The inositol 1,4,5-trisphosphate receptor is localized on specialized sub-regions of the endoplasmic reticulum in rat liver

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Inositol 1,4,5-trisphosphate (InsP_3) is involved in the mobilization of Ca^{2+} from intracellular non-mitochondrial stores. In rat liver, it has been shown that the InsP_3 -binding site copurifies with the plasma membrane. This suggests that in the liver the InsP_3 receptor (InsP_3R) associates with plasma membrane. We studied the subcellular distribution of the liver InsP_3R by measuring the maximal binding capacity of [^3H] InsP_3 and using antibodies against the 14 C-terminal residues of the type 1 InsP_3R . The antibodies recognized a large amount of an InsP_3R protein of 260 kDa in a membrane fraction which is also enriched with [^3H] InsP_3 -binding sites and with markers of the basal, the lateral and the bile-canalicular membrane and the plasma-membrane Ca^{2+} pump (PMCA). The fractions enriched in markers of the endoplasmic reticulum (ER) and the Ca^{2+} pump of the ER (SERCA2b) contained low levels of InsP_3 receptors. The immunofluorescent labelling of cultured hepatocytes with anti- InsP_3R antibodies indicated that the receptor is concentrated

in the perinuclear area and in some regions near the plasma membrane. The fraction enriched with InsP_3R is also contaminated with markers of the ER and with SERCA2b. It was exposed to alkaline medium (pH 10.5) to extract endogenous actin and membrane-associated proteins before being subfractionated by Percoll-gradient centrifugation. The alkaline treatment allowed partial separation of the markers of the ER from the markers of the plasma membrane. The InsP_3R was recovered in the heavy subfraction, which was also enriched with markers for the ER and with the SERCA2b and contained low levels of markers of the plasma membrane. These data indicate that the InsP_3R is neither localized on the plasma membrane itself nor homogeneously distributed on the ER membrane. This supports the view that part of the receptor is localized on a specialized sub-region of the ER which interacts with the plasma membrane.

INTRODUCTION

The mobilization of Ca^{2+} from intracellular stores is a key event in the transduction of the signal of several hormones and mediators. Over the last few years there has been considerable progress in understanding the molecular events involved in the accumulation, the sequestration and the release of Ca^{2+} from intracellular compartments. The molecular properties of the Ca^{2+} pump of the plasma membrane (PMCA) involved in the extrusion of Ca^{2+} to the extracellular medium have been partly elucidated, and several genes have been identified. Also, different isoforms of the sarco(endo)plasmic-reticulum Ca^{2+} -ATPase (SERCA) have been identified and localized in different muscle and non-muscle tissues (see Missiaen et al., 1991). Several members of the family of the high-capacity Ca^{2+} -binding proteins necessary for the accumulation of Ca^{2+} inside the stores have been identified (see Meldolesi et al., 1990). In many cell types, Ca^{2+} is mobilized by inositol 1,4,5-trisphosphate (InsP_3), which is the product of hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate. InsP_3 binds to a specific receptor/channel located on the membrane of an intracellular Ca^{2+} -storing organelle (Berridge and Irvine, 1989; Ferris and Snyder, 1992; Mikoshiba, 1993). Different isoforms of this receptor have been identified and are produced by three or four different genes and by alternative splicing of mRNA (Südhof et al., 1991; Nakagawa et al., 1991; Ross et al., 1992). The spatial organization and the intracellular localization of the rapidly exchanging Ca^{2+} -storage organelle comprising the different components involved in Ca^{2+} sequestration and release are not

yet fully elucidated. InsP_3 releases Ca^{2+} from a non-mitochondrial pool, which was first identified as being the whole endoplasmic reticulum (ER) (Berridge and Irvine, 1984). This view has been questioned since 1988, when the Ca^{2+} -binding protein calsequestrin was found to be non-homogeneously distributed in the cytoplasm, suggesting that the whole ER is not the Ca^{2+} -sequestering organelle (Volpe et al., 1988). It was therefore of interest to compare the localization of the InsP_3 receptor (InsP_3R) with that of the Ca^{2+} -binding proteins. Various experimental approaches have been used to identify and purify the intracellular InsP_3 -sensitive organelle. The high density of InsP_3R in the Purkinje cells of the cerebellum allowed the localization *in situ* of this receptor, the Ca^{2+} -binding proteins and the Ca^{2+} pump. InsP_3R was found unevenly distributed on the ER, with labelling on cisternae and smooth-surfaced structures in direct membrane continuity with the rest of the ER. These structures appear beneath the plasma membrane or in the perinuclear region. The Ca^{2+} pump was widely distributed across the ER, and calsequestrin was found at low concentrations in the ER cisternae but at high concentration in small vacuoles distributed throughout the ER (Mignery et al., 1989; Otsu et al., 1990; Satoh et al., 1990; Meldolesi et al., 1992). These results and recent data from immunochemical analysis of specific markers of the cellular membranes led to the idea that the ER is a patchwork of specialized sub-regions (Sitia and Meldolesi, 1992). In non-neural cells, direct localization of InsP_3R by immunohistochemistry is difficult because of the low receptor density. Subcellular fractionation of the tissues, followed by the identification of the fractions, indicated that the different

Abbreviations used: ER, endoplasmic reticulum; RER, rough ER; InsP_3 , D-myo-inositol 1,4,5-trisphosphate; InsP_3R , InsP_3 receptor; PMCA, plasma-membrane Ca^{2+} pump; SERCA, sarco(endo)plasmic-reticulum Ca^{2+} pump.

