Intracellular calcium stores and inositol 1,4,5-trisphosphate receptor in rat liver cells

Jean-Philippe LIÈVREMONT*, Anne-Marie HILL*, Dien TRAN*, Jean-François COQUIL*, Nicole STELLY† and Jean-Pierre MAUGER*‡

*INSERM U274, Physiologie et Pharmacologie Cellulaire, Bâtiment 443, Université Paris Sud, F-91405 Orsay Cedex, France, and †Laboratoire de Biologie Cellulaire 4, CNRS URA 1134, Bâtiment 444, Université Paris Sud, F-91405 Orsay Cedex, France

The D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] receptor was localized by immunofluorescence experiments *in situ* in liver cryosections. Two anti-Ins(1,4,5)P_3 receptor antibodies (against the 14 C-terminal residues of the type 1 receptor or against the entire cerebellar receptor) weakly decorated the whole cytoplasm, and a more intense labelling was observed at the periphery of the hepatocytes, particularly beneath the canalicular and the sinusoidal domains of the plasma membrane (PM). Antibodies against calreticulin, the Ca^{2+} pump (SERCA2b) or endoplasmic reticulum (ER) membranes homogeneously labelled the cytoplasm and the subplasmalemmal area. These data indicate that the ER can be divided into at least two specialized subregions: one is located throughout most of the cytoplasm and contains markers of the rough ER (RER), calreticulin, SERCA2b and a low density of Ins(1,4,5)P_3 receptor, and the other is confined to the periphery of the cells and contains calreticulin, Ca^{2+} pump, RER markers and a high density of Ins(1,4,5)P_3 receptor. A membrane fraction enriched in Ins(1,4,5)P_3 receptor and in markers of the PM was immuno-adsorbed with the antibody against the C-terminal end of the Ins(1,4,5)P_3 receptor and pelleted with Sepharose protein A. The immuno-isolated material was enriched in Ins(1,4,5)P_3 receptor, but none of the markers of the ER or of the PM could be detected. This suggests that the Ins(1,4,5)P_3 receptor is localized on discrete domains of the ER membrane beneath the canalicular and the sinusoidal membranes, where it was found at higher densities than the other markers.

INTRODUCTION

The mobilization of intracellular Ca^{2+} under hormonal stimulation involves the formation of the messenger inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which binds to specific receptors and opens a Ca^{2+} channel [1]. The endoplasmic reticulum (ER) is the intracellular Ca^{2+} store sensitive to Ins(1,4,5)P_3 [2]. However, several features of the spatial organization of the Ca^{2+} store have remained unresolved. They particularly concern the location of the sites where Ca^{2+} is accumulated by the Ca^{2+} pumps, where it is stored by binding to high-capacity binding proteins, and where it is released during hormonal stimulation, through the Ca^{2+} channels. The spatial organization of the Ca^{2+} store could have some roles in the physiology of the cell because it determines the area where Ca^{2+} is stored and where it is released. The intraluminal Ca^{2+} concentration has been implicated in the regulation of functions as diverse as protein synthesis and cell proliferation [3] and the regulation of Ca^{2+} fluxes through the plasma membrane (PM) [4]. The localization of the Ca^{2+} channel sensitive to Ins(1,4,5)P_3 in relation to the site of production of Ins(1,4,5)P_3 could determine the efficiency, or the sensitivity, of the Ins(1,4,5)P_3-induced Ca^{2+} release during hormonal stimulation. The spatial localization of the Ca^{2+} release channel will also determine where Ca^{2+} is discharged in the cytosol and therefore where it will be able to activate some specific functions, because it is believed that Ca^{2+} actions are short range. The visualization of the Ca^{2+} waves in stimulated single cells illustrates this spatial aspect of Ca^{2+} release [5]. In some cases, the Ca^{2+} wave started from a specific initiation locus, from where the wave propagated throughout the cytosol [5–7]. Such a locus could be due to a high local concentration of Ins(1,4,5)P_3 receptors.

