 60% of the RNA and 85% of the binding sites measured in the total homogenate (results not shown).

Analysis of microsomal fractions by isopycnic centrifugation

In these experiments, we measured the sub-microsomal distributions of protein, RNA and the three binding sites, and, in addition, of several enzyme activities, in particular two Ca^{2+}-ATPase activities, which were distinguished on the basis of their sensitivity to 50 nM thapsigargin (Thastrup et al., 1990). Under our experimental conditions, inhibition of the total Ca^{2+}-ATPase activity by thapsigargin was almost maximal at a concentration of 10 nM and did not increase significantly at concentrations up to 200 nM. The thapsigargin-sensitive and -resistant activities in microsomes amounted to 60.7 ± 8.7 and 124.2 ± 11.4 nmol/min per mg of protein respectively (6 experiments). The same proportion of the total activity was inhibitable by cyclopiazonic acid (20–30 μM).

Density-gradient experiments were carried out on P′ fractions (see the Materials and methods section), which contained about 60% of the protein, and 75–80% of the RNA, receptors and enzymes measured in the P fraction. In a first series of three experiments, gradients were centrifuged for 2.5 h at 100000 *g* (Figure 3). Two clearly different distribution patterns can be recognized. (1) [^{3}H]PN200-110 binding (shaded histogram, left panels) and the thapsigargin-resistant Ca^{2+}-ATPase activity (Figure 3a) showed a relatively sharp and symmetrical distribution, with a peak at low densities (1.11–1.13 g/ml). (2) [^{3}H]Ryanodine binding (shaded histogram, right panels), [^{3}H]Ins(1,4,5)P_3 (high-affinity) binding (Figure 3c) and the thapsigargin-sensitive Ca^{2+}-ATPase activity (Figure 3d) had peaks at high densities (1.17–1.19 g/ml) and their distributions were skewed towards low densities. The distribution pattern of RNA (Figure 3e) resembled that of the second group, but its peak was slightly shifted towards high densities. The distribution pattern of protein (Figure 3b) was the most flattened, with a peak at 1.12–1.14 g/ml and a pronounced shoulder at high densities. Median equilibrium densities are listed in Table 4, under the heading '2.5 h; 100000 *g*'.

As illustrated in Figure 3, the distribution of [^{3}H]Ins(1,4,5)P_3 binding was almost identical with that of the thapsigargin-sensitive Ca^{2+}-ATPase, but differed slightly from that of [^{3}H]ryanodine binding; there was a slight excess of [^{3}H]ryanodine binding in low-density subfractions, and a corresponding excess of [^{3}H]Ins(1,4,5)P_3 binding in high-density subfractions. This difference was confirmed and shown to be statistically significant ($P < 0.01$) in separate experiments ($n = 5$) in which the pro-

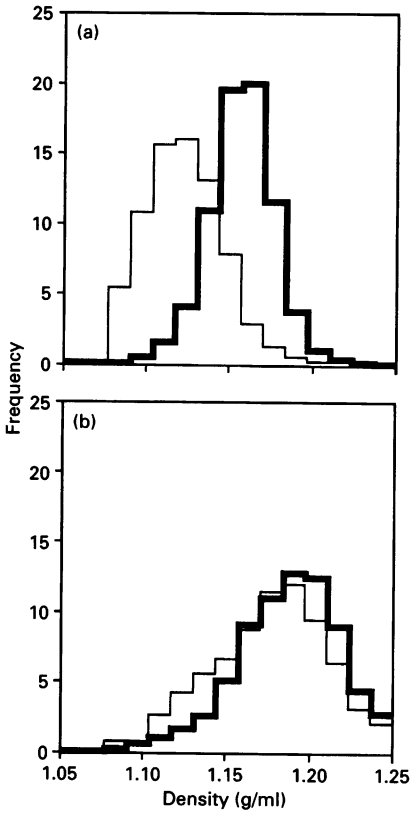

Figure 4 Effect of digitonin on density distributions of [^{3}H]PN200-110 and [^{3}H]ryanodine binding

Average density distributions (two experiments) of [^{3}H]PN200-110 binding (**a**) and [^{3}H]ryanodine binding (**b**) were obtained from untreated (thin-line histograms) and digitonin-treated (thick-line histograms) microsomes as described in the Materials and methods section. Gradients were centrifuged for 12 h at 50000 *g*. Average recoveries with respect to total microsomes were 95% (**a**) and 99% (**b**). Median densities are listed in Table 4.