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components required for Ca^{2+} accumulation and release are not homogeneously distributed. Particularly, the fractions sensitive to InsP_3 or enriched in InsP_3R do not co-purify with markers of the ER (Rossier et al., 1989; Van Delden et al., 1992; Sharp et al., 1992; Enyedi et al., 1993). Furthermore, in the liver, it has been found that InsP_3R co-purifies with the plasma membrane (Guillemette et al., 1988; Piétri et al., 1990a; Nunn and Taylor, 1990; Rossier et al., 1991; Feng et al., 1992; Sharp et al., 1992; Enyedi et al., 1993). This suggests that the InsP_3R is located either on the plasma membrane itself or on an intracellular organelle which associates with plasma membrane (Rossier and Putney, 1991). The putative interaction of the InsP_3 -sensitive store with the plasma membrane has led to models linking the release of intracellular Ca^{2+} to the stimulation of Ca^{2+} influx from the extracellular medium (Putney, 1986; Irvine, 1990). We report the subcellular distribution of the InsP_3R in the rat liver, assessed by measuring the maximal binding capacity of $[\text{H}]\text{InsP}_3$ and using antibodies against type 1 InsP_3R . Different subcellular fractions were characterized by specific markers of the ER and of the plasma membrane. The receptor was found in a particular sub-region of the ER which co-purified with the plasma membrane.

MATERIALS AND METHODS

Chemicals

$[\text{H}]\text{InsP}_3$ was obtained from Du Pont–New England Nuclear. InsP_3 was obtained from Calbiochem. All other reagents were obtained from Sigma or Boehringer Mannheim.

Antibodies

Synthetic peptide (GHPPHMNVNPQQPA) corresponding to the 14 C-terminal residues (2737–2750) of the type 1 InsP_3R (Furuichi et al., 1989) was prepared by Neosystem (Strasbourg, France). The N-terminal amino acid was cross-linked to ovalbumin by glutaraldehyde. Polyclonal antibodies against the C-terminal peptide were produced in rabbit and purified on a matrix containing the peptide coupled to Affigel 15 (Bio-Rad). Purified antibodies were kept at 1 mg/ml in PBS containing 40 % glycerol at -20°C .

The following antibodies used in this study have been described elsewhere: anti-SERCA2a and anti-SERCA2b polyclonal rabbit antibodies (Wuytack et al., 1989; Eggermont et al., 1990); anti-RER (rough ER) polyclonal antibodies raised against dog pancreas RER membrane (Louvard et al., 1982); anti-PMCA monoclonal antibodies (5F10) raised against erythrocyte Ca^{2+} -ATPase (Kessler et al., 1990). The polyclonal and monoclonal antibodies B1 and B10 raised against markers of the lateral and the bile-canalicular membrane, respectively, have been described (Maurice et al., 1985, 1988).

Preparation of the membrane fractions

Livers from female Wistar rats (200–250 g) were homogenized with a Dounce homogenizer in an ice-cold homogenization medium containing 250 mM sucrose, 5 mM Hepes/KOH (pH 7.4), 1 mM EGTA and supplemented with 1 mM dithiothreitol, 0.2 mM phenylmethanesulphonyl fluoride and 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 μM pepstatin, 2 μM benzamidin, 5 $\mu\text{g}/\text{ml}$ aprotinin, 50 $\mu\text{g}/\text{ml}$ trypsin inhibitor and 1 $\mu\text{g}/\text{ml}$ *o*-phenanthroline, as previously described (Piétri et al., 1990a). The high-speed pellet (HSP) was obtained by centrifuging the homo-

genate at 100 000 *g* for 60 min. The other fractions were obtained by differential centrifugation. The homogenate was centrifuged for 10 min at 1500 *g*, yielding the low-speed pellet (LSP). The supernatant was centrifuged for 20 min at 8000 *g* to obtain the mitochondrial fraction. The heavy and the light microsomal fractions (HM and LM) were obtained by centrifuging the 8000 *g* supernatant for 30 min at 35 000 *g* and the 35 000 *g* supernatant for 60 min at 100 000 *g*, respectively. The plasma-membrane fraction (PM) was prepared by Percoll-gradient centrifugation of the low-speed pellet as described by Prpic et al. (1984). The fractions were washed and resuspended in the following ice-cold washing medium: 250 mM sucrose, 25 mM Hepes/KOH (pH 7.4), 1 mM dithiothreitol, and the protease-inhibitor cocktail.

Rat cerebellar membranes were prepared as previously described (Piétri et al., 1990b).

The 5'-nucleotidase and glucose-6-phosphatase activities were measured as described by Aronson and Touster (1974).

Alkaline treatment of the PM fraction

The PM fraction was washed at 4°C in 2 mM Tris/HCl (pH 7.5)/0.5 mM CaCl_2 /0.5 mM dithiothreitol. The membranes were then incubated for 30 min in 1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.5, as described by Tranter et al. (1989). The membranes were centrifuged for 30 min at 35 000 *g* and washed once in the carbonate buffer and twice in 250 mM sucrose/25 mM Hepes/KOH, pH 7.4 at 4°C , giving the f_0 fraction. All the media were supplemented with the protease-inhibitor cocktail. Percoll (25 %, v/v) was added to the membrane suspension, which was then distributed in 15 ml Corex centrifugation tubes and centrifuged for 30 min at 35 000 *g*. Four fractions (f_1 – f_4) were collected, washed six times and resuspended in the washing medium.