The structural organization of the intracellular Ca^{2+} store has been indirectly studied by an immunohistochemical approach, labelling with specific antibodies the proteins involved in the Ca^{2+} homoeostasis, namely the Ca^{2+} pumps, the low-affinity Ca^{2+} binding proteins that buffer Ca^{2+} inside the store, and the Ca^{2+} release channel. Among the different tissues that have been investigated, the cerebellum is of particular interest because of the high density of Ins(1,4,5)P_3 receptors found in the Purkinje cells. The proteins involved in Ca^{2+} homoeostasis followed a widespread cytoplasmic distribution resembling that of the resident markers of the ER. Observations at the electron microscopic level revealed differences in the distributions of the Ins(1,4,5)P_3 receptor, the Ca^{2+} pump of the ER (SERCA), and calsequestrin, which suggest that the ER is divided into different compartments; this supports the existence of distinct Ca^{2+} pools [8,9].

Among the non-neuronal cells, the smooth muscle cells have been studied at the electron microscopic level. In the vas deferens the calreticulin, which is considered to be a low-affinity binding protein [10], is evenly distributed like other markers of the ER including the immunoglobulin binding protein and protein disulphide isomerase; however, calsequestrin and the Ins(1,4,5)P_3 receptor are found on peripheral surface elements [11,12]. In the aorta, the Ins(1,4,5)P_3 receptor distribution was similar to that of the other markers of the sarcoplasmic reticulum [12]. A specialized distribution of the Ins(1,4,5)P_3 receptor has also been described in *Xenopus laevis* oocytes, where it was found in the

Abbreviations used: ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; PM, plasma membrane; SERCA, sarco(endo)plasmic reticulum Ca^{2+} pump.

‡ To whom correspondence should be addressed.

cortical layer and the perinuclear ER [13]. Thus the specialization of the ER in different subcompartments can vary according to the cell type and could correspond to specific functions.

The epithelial cells offer a particularly interesting model for studying the spatial organization of the Ca^{2+} store in relation to the polarization of the cell in apical and basal poles separated by a lateral domain. In these cells, calreticulin appears evenly distributed as in most cell types [10,14]. Few data are available concerning the localization of SERCA2b, which is the predominant isoform in these cell types [15]. We previously found that in liver membrane fractions SERCA2b followed the same distribution as the other markers of the ER, suggesting that it has a widespread distribution [16]. Different types of the Ins(1,4,5)P_3 receptor were characterized [17–20] and several of them were found in the epithelial cells [21]. Immunocytochemical experiments indicated that irrespective of the receptor types the Ins(1,4,5)P_3 receptors were found at high density at the periphery of the cells but their distribution relative to the cell polarity depended on the cell type. The type 3 receptor was found with the highest concentration on the ER at the apical brush border of intestinal cells [22] or at the extreme apex of pancreatic acinar cells [23]. The type 1 receptor was found on the ER near the basal and the lateral domains of the PM of Madin-Darby canine kidney (MDCK) cells or kidney tubule cells [24].

It has been found that the liver Ins(1,4,5)P_3 receptor co-purified with markers of the PM [25–28] and the receptor purified from this fraction displayed ion-channel activity [29]. We found that in liver cells the type 1 receptor is localized on an ER subcompartment that co-purified with the PM [16]. However, these experiments did not permit localization of the Ins(1,4,5)P_3 receptor in relation to the cell polarity. In the present work we used immunocytochemistry to localize the Ins(1,4,5)P_3 receptor on liver sections. We found that it is aggregated on discrete domains of the ER associated with the different domains of the PM.

MATERIALS AND METHODS

Chemicals

Ins(1,4,5)P_3 was obtained from Calbiochem, and all other reagents were obtained from Sigma or Boehringer Mannheim.