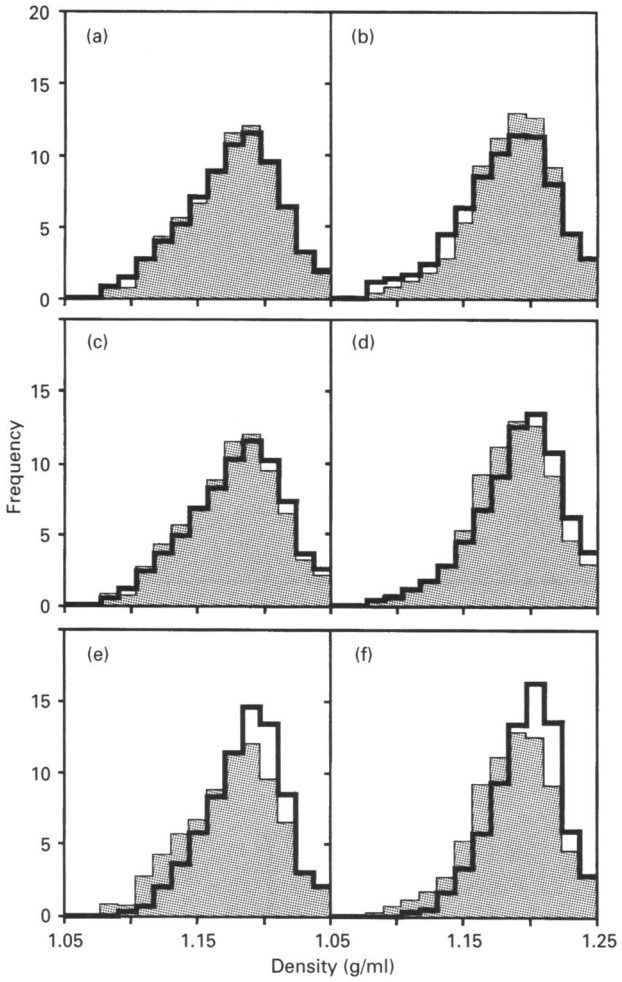

Figure 5 Subfractionation of untreated and digitonin-treated microsomal fractions by density-gradient centrifugation

Average density distributions (two experiments) obtained from untreated and digitonin-treated microsomes are shown in the left and right panels respectively: [^{3}H]ryanodine binding (shaded histograms, reproduced in each panel), NADPH:cytochrome *c* reductase (**a** and **b**), sulphatase C (**c** and **d**), [^{3}H]Ins(1,4,5)P_3 binding (**e** and **f**). Average recoveries with respect to total microsomes ranged from 98 to 107%. Median densities are listed in Table 4.

portion of microsomal sites recovered in light microsomes (P_1) was determined: it amounted to 23.9% (±1.5, S.E.M.) for [^{3}H]ryanodine binding and 18.3% (±0.5) for [^{3}H]Ins(1,4,5)P_3 binding.

In a second series of two density-gradient centrifugation experiments, untreated P′ fractions and fractions treated with a small concentration of digitonin were analysed in parallel (Figures 4 and 5; Table 4). As shown in Figure 4, the digitonin treatment induced a marked density shift (0.035 density unit; Table 4) of the dihydropyridine receptor ([^{3}H]PN200-110 binding), in agreement with previous results (Godfraind and Wibo, 1985), but increased the density of [^{3}H]ryanodine receptors only moderately. NADPH:cytochrome *c* reductase, sulphatase C and [^{3}H]Ins(1,4,5)P_3 binding also displayed a similar and moderate density shift (0.005–0.014; see Table 4). In these experiments, gradients were centrifuged for 12 h at 50000 ***g***. As for untreated microsomes, the modified centrifugation conditions did not change the equilibrium density of [^{3}H]PN200-110 binding and that of the thapsigargin-resistant Ca^{2+}-ATPase (Table 4). In contrast, the median densities of [^{3}H]ryanodine and [^{3}H]Ins(1,4,5)P_3 binding, and of the thapsigargin-sensitive Ca^{2+}-ATPase, were slightly higher in the second series of experiments, indicating that the equilibrium position of the host particles were not completely reached after 2.5 h at 100000 ***g***. The distributions of NADPH:cytochrome *c* reductase (Figure 5a) and sulphatase C (Figure 5c) were found to be quite superimposable on that of [^{3}H]ryanodine binding (shaded), whereas [^{3}H]Ins(1,4,5)P_3 binding (Figure 5e) and the thapsigargin-sensitive Ca^{2+}-ATPase (not shown) equilibrated at slightly higher densities. After digitonin treatment, the distribution patterns of the two enzymes (Figures 5b and 5d) again coincided fairly well with that of [^{3}H]ryanodine binding (shaded), whereas the distribution of [^{3}H]Ins(1,4,5)P_3 binding (Figure 5f) remained distinctly shifted towards higher densities. Unfortunately, Ca^{2+}-ATPase activities could not be measured reliably after digitonin treatment.