Cultured hepatocytes

Hepatocytes were isolated from female Wistar rats weighing 200–250 g as previously described (Piétri et al., 1990a). The cells were resuspended in Williams' medium E supplemented with 2 mM glutamine, 100 i.u./ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. They were grown on glass coverslips previously coated with 1 mg/ml collagen at a cell density of $2 \times 10^5 \text{ ml}^{-1}$.

Equilibrium $[\text{H}]\text{InsP}_3$ -binding studies

Membranes were preincubated for 10 min at 37°C as previously described (Piétri et al., 1990b) in the incubation (cytosol-like) medium consisting of: 110 mM KCl, 20 mM NaCl, 25 mM Hepes/KOH (pH 7.4), 1 mM NaH_2PO_4 , 1 mM EDTA, 1 mg/ml BSA. $[\text{H}]\text{InsP}_3$ (17–21 Ci/mmol) binding was measured on ice by adding 100 μl of the membrane fraction to 900 μl of the incubation (cytosol-like) medium supplemented with $(0.5\text{--}1) \times 10^4$ c.p.m. of $[\text{H}]\text{InsP}_3$ and the indicated concentrations of unlabelled ligand. Non-specific binding was determined in the presence of 5 μM InsP_3 . After incubation for 5 min, 0.8 ml of the sample was layered on a Whatman GF/C glass-fibre filter and washed with 1 ml of an ice-cold medium consisting of 250 mM sucrose, 10 mM NaH_2PO_4 , and 4 mM EGTA (pH 7.4). This washing procedure took less than 1 s and minimized the dissociation of the ligand–receptor complex. Radioactivity retained on the filters was counted by scintillation spectrometry.

Inplot software (GraphPAD) was used to fit the inhibition binding curves to data points by non-linear regression analysis

according to models involving one or two binding sites. The two fits to the same data were compared by calculating the sum of square values. The maximal binding capacities were estimated as described by Swillens (1992).

SDS/PAGE and Western blotting

The membrane fractions were resuspended in SDS sample buffer in reducing conditions as described by Laemmli (1970). SDS/PAGE was performed on 4–10% gradient gels, and the separated proteins were electro-transferred as described by Towbin et al. (1979) to a Hybond C-Super nitrocellulose membrane (Amersham). The blots were blocked with PBS containing 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween 20 for 1 h at 37 °C. The blots were then incubated with the different antibodies at the indicated dilution in PBS containing 2.5% (w/v) non-fat dry milk and 0.1% (v/v) Tween 20 for 1 h at room temperature. After five washes with PBS, the nitrocellulose membranes were incubated for 30 min at room temperature with either peroxidase-conjugated goat IgG against rabbit IgG (1:2000) or peroxidase-conjugated goat IgG against mouse IgG (1:2000) (both from Diagnostics Pasteur, Marnes-la-Coquette, France). Blots were then washed five times and finally developed with the enhanced chemiluminescence (ECL) Western-blotting system using Hyperfilm (Amersham).

Immunofluorescence

Cells cultured for 20–24 h were fixed with PBS containing 4% paraformaldehyde for 20 min and permeabilized by incubation for 4 min with 0.1% (w/v) Triton X100 in PBS. The cells were then washed twice with PBS containing 50 mM NH₄Cl and treated with an avidin–biotin kit (Biogenex Laboratories) for blocking the endogenous biotin. After washing three times in PBS, the cells were incubated with immunopurified anti-InsP₃R rabbit antibodies used at 50 µg/ml or the anti-ER rabbit antiserum diluted at 1:1000, then with anti-rabbit IgG biotinylated donkey antibodies (1:200) and finally with avidin fluorescein (1:100) (Amersham). The cells were observed by using epifluorescence on a C Ziess Axoscope with a 40 × oil-immersion lens.

RESULTS

Distribution of the InsP₃-binding sites among rat liver subcellular fractions

Several reports have indicated that the InsP₃ receptor can be recovered from a liver membrane fraction enriched in plasma membrane (Guillemette et al., 1988; Piétri et al., 1990a; Nunn and Taylor, 1990; Rossier et al., 1991; Feng et al., 1992; Sharp et al., 1992; Enyedi et al., 1993). However, these studies mainly described the distribution of a high-affinity binding site which is not coupled to Ca²⁺ release. We have previously defined the experimental conditions minimizing the rapid dissociation of the InsP₃R complex and labelling a low-affinity site more likely to be involved in Ca²⁺ release (Piétri-Rouxel et al., 1992). We then determined the maximal [³H]InsP₃-binding capacities (*B*_{max}) in subcellular fractions prepared from a rat liver homogenate. The *B*_{max} was determined in each fraction by homologous competition experiments in accordance with the method described by Swillens (1992). The *K*_d values for the InsP₃ binding were 40–65 nM and were not significantly different in the different fractions. The low-speed pellet (LSP) was enriched about 2-fold in InsP₃-binding sites and contained 73% of the binding sites