Antibodies

A polyclonal antibody against the 14 C-terminal residues (2737–2750) of the type 1 Ins(1,4,5)P_3 receptor was previously described and characterized [16]. An antibody against the entire Ins(1,4,5)P_3 receptor was prepared as follows. Ins(1,4,5)P_3 receptor was solubilized and purified from sheep cerebellar microsomes by the method of Maeda et al. [30]. The receptor was further purified by electrophoresis on 4–10 % (w/v) polyacrylamide gels under reducing conditions as described by Laemmli [31], and the gel was stained with Coomassie Blue. The band that migrated at 260 kDa was cut out, dialysed and homogenized. The receptor (100 μg) was injected with complete Freund's adjuvant into a rabbit. Every 3 weeks, 100 μg of receptor was injected with incomplete Freund's adjuvant. After the seventh injection the rabbit was bled. The antibody was purified from the antiserum on a matrix of Affigel 15 coupled to purified Ins(1,4,5)P_3 receptor. The antibody was kept at -20 °C at 0.5 mg/ml in PBS containing 40 % (v/v) glycerol.

The following antibodies have been described elsewhere: anti-SERCA2b polyclonal rabbit antibody [32,33], anti-RER polyclonal antibody raised against dog pancreas RER membranes [34], and the polyclonal antibodies B1 and B10 raised against markers of the lateral and the bile canalicular membrane of rat hepatocytes respectively [35,36]. Polyclonal anti-calreticulin antibody was from Affinity BioReagent.

Immunofluorescence microscopy

Livers from female Wistar rats (200–250 g) were fixed by perfusion with 4 % (w/v) paraformaldehyde in PBS following the procedure of Pignal et al. [37]. The liver was then cut into 2–3 mm slices, fixed again in the 4 % (w/v) paraformaldehyde solution for 1 h at 4 °C and kept in PBS at 4 °C. The slices were washed and immersed in PBS containing 10 % glycerol for 3 h and then frozen in liquid nitrogen. Sections (5–7 μm) obtained with a cryostat at -20 °C were collected on gelatin-coated slides, dried for 1 h at room temperature, and stored at -20 °C. The slides were used within 1 month.

The liver sections were rapidly thawed and immersed in PBS containing 50 mM NH_4Cl and permeabilized in 0.1 % Triton X-100. The slides were washed twice with PBS and incubated overnight at 4 °C with the primary antibodies diluted as indicated in PBS containing 0.3 % BSA. The slides were washed five times with PBS and incubated for 1 h with fluorescein-labelled anti-rabbit IgG donkey antibody (Jackson Laboratories) diluted 1:100 in PBS with 0.3 % BSA. After five washes, the slides were mounted in Citifluor and examined with a Biorad Lasersharp MRC 600 confocal scanning microscope equipped with an argon ion laser.

Preparation of 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, containing 1 mM EGTA, supplemented with 1 mM dithiothreitol, 0.2 mM PMSF, 10 μg/ml leupeptin, 10 μM pepstatin, 2 μM benzamidine, 5 μg/ml aprotinin, 50 μg/ml trypsin inhibitor and 1 μg/ml *o*-phenanthroline, as previously described [26]. The high-speed pellet was obtained by centrifuging the homogenate at 100000 ***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. The supernatant was centrifuged for 20 min at 8000 ***g*** to obtain the mitochondrial fraction. The heavy and the light microsomal fractions were obtained by centrifuging the 8000 ***g*** supernatant for 30 min at 35000 ***g*** and the 35000 ***g*** supernatant for 60 min at 100000 ***g*** respectively. The PM fraction was prepared by Percoll gradient centrifugation of the low-speed pellet as described by Prpic et al. [38]. 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 cerebella were homogenized with an Ultra-Turrax in ice-cold homogenization medium. The homogenate was centrifuged for 10 min at 1500 ***g*** and the resulting supernatant for 30 min at 50000 ***g***. The pellet was washed and resuspended in the washing medium containing 250 mM sucrose, 25 mM Hepes/KOH, pH 7.4, supplemented with the protease inhibitor cocktail. The microsomes were resuspended at 5 mg/ml in the washing medium.

Immunoprecipitation of the solubilized Ins(1,4,5)P_3 receptor

The liver PM fraction was washed and resuspended at 5 mg/ml in the incubation medium containing dithiothreitol and the protease inhibitor cocktail and incubated for 30 min on ice in the presence of 1 % (w/v) Triton X-100. The mixture was centrifuged

for 1 h at 20 000 ***g***. The supernatant was dialysed against the incubation medium containing 0.1 % Triton X-100.