DISCUSSION

Identification of [^{3}H]Ins(1,4,5)P_3- and [^{3}H]ryanodine-binding sites

The high-affinity [^{3}H]Ins(1,4,5)P_3-binding site reported here shares several properties (K_D, heparin-sensitivity, pH-dependency) with those described previously in membrane fractions or purified Ins(1,4,5)P_3-receptor preparations from smooth-muscle tissues (Chadwick et al., 1990; Mourey et al., 1990; Varney et al., 1990). No indication in favour of the existence in smooth muscle of a second binding site of lower affinity but higher capacity has ever been reported. In contrast, two binding sites, with affinities similar to those that we found, were found in an ER fraction from WRK_1 cells (Mouillac et al., 1992). In microsomes from nervous tissue, an inositol polyphosphate receptor, displaying K^+-channel activity and closely related to clathrin assembly protein AP-2, has been recently identified and shown to bind Ins(1,4,5)P_3 with low-affinity (Chadwick et al., 1992; Timerman et al., 1992). In addition, a sialic acid-rich Ins(1,4,5)P_3-receptor-like protein, but with a rather high K_D for [^{3}H]Ins(1,4,5)P_3, has been described in the plasma membrane of lymphocytes (Khan et al., 1992). In our experiments, low-affinity binding was not detectable in light microsomes, in which plasma membranes were purified (see below). This observation seems also to rule out the possibility that low-affinity binding could be associated with Ins(1,4,5)P_3 3-kinase, which has comparable affinity for Ins(1,4,5)P_3 and sensitivity to heparin, and is partly bound to plasma membranes in some cell types (Morris et al., 1987). Low-affinity binding is also unlikely to be due to Ins(1,4,5)P_3 5-phosphatase, since (i) no detectable hydrolysis of [^{3}H]Ins(1,4,5)P_3 occurred under our incubation conditions and (ii) the 5-phosphatase activity is little sensitive to heparin (Worley et al., 1987). In cardiomyocytes, Ins(1,4,5)P_3 has been shown recently to bind almost exclusively to intercalated discs, in particular in the region of adherens junctions (Kijima et al., 1993). Although the K_D of [^{3}H]Ins(1,4,5)P_3 binding to intercalated discs of cardiomyocytes was not reported, it is tempting to speculate that low-affinity [^{3}H]Ins(1,4,5)P_3-binding sites of smooth-muscle cells might be associated to adherens junctions, which serve as anchorage sites for the actin cytoskeleton and might sediment in the N and M fractions, together with attached contractile proteins. However, immunolocalization studies on smooth-muscle cells have revealed the presence of an Ins(1,4,5)P_3-receptor-like protein (of unknown affinity) in caveolae, rather than in adherens junctions (Fujimoto et al., 1992).

As for [^{3}H]ryanodine binding, its K_D (0.8 nM) was quite similar to that measured under identical conditions in brain tissue by McPherson and Campbell (1990). The K_D of the

ryanodine receptor is sensitive to ionic strength, Ca^{2+} concentration and adenine nucleotides, which probably accounts for the higher K_D value (4–8 nM) found recently in rat vas deferens (Zhang et al., 1993). The $B_{max.}$ value was rather low, 9–10 times less than that of high-affinity Ins(1,4,5)P_3 binding. A low level of ryanodine receptors in the longitudinal smooth muscle of guinea-pig intestine is in agreement with previous findings on other smooth-muscle tissues, except vas deferens (Zhang et al., 1993).