present in the high-speed pellet (HSP), which is assumed to contain all the cell membranes. The LSP was submitted to a Percoll-gradient centrifugation as described by Prpic et al. (1984). The second layer from the top of the tube (PM fraction) was enriched 10-fold in InsP₃-binding sites (Figure 1a) and contained about half of the receptors present in the LSP. The remaining receptors were recovered, without enrichment, with all other material on the bottom of the tube. The fraction enriched in InsP₃R is also enriched in 5'-nucleotidase activity (Figure 1b). There was no enrichment in InsP₃-binding sites in the mitochondrial (Mit) and microsomal fractions (HM and LM, Figure 1a), which contain a low 5'-nucleotidase activity (Figure 1b). There was no correlation between the InsP₃-binding capacities and the glucose-6-phosphatase activities (a marker of the ER) of the fractions: glucose-6-phosphatase was found in all the fractions, and the HM fraction displayed the highest activity (Figure 1c). Therefore, about one-third of the liver InsP₃R was found at high levels in a fraction rich in plasma membranes and two-thirds were scattered in all the other subcellular fractions.

Figure 1 Distribution of [³H]InsP₃-binding sites and enzyme markers in rat liver subcellular fractions

The high-speed pellet (HSP), the low-speed pellet (LSP), the plasma-membrane fraction (PM), the mitochondrial fraction (Mit), the heavy microsomes (HM) and the light microsomes (LM) were prepared as described in the Materials and methods section. (a) The membrane fractions (0.4 mg/ml for PM and 1.8–2.2 mg/ml for the other fractions) were incubated in 1 ml of incubation medium in the presence of 11 500 c.p.m. of [³H]InsP₃ and increasing concentrations (1 nM–5 µM) of unlabelled InsP₃. The maximal binding capacities were determined as described in the Materials and methods section. (b) The 5'-nucleotidase activities in the different fractions were determined. Results are expressed in µmol of P_i formed in 10 min per mg of protein. (c) The glucose-6-phosphatase activities in the different fractions were determined. Results are expressed in µmol of P_i formed in 10 min per mg of protein. Data are means of triplicate determinations from one typical experiment. [³H]InsP₃ binding, 5'-nucleotidase activity and glucose-6-phosphatase activity were all determined in the same membrane preparations from a single subcellular fractionation.

Figure 2 Immunodetection of the InsP_3R in rat liver subcellular fractions

The subcellular fractions (30 μg per lane) prepared as described in Figure 1 were submitted to SDS/PAGE (4–10% gradient) and then stained with Coomassie Blue (a) or transferred to a nitrocellulose membrane (b). Blots were incubated with the immunopurified anti- InsP_3R antibodies (5 $\mu\text{g}/\text{ml}$) and with secondary antibodies as described in the Materials and methods section. The blot was developed with ECL Western blotting system. Abbreviations: Cer, cerebellar membranes; for others, see legend to Figure 1.

These data confirm previous observations that part of the InsP_3R of the liver co-purifies with the plasma membrane prepared by various protocols.

Distribution of immunoreactive InsP_3R

The association of the InsP_3R with the plasma membrane seems to be a specific feature of the liver. This could result from an artifactual identification of InsP_3 -binding sites not related to the receptor involved in Ca^{2+} release. It is also possible that most of the InsP_3Rs present in other subcellular fractions could have lost their binding properties during preparation of the membrane fractions. To test these possibilities, we screened different subcellular fractions for InsP_3R , using anti- InsP_3R antibodies. We used antibodies raised against the 14 C-terminal residues of the type 1 InsP_3R . This sequence has been previously shown to be immunoreactive in different tissues (Mignery et al., 1989). The type 1 InsP_3R from the cerebellum has been found to bind InsP_3 and induce Ca^{2+} release when expressed in a fibroblast cell line (Miyawaki et al., 1990). The immunopurified antibodies recognized a protein of 260 kDa that is abundant in cerebellar membranes (Figure 2), in agreement with the high receptor density found in this tissue. The antibodies also detected a protein in liver membranes which has the same molecular mass as the receptor present in the cerebellum. This strongly suggests

Figure 3 Characterization of the rat liver subcellular fractions by immunoblotting with antibodies specific for markers for the different cellular membranes

The subcellular fractions (abbreviations as for Figure 1; 30 μg on each lane) were prepared and immunoblotted as described in Figure 2. The blots were incubated with the following first antibodies: immunopurified anti- InsP_3R antibodies (5 $\mu\text{g}/\text{ml}$) (InsP_3R); polyclonal antiserum B1 raised against a marker of the basal and the lateral domain of the hepatocyte plasma membrane, diluted 1:5000 (LS); polyclonal antiserum B10 raised against a marker of the bile-canalicular domain of the hepatocyte plasma membrane, diluted 1:5000 (BC); monoclonal antibodies mAb-5F10, raised against the erythrocyte Ca^{2+} pump, diluted 1:2000 (PMCA); polyclonal antibodies raised against RER from dog pancreas, diluted 1:5000 (ER); and polyclonal antiserum raised against the SERCA2b Ca^{2+} pump, diluted 1:500 (SERCA).

that the type 1 InsP_3R is also present in liver membranes and has a molecular mass of about 260 kDa. Antibodies preabsorbed with the C-terminal peptide did not label the 260 kDa band (results not shown). The antibodies are highly specific for the InsP_3R , since they did not recognize other proteins in the crude membrane fraction: only two weak bands, with molecular masses of 140 and 150 kDa, were revealed in the LM fraction. The 260 kDa protein was detected in each subcellular fraction and was particularly concentrated in the PM fraction, which was also enriched in 5'-nucleotidase. These data confirm the presence of the type 1 InsP_3R in the liver membranes, with the same subcellular distribution as the [^3H] InsP_3 -binding sites. We used several antibodies specific for different markers of the three domains of the hepatocyte plasma membrane and specific for the ER, to characterize further the different membrane fractions prepared from the liver homogenate. The membrane fraction enriched in InsP_3R and in 5'-nucleotidase activity was also enriched in the three domains of the liver plasma membrane (Figure 3). Antibodies B1, which specifically label the lateral and the basal domain of the hepatic plasma membrane, detected a 90 kDa polypeptide present in the PM fraction. This 90 kDa polypeptide is a major degradation product of a 100 kDa protein present in the basal and the lateral plasma membrane (Maurice

Figure 4 Immunofluorescence detection of hepatocyte InsP₃R by using anti-InsP₃R antibodies

The cultured hepatocytes were fixed, permeabilized and stained as described in the Materials and methods section. (a) Immunopurified anti-InsP₃R antibodies (50 µg/ml) labelled the whole ER and a perinuclear area, including the perinuclear membrane. More intense fluorescence appeared near, but not all over, the plasma membrane (arrowheads). (b) By comparison, the anti-ER antibodies, diluted 1:1000, homogeneously labelled the whole ER. Bar = 25 µm.

et al., 1988). Antibodies B10, which specifically label the bile-canicular membrane (Maurice et al., 1985, 1988), detected a 150 kDa polypeptide present only in the PM fraction. These markers were not detected in the other subcellular fractions, in the HSP or the LSP (Figure 3). Since the same amount of protein was applied in each lane, this suggests that the markers of the plasma membrane were present at concentrations below the detection limit. We used monoclonal antibodies (mAb-5F10) against PMCA, which react with the Ca²⁺ pump of the liver located on the basal membrane (Kessler et al., 1990). The antibodies detected a 140 kDa protein present at high levels in the PM fraction. This protein was also detected at lower level in all the other fractions, consistent with contamination by plasma membrane.

Polyclonal antibodies raised against the RER from dog pancreas (Louvard et al., 1982) detected polypeptides of apparent molecular masses 58 and 90 kDa in all the membrane fractions. The band of 90 kDa has been identified as a Ca²⁺-binding protein and called calnexin (Wada et al., 1991). Calnexin and the 58 kDa protein were strongly labelled in the LM and HM fractions, and were present in about the same amounts in the other fractions and specially in the PM fraction (see Figure 3). The other polypeptides, of apparent molecular masses 29 and 66 kDa, recognized by these antibodies and initially identified in rat kidney cells were not detected in the liver membranes. Two other bands of 80 and 100 kDa were also weakly labelled, which follow the same distribution as the 58 and 90 kDa polypeptides.

Several isoforms of the Ca²⁺-transporting ATPase (SERCA) located in intracellular organelles have been described. In particular, the SERCA2a isoform is found in cardiac, slow skeletal and some smooth muscles, and the SERCA2b isoform is found in smooth muscle and most non-muscle tissues (see Missiaen et al., 1991). Accordingly, anti-SERCA2b antibodies detected a 115 kDa polypeptide in all the fractions tested (Figure 3), and anti-SERCA2a antibodies did not bind any polypeptides in liver membranes (results not shown). The distribution in the different subcellular fractions of the intracellular Ca²⁺ pump SERCA2b involved in Ca²⁺ accumulation into the intracellular Ca²⁺ store was the same as the RER markers and the glucose-6-phosphatase

activity (Figures 1 and 3). It is present at high level in the microsomal fractions, and is present in all the other fractions, particularly the PM fraction. Calnexin and the markers of the ER, SERCA2b and the glucose-6-phosphatase activity, are similarly distributed in the various subcellular fractions prepared from liver homogenate. This suggests that these markers are homogeneously distributed throughout the ER membrane.

The microsomal fractions (HM and LM) enriched in ER markers are not enriched in InsP₃R. The PM fraction which contains the three domains of the liver plasma membrane is enriched in InsP₃R and contains contaminant ER membrane. Thus the receptor could be localized (i) on one or several domains of the plasma membrane, or (ii) on a specialized sub-region of the ER which co-purified with the plasma membrane, or (iii) on a specialized sub-region of the ER associated with the plasma membrane.

Immunofluorescence

To determine whether the receptor is associated with the plasma membrane or is located in an independent organelle having the same density as the plasma membrane, we localized InsP₃R in unbroken cultured hepatocytes. We used hepatocytes maintained in primary culture for 20–24 h which are responsive to hormones and generate Ca²⁺ waves (Rooney et al., 1989). Immunofluorescence labelling of permeabilized cultured hepatocytes with anti-InsP₃R antibodies revealed a heterogeneous distribution of the receptor inside the cells (Figure 4a). There was a weak labelling throughout the cytoplasm, but more intense labelling in the perinuclear area, including the perinuclear membrane. In addition, some regions near the plasma membrane were more intensely labelled. Anti-InsP₃R antibodies preincubated with the C-terminal peptide did not label any of these zones (results not shown). By comparison, anti-ER antibodies labelled the cytoplasm homogeneously: neither the periphery of the cells nor the perinuclear region was more intensely labelled (Figure 4b). This confirms that the InsP₃R is not homogeneously distributed throughout the ER. The more intense labelling near the plasma membrane confirms the results of the subcellular fractionation.