Subcellular localization of [^{3}H]Ins(1,4,5)P_3- and [^{3}H]ryanodine-binding sites

Nearly half of the sedimentable ryanodine and Ins(1,4,5)P_3 receptors were recovered in the microsomal (P) fraction, and their sub-microsomal distribution pattern (without or after treatment with digitonin) followed closely those of NADPH:cytochrome *c* reductase and sulphatase C, which are putative ER enzymes in smooth-muscle tissue (Morel et al., 1981; Daniel et al., 1982; Godfraind and Wibo, 1985). Moreover, after density equilibration, ryanodine and Ins(1,4,5)P_3 receptors were distributed like the thapsigargin-sensitive Ca^{2+}-ATPase activity, and the sensitivity to thapsigargin is a specific property of the SR or ER Ca^{2+}-ATPase (SERCA) family (Lytton et al., 1991). In contrast, the distribution pattern and the response to digitonin clearly distinguished ryanodine and Ins(1,4,5)P_3 receptors from the dihydropyridine receptor ([^{3}H]PN200-110 binding), i.e. the L-type voltage-dependent Ca^{2+} channel of the plasma membrane (Godfraind and Wibo, 1985), and from the thapsigargin-insensitive Ca^{2+}-ATPase activity. Part at least of this latter activity must correspond to the Ca^{2+} pump that has been demonstrated in the plasma membrane of intestinal smooth muscle (Wibo et al., 1981). Thus our subcellular-fractionation data indicate that most of the ryanodine and Ins(1,4,5)P_3 receptors in intestinal-smooth-muscle cells are not localized in the plasma membrane, but are most probably associated with the ER. The same conclusion, limited, however, to ryanodine receptors, has been reached by Bourreau et al. (1991) on rat vas deferens.

The important density shift experienced by digitonin-treated plasma membranes has been attributed to the equimolecular binding of digitonin to the cholesterol of those membranes (Amar-Costesec et al., 1974). The weak shift of the presumed ER constituents in our experiments could be due to the presence of a small amount of cholesterol in the ER membrane of smooth-muscle cells, or could reflect some degree of 'non-specific' binding of digitonin to these membranes. Alternatively, this shift might suggest that ER vesicles were partly linked to plasma-membrane fragments. If such were the case, one would expect a distinct narrowing of the distribution pattern of ER vesicles after digitonin treatment. Indeed, most of the ER vesicles physically linked to plasma membranes should equilibrate at densities lower than 1.20 g/ml, the upper density limit of the distribution of digitonin-treated plasma membranes, whereas 'free' ER vesicles would remain unshifted. In contrast, the proportion of ryanodine-binding sites and other ER constituents recovered at densities higher than 1.20 g/ml increased after digitonin treatment (see Figures 4 and 5), suggesting that the weak shift of ER constituents resulted from a modification affecting most of the ER vesicles, rather than from a physical association of some ER membranes with plasma membranes. Quasi-periodic structures bridging the gap between sub-plasmalemmal ER cisternae and the plasma membrane have been reported in smooth-muscle cells (Somlyo and Franzini-Armstrong, 1985); those structures resemble the 'feet' connecting junctional SR and transverse tubules in cardiac and skeletal muscle, which have been clearly identified

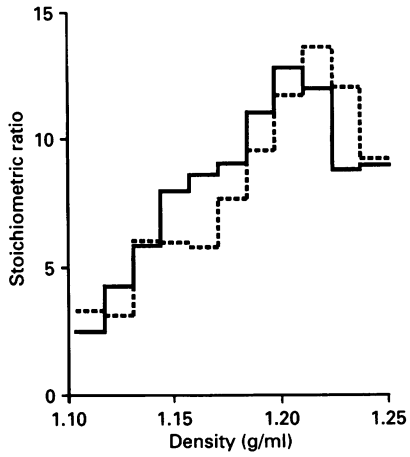

Figure 6 Stoichiometric ratio of Ins(1,4,5)P_3 to ryanodine receptors in microsomal sub-fractions as a function of density

Frequency ratios computed from Figure 5 were multiplied by 9.1, the stoichiometric ratio in total microsomes (Table 2), to yield stoichiometric ratios in subfractions of increasing density. Continuous and broken lines refer to untreated and digitonin-treated microsomes respectively.

as ryanodine receptors (Lai and Meissner, 1989). It has also been proposed that the large cytoplasmic head of the Ins(1,4,5)P_3 receptor might establish a connection with a Ca^{2+} channel in the plasma membrane and be involved in the Ca^{2+} entry stimulated by store depletion (reviewed by Berridge, 1993). If feet-like connections do exist in intact smooth-muscle cells, either they are too uncommon to affect density distributions, or they do not survive tissue homogenization, unlike junctional structures of skeletal or cardiac muscle (Wibo et al., 1991).