Figure 5 Distribution of the [³H]InsP₃-binding sites and the enzyme markers in subfractions obtained after alkaline treatment of the PM fraction

The PM fraction was incubated in carbonate buffer, pH 10.5, as indicated in the Materials and methods section. The treated fraction f₀ was submitted to Percoll-gradient centrifugation, and four fractions (f₁, f₂, f₃ and f₄, from the lightest to the heaviest) were collected. (a) [³H]InsP₃ binding was determined as indicated in the Materials and methods section by incubating the membranes (0.2–0.3 mg/ml) in the presence of 1 nM [³H]InsP₃. Non-specific binding was determined in the presence of 5 μM unlabelled InsP₃. (b) The 5'-nucleotidase activities in the different fractions were determined. Results are expressed in μmol of P_i formed in 10 min per mg of protein. (c) The glucose-6-phosphatase activities in the different fractions were determined. Results are expressed in μmol of P_i formed in 10 min per mg of protein. Data are means of triplicate determinations in one typical experiment. [³H]InsP₃ binding, 5'-nucleotidase and glucose-6-phosphatase activities were determined in the same membrane preparations from a single subcellular fractionation.

We can conclude that InsP₃R is associated with the plasma membrane either directly or indirectly.

Alkaline treatment of the plasma-membrane fraction

It has been suggested that the InsP₃R is located in an intracellular organelle which associates with the plasma membrane through elements of the cytoskeleton. To determine whether the InsP₃R is located on the plasma membrane itself or on an organelle associated with the plasma membrane, we treated the fraction with alkaline medium. This treatment has been shown to eliminate filaments associated with the PM fraction and to allow a better separation of ER and plasma membrane (Hubbard and Ma, 1983; Payrastré et al., 1988; Tranter et al., 1989). The PM fraction was incubated in low-ionic-strength medium and with 1 M Na₂CO₃/NaHCO₃, pH 10.5. This treatment resulted in the loss of 50% of the proteins from the membrane fraction. The sample was then submitted to Percoll-gradient centrifugation as

Figure 6 Characterization of the subfractions obtained after alkaline treatment of the PM fraction by immunoblotting with antibodies specific for markers for different cellular membranes

The PM fraction was incubated in carbonate buffer, pH 10.5, as indicated in the Materials and methods section. The treated fraction f₀ was submitted to Percoll-gradient centrifugation, and four fractions (f₁, f₂, f₃ and f₄, from the lightest to the heaviest) were collected. The membrane fractions (30 μg on each lane) were immunoblotted and incubated with the first and the secondary antibodies as described in Figure 3.

described in the Materials and methods section, and four fractions were collected. They were extensively washed (to remove Percoll) before further analysis. The fractions were called f₁, f₂, f₃ and f₄ from the top to the bottom of the tube, and contained 21, 32, 16 and 32% of the protein collected from the gradient, respectively. The fractions of higher density contained more InsP₃R, as assessed by [³H]InsP₃-binding data (Figure 5a). Percoll-gradient centrifugation resulted in a decrease in total [³H]InsP₃ binding, presumably due to modification of the binding properties of the InsP₃R by the high concentration of Percoll used (Figure 5a). The 5'-nucleotidase activity was decreased by about 50% after the treatment at pH 10.5. The remaining activity was homogeneously distributed in the four fractions (Figure 5b). The glucose-6-phosphatase activity was not modified by the alkaline treatment of the membrane fraction, and a 50% increase in the specific activity in the f₀ fraction is due to the removal of peripheral proteins. The specific glucose-6-phosphatase activity was increased in the fractions of the highest density (Figure 5c). Particularly, the fraction f₄, which is enriched in InsP₃-binding sites, displayed the highest specific glucose-6-phosphatase activity among the different fractions prepared from the liver homogenate. These data suggest that, after treatment of the PM fraction at alkaline pH, InsP₃R co-purified with the ER markers.

The subfractions prepared from the PM fractions were further characterized with antibodies against different markers of the plasma membrane and of the ER (Figure 6). The anti-InsP₃R antibodies recognized a 260 kDa protein enriched in fraction f₄, in agreement with the data from the binding experiments. The SERCA2b and the ER markers were mainly in the fractions of highest density, suggesting that, after the treatment at pH 10.5, the InsP₃R co-purified with the ER. The markers of the different domains of the plasma membrane, labelled with the B1 and B10 antibodies, were more homogeneously distributed among the

different membrane fractions. The PMCA was enriched in the light fraction f_1 and was not detectable in the heavy fraction f_4 . This indicates that the PMCA and the InsP₃R completely segregated after the alkaline treatment.

DISCUSSION

The localization of the InsP₃R in the target cells has been the subject of several investigations during the last few years. The high receptor density found in the Purkinje cells of the cerebellum has allowed the localization in intracellular compartments involving rough and smooth ER. In non-neural tissues, little is known about the intracellular localization of the receptor. Only subcellular fractionation of the tissues has given some information on the distribution of the receptor. In most tissues, the InsP₃R has been recovered from microsomal fractions more or less enriched in markers of the ER, suggesting that the InsP₃-sensitive Ca²⁺ store could be a specialized sub-region of the ER or a specialized organelle called a 'calciosome' (Volpe et al., 1988; Sitia and Meldolesi, 1992). During subcellular fractionation of the liver homogenate, the InsP₃R co-purified with the markers of the plasma membrane. This result suggested that the receptor is localized on the plasma membrane itself, and it could thus be directly involved in the Ca²⁺ influx, as has been suggested in olfactory neurons or T-cells (Kalinowski et al., 1992; Khan et al., 1992). Alternatively, the receptor could be localized on the membrane of an intracellular organelle in close association with the plasma membrane, and it could participate in stimulation of the Ca²⁺ influx by interacting with the plasma membrane (Rossier et al., 1991; Irvine, 1990).

Characterization of the subcellular fractions

In this study, we separated various membrane fractions from a rat liver homogenate. The low-speed pellet contains about one-third of the membrane proteins, and is enriched in 5'-nucleotidase activity and PMCA. A membrane fraction (PM) prepared from this pellet is enriched in markers specific for each of the three domains of the plasma membrane of hepatocytes. Electron microscopy of the liver plasma-membrane fraction indicated the presence of large sheets containing the different domains of the membrane (results not shown). This confirms that gentle homogenization of the liver in sucrose medium yielded membrane sheets and allowed the recovery of all three major domains of the hepatocyte plasma membrane (Hubbard et al., 1983). This fraction also contains the glucose-6-phosphatase activity and the markers recognized by the anti-ER antibodies and the SERCA2b, indicating a contamination of this fraction by ER membrane. We have previously found that this PM fraction accumulated Ca²⁺ mainly into inside-out plasma-membrane vesicles sensitive to low concentrations of saponin. Accumulation of Ca²⁺ into a saponin-insensitive compartment, probably ER, represented less than 20% of the accumulated Ca²⁺. This suggests that the contaminating ER has a low capacity of Ca²⁺ accumulation as compared with the microsomal fraction (Dargemont et al., 1988; Piétri et al., 1990a).

The two microsomal fractions (HM and LM) obtained by differential centrifugation were enriched in glucose-6-phosphatase activity and in the 90 kDa and 58 kDa markers recognized by the antibodies raised against the ER. The 90 kDa marker has been identified as the Ca²⁺-binding protein calnexin (Wada et al., 1991) and was present at high level in the HM fraction. This protein is homogeneously distributed throughout the ER membrane and can thus be used as a general ER marker. The HM and LM fractions were not enriched in the markers of the plasma

membrane: the B1 and B10 antibodies did not detect any peptides. These fractions contained low 5'-nucleotidase activity and small amounts of PMCA, indicating a weak contamination with plasma membrane. The anti-SERCA2b antibodies recognized a protein of 115 kDa enriched in the HM and the LM fractions. The distribution of the Ca²⁺-ATPase is the same as that of the other markers of the ER, suggesting that it is distributed throughout the ER membrane. It has been postulated that at least three proteins should be present in non-muscle Ca²⁺ stores: a high-affinity Ca²⁺-ATPase, a high-capacity Ca²⁺-binding protein and a Ca²⁺ channel. It has been reported that the Ca²⁺-binding proteins (calsequestrin, calreticulin or others) are recovered in liver microsomal fractions enriched in ER membrane (Nguyen Van et al., 1989; Milner et al., 1991) and that calsequestrin and a 100 kDa Ca²⁺-ATPase are found in liver ER membrane (Damiani et al., 1988). Therefore, two of the proteins required to constitute a rapidly mobilizable Ca²⁺ store, i.e. a Ca²⁺-ATPase and a Ca²⁺-binding protein, are present in the microsomal fractions containing ER membranes.

Association of the InsP₃R with the plasma membrane

The presence of InsP₃R in different subcellular fractions was investigated by measuring the maximal binding capacities of [³H]InsP₃ and by using antibodies against the 14 C-terminal residues of the type 1 receptor. The antibodies recognized a 260 kDa protein abundant in cerebellar membranes, consistent with the high receptor density found in this tissue. This indicates that the antibodies recognize the monomeric form of the InsP₃R. This 260 kDa protein was also detected in the different fractions prepared from the liver homogenate and was present at high levels in the PM fraction. InsP₃-binding sites were found in all the different subfractions, but were 10 times more abundant in the PM fraction. There was a good correlation between the intensity of the labelling by the antibodies in Western blots and the maximal binding capacities determined with [³H]InsP₃ in the different membrane preparations. Thus the two methods validate each other. The fact that the [³H]InsP₃ binding site and the type 1 receptor show the same subcellular distribution indicates that either the type 1 receptor is the main isoform found in hepatocytes or, if there are large amounts of other isoforms, they have the same subcellular distribution as the type 1 receptor.