It is clear from Figure 3 that the RNA/protein ratio increased with equilibrium density, suggesting that, as in liver microsomes (Amar-Costesec et al., 1974), the number of ribosomes per unit surface area of ER vesicles is an important determinant of their equilibrium density. The density distributions of Ins(1,4,5)P_3 and ryanodine receptors, which were quite similar to those of NADPH:cytochrome *c* reductase, sulphatase C and the thapsigargin-sensitive Ca^{2+}-ATPase, suggest that both receptors were present in rough and smooth ER vesicles. However, Ins(1,4,5)P_3 receptors and the thapsigargin-sensitive Ca^{2+}-ATPase equilibrated at slightly higher densities than did ryanodine receptors, NADPH:cytochrome *c* reductase and sulphatase C. In the experiments reported in Figure 5, the stoichiometric ratio of Ins(1,4,5)P_3 receptors to ryanodine receptors increased with density from about 3 to 14 (Figure 6), suggesting that the Ins(1,4,5)P_3 receptor (and the thapsigargin-sensitive Ca^{2+} pump) might be preferentially localized on the ribosome-coated portions of the ER, in contrast with the other ER constituents. A concentration of Ins(1,4,5)P_3 receptors in rough ER has been reported previously in DDT_1MF-2 cells derived from hamster vas deferens (Ghosh et al., 1989) and in dog pancreas (Sharp et al., 1992). The situation might be different in other cell types, as suggested by subcellular-fractionation studies on parotid (Henne et al., 1987), adrenal cortex (Rossier et al., 1989) and liver (Shoshan-Barmatz et al., 1990; Rossier et al., 1991). In cerebellar Purkinje neurons, Ins(1,4,5)P_3 receptors are most abundant in stacks of smooth cisternae, which are in continuity with the rough ER (Satoh et al., 1990; Otsu et al., 1990).

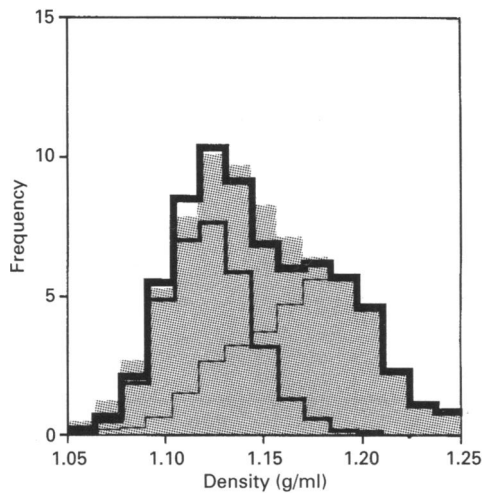

Figure 7 Analysis of the density distribution of microsomal protein

The measured distribution (shaded), taken from Figure 3, is compared with the fitted distribution (shown by the thick-line histogram) obtained from those of [^{3}H]PN200-110 and [^{3}H]ryanodine binding. The intermediate-line histogram was constructed from the frequencies of [^{3}H]PN200-110 binding, multiplied by 0.45, and the thin-like histogram from the frequencies of [^{3}H]ryanodine binding, multiplied by 0.5.

Estimated surface density of ryanodine and Ins(1,4,5)P_3 receptors in ER membranes

To determine the surface density of receptors in ER membranes, we should relate the numbers of receptors measured to the surface area of ER membrane in the microsomal fraction. In the absence of morphometric data on microsomes from smooth muscle, this surface area may be roughly estimated from (i) the amount of microsomal protein attributable to ER, and (ii) the ratio of membrane surface area to protein, as determined in a previous morphometric study on liver microsomes (Wibo et al., 1971).

The distribution pattern of microsomal protein (Figure 3) is expected to reflect two main contributions, those of plasma membranes and ER. As shown in Figure 7, assuming that plasma-membrane protein is distributed like [^{3}H]PN200-110 binding and ER protein like [^{3}H]ryanodine binding, the protein distribution can be satisfactorily fitted by ascribing 45% and 50% of the microsomal protein to plasma membranes and ER, respectively. Protein not accounted for (5%) is found mainly at densities 1.15–1.17 g/ml and could belong to small mitochondria. Indeed, in rat aorta microsomal fractions, the median density of the mitochondrial marker cytochrome *c* oxidase is about 1.16 g/ml (Wibo et al., 1980).

Assuming a proportion of 50% of ER protein in microsomal fractions, the average concentration of Ins(1,4,5)P_3 and ryanodine receptors may be calculated from Table 2 to be about 1.5 and 0.16 pmol/mg of ER protein, respectively. In liver microsomes, which are made up mostly of ER-derived vesicles, the membrane surface area is 0.15 m^2/mg of protein (Wibo et al., 1971). We may thus estimate the surface density of Ins(1,4,5)P_3 and ryanodine receptors to be 10 and 1.1 pmol/m^2 of ER membrane, i.e. 6 and 0.64 receptors/μm^2 respectively. As discussed above, Ins(1,4,5)P_3 receptors appear more concentrated in rough ER, where their density could reach a value of 9 receptors/μm^2. This estimation agrees with that obtained in immunocytochemical studies on cerebellar Purkinje neurons. Indeed, Satoh et al. (1990) counted 0.5 Ins(1,4,5)P_3 receptor/μm of membrane profile at the level of the rough ER in 0.1 μm-thick cryosections; a lower limit of 5 receptors/μm^2 is suggested by this result, since only antigen molecules exposed at the surface of cryosections are revealed by this method.

Functional implications

In their ultrastructural studies on smooth-muscle cells, Devine et al. (1972) distinguished the 'peripheral' SR, a network of predominantly smooth tubules and cisternae located close to the inner surface of the plasma membrane, from the 'deep' ER, comprising mainly rough ER located in the interior of the cell and connected with the nuclear envelope. It is generally accepted that the peripheral SR is the main agonist-sensitive Ca^{2+} store in smooth muscle (Bond et al., 1984). However, as shown here and in a previous study (Ghosh et al., 1989), the rough ER of smooth muscle seems to be well equipped with Ca^{2+}-release channels and Ca^{2+}-pumping ATPase. In line with this observation, agonist stimulation of DDT_1MF-2 smooth-muscle cells evokes an early Ca^{2+} rise in the vicinity of the nuclear envelope (Himpens et al., 1992), suggesting the release of Ca^{2+} stored in the envelope (Nicotera et al., 1990) and in the perinuclear ER. A perinuclear localization of the agonist- [and Ins(1,4,5)P_3-] sensitive Ca^{2+} store, as opposed to a more diffuse cytoplasmic distribution of the caffeine- (and ryanodine-) sensitive store has also been described in bovine chromaffin cells (Burgoyne et al., 1989). Possibly, deeply located Ins(1,4,5)P_3 receptors cannot be activated after agonist stimulation in many cell types, because the Ins(1,4,5)P_3 generated at the plasma membrane is rapidly attacked by metabolic enzymes, leaving the peripheral SR as the main Ca^{2+} store actually mobilized by agonists (see van Breemen and Saida, 1989).

In this analytical-fractionation study, we could not sharply differentiate ryanodine and Ins(1,4,5)P_3 receptors on the basis of sedimentation velocity, equilibrium density in sucrose solution and response to digitonin, although small, but reproducible, differences in the density distributions led us to conclude that the stoichiometric ratio of Ins(1,4,5)P_3 to ryanodine receptors could be distinctly higher in rough ER than in smooth ER. These results are certainly compatible with functional models of smooth-muscle cells, according to which both receptors would be largely co-localized in all parts of the ER (van Breemen and Saida, 1989). They do not, however, rule out the possibility that Ins(1,4,5)P_3 and ryanodine receptors could belong to separate ER sub-compartments, both comprising rough and smooth domains, though maybe in different proportions. From their Ca^{2+}-release studies on saponin-permeabilized smooth-muscle tissues, Iino et al. (1988) postulated two Ca^{2+}-storage compartments, one (Sα) endowed with both ryanodine- and Ins(1,4,5)P_3-sensitive channels, and the other (Sβ) containing only Ins(1,4,5)P_3-sensitive channels. This situation might result from the very low surface density of ryanodine receptors in the ER membrane of most smooth-muscle cell types, provided that all parts of the ER are not interconnected. In this respect, it has been reported that the saponin treatment may induce fragmentation of the ER (Bird et al., 1992), which could promote the artefactual formation of a Sβ compartment containing very few, if any, ryanodine receptors but an appreciable number of Ins(1,4,5)P_3 receptors and Ca^{2+} pumps. That ER fragmentation is likely to give rise to ER compartments devoid of ryanodine receptors is supported by calculations showing that, in our microsomal fractions, only one out of 23 vesicles (assuming an average surface area of 0.06 μm^2; Wibo et al., 1971) would be equipped with a ryanodine receptor.

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