Both subcellular fractionation and immunofluorescent staining of permeabilized cultured hepatocytes indicate that InsP₃R is not distributed in the same way as the ER markers. Anti-InsP₃R antibodies labelled the outline of the cells and the perinuclear area more intensely than other areas. By comparison, the anti-ER antibodies displayed a homogeneous labelling throughout the cytoplasm. This distribution of InsP₃R is similar to what has been found in the Purkinje cells of the cerebellum, where the receptor is located in the perinuclear region and also on stacks beneath the plasma membrane (Otsu et al., 1990; Satoh et al., 1990; Meldolesi et al., 1992). The InsP₃R from the liver homogenate was scattered among the different subcellular fractions and was found at low density in the subcellular fractions enriched in ER markers or SERCA2b. One-third of the receptor was concentrated in the PM fraction, in agreement with the more intense labelling of the plasma membrane by anti-InsP₃R immunofluorescence. This fraction was not enriched in SERCA2b, which allows the Ca²⁺ accumulation inside the Ca²⁺ store: thus it does not fulfil the criteria of a rapidly mobilizable Ca²⁺ store.

The PM fraction contains inside-out plasma-membrane vesicles, which accumulate Ca²⁺ in the presence of ATP (Prpic et al., 1984). This Ca²⁺ cannot be released by the addition of InsP₃

to the medium (Dargemont et al., 1988). Thus presumably the plasma membrane does not contain functional InsP_3R , and the receptor is localized on vesicles having a low Ca^{2+} -storage capacity which are associated with the plasma membrane. Accordingly, alkaline treatment of the PM fraction followed by Percoll-gradient centrifugation allowed the partial segregation of the plasma membrane and the InsP_3R . Four fractions, f_1 – f_4 , were recovered from the top to the bottom of the tube. The PMCA was present at high levels in f_1 , and the other markers of the plasma membrane, B1 and B10, were homogeneously distributed among the different fractions and do not co-purify with the InsP_3R or the proteins of the ER. The basal membrane found in the light fraction f_1 , and the lateral and bile-canalicular membranes were found in the intermediate fractions f_2 and f_3 . The ER membrane and the InsP_3R were recovered in the heavy fraction f_4 , which displays a higher enrichment in glucose-6-phosphatase activity than the microsomal HM fraction. This strongly suggests that the InsP_3R is localized on the ER membrane, which was dissociated from the plasma membrane by the alkaline treatment. The link between the two membranes has not been identified. However, it has been previously suggested that actin could be involved in the association between plasma membrane and intracellular organelles (Rossier et al., 1991). The alkaline treatment has previously been described to extract actin from liver plasma membrane (Tranter et al., 1989; Hubbard and Ma, 1983). Our data support the view that elements of the cytoskeleton could be involved in the association of the InsP_3R with the plasma membrane. In agreement with this idea, recent data described an interaction between the InsP_3R and proteins of the cytoskeleton such as actin or ankyrin (Joseph and Samanta, 1993).

InsP_3 releases more Ca^{2+} from the mitochondrial and the microsomal fractions than from the PM fraction (Dawson and Irvine, 1984; Benedetti et al., 1988), which contains the highest density of InsP_3R (Figures 1 and 3). This suggests that only some of the InsP_3R detected in the subcellular fractions is coupled to Ca^{2+} release. This could be due to heterogeneity of the properties of the receptor. However, neither the binding experiments nor the immunochemical studies indicated such heterogeneity. Another hypothesis is that, after cell fractionation, some of the InsP_3R could not be associated with a Ca^{2+} store. In permeabilized hepatocytes, InsP_3 releases more Ca^{2+} from a compartment containing the marker glucose-6-phosphatase (more likely the ER), than from the microsomal fractions enriched in ER vesicles (Benedetti et al., 1987). This suggests that the three components required to form a functional Ca^{2+} -accumulating organelle are associated in intact cells and are dissociated upon cell fractionation, as has been proposed by Dawson and Comerford (1989). This supports the view that the ER is not structurally homogeneous: InsP_3R is localized on a specialized subcompartment of the ER which is recovered in the PM fraction. This fraction is weakly responsive to InsP_3 , suggesting that in intact cells the subcompartment enriched in InsP_3R is contiguous with the other regions of the ER to form a functional organelle allowing Ca^{2+} accumulation and release from the same store. This situation is well described in the skeletal muscle, where the Ca^{2+} -ATPase and the Ca^{2+} channel are physically dissociated on different membrane regions, but communicate functionally (see Lai and Messner, 1989). The components required to form a Ca^{2+} -storing organelle, i.e. the Ca^{2+} pump, the Ca^{2+} -binding proteins and the InsP_3R , can be found in intact ER and form a functional Ca^{2+} store made up of different specialized membranes delimiting a single luminal compartment. However, the disruption of cell integrity and the fractionation of the membranes can lead to an artifactual

segregation of the components forming the Ca^{2+} -sequestering organelle. Therefore, different markers of the ER can be recovered in different subcellular fractions and lead to misinterpretation of the localization of intracellular proteins.

Our study indicates that the InsP_3R is concentrated in particular areas of the hepatocyte, beneath the plasma membrane and around the nucleus. The specialized localization of the InsP_3R could lead to the release of Ca^{2+} in particular regions of the cytoplasm where it could rapidly interact with different effector systems. Such a localized Ca^{2+} release could be more efficient than a dispersed release in the whole cytoplasm. On the other hand, the high receptor density found near the plasma membrane is also consistent with interaction with proteins of the plasma membrane in addition to the Ca^{2+} -channel activity. Such a mechanism has been proposed to explain the stimulation of Ca^{2+} influx under agonist stimulation (Putney, 1986; Irvine, 1990).

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