The solubilized proteins (1 ml at 3–5 mg/ml) were incubated overnight at 4 °C with either 7 μg of the affinity purified antibodies directed against the C-terminal extremity of the type 1 Ins(1,4,5)P_3 receptor or 7 μg of non-specific immunoglobulins G. The mixture was incubated with 100 μl of Sepharose protein A beads for 2 h and pelleted by centrifugation at 2000 ***g*** for 5 min. The pellet was washed five times with the incubation medium containing 0.1 % Triton X-100. The immunoprecipitated material was removed from the Sepharose beads by boiling the sample in an equal volume of SDS gel sample buffer [31] and analysed by Western blotting.

Immuno-adsorption of membranes

The liver PM fraction was resuspended at 5 mg/ml in the washing medium supplemented with the protease inhibitor cocktail. The fraction was sonicated at 0 °C three times for 10 s with 10 s intervals, with a Branson sonificator set at 50 W. The efficiency of the sonication was followed by examining the membranes with a phase-contrast microscope.

The PM fraction or the sonicated PM fraction, 1 ml at 5 mg/ml, was incubated for 1 h at 4 °C with either 7 μg of the affinity-purified antibodies directed against the C-terminal end of the type 1 Ins(1,4,5)P_3 receptor or 7 μg of non-specific immunoglobulins G. The mixture was incubated with 100 μl of Sepharose protein A beads for 2 h and pelleted by centrifugation at 70 ***g*** for 5 min. The pellets were washed five times with the washing medium supplemented with 0.5 % BSA. The immunoprecipitated material was removed from the Sepharose beads by boiling the sample in an equal volume of SDS gel sample buffer [31] and analysed by Western blotting.

Electron microscope observation of the PM fraction

The pellets of the PM fraction before and after sonication were fixed in 1 % glutaraldehyde in 25 mM Hepes buffer, pH 7.4, for 15 min at 4 °C. They were rinsed with the same buffer and postfixed with 1 % (w/v) OsO_4 in the same Hepes buffer for 1 h at room temperature. They were dehydrated in an aqueous acetone series and embedded in Epon Araldite. The ultrathin sections, obtained with the LKB ultramicrotome, were stained with alcoholic uranyl acetate (2 %, w/v) for 10 min and Reynold's lead citrate for 5 min at room temperature. The sections were observed by transmission electron microscopy (Siemens Elmiskop 102).

SDS/PAGE and Western blotting

The membrane fractions were resuspended in SDS sample buffer in reducing conditions as described by Laemmli [31]. SDS/PAGE was performed on 4–10 % (w/v) gradient gels, and the separated proteins were electro-transferred, as described by Towbin et al. [39], 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 peroxidase-conjugated goat IgG against rabbit IgG (1:2000) (from Diagnostics Pasteur, Marnes-la-Coquette, France). Blots were then washed five times and developed with the enhanced chemiluminescence (ECL) Western blotting system using Hyperfilm (Amersham).

RESULTS

Characterization of the antibodies

We previously found that the Ins(1,4,5)P_3 receptor was localized near the PM in cultured hepatocytes. This observation was confirmed because the receptor co-purified with markers of the PM during a cell fractionation protocol. In the present work we localized the Ins(1,4,5)P_3 receptor *in situ* in sections obtained from rat liver fixed by perfusion. Two different polyclonal antibodies were used for these studies. One is directed against the 14-residue C-terminal peptide of the type 1 receptor and recognized a single band at about 260 kDa in cerebellar and liver membranes in Western blot experiments [16]. The second antibody obtained against the entire sheep cerebellar Ins(1,4,5)P_3 receptor has been characterized by Western blot experiments by using crude cerebellar and liver membranes and subcellular fractions prepared from a liver homogenate. A single band was recognized at about 260 kDa in the cerebellar membrane (results not shown) and the crude liver fraction obtained by high-speed centrifugation (Figure 1). This indicated that this is the major protein recognized by the purified antibody in the crude liver membranes. Thus the purified antibody against the entire Ins(1,4,5)P_3 receptor can be used for immunocytochemical experiments. This band was also present in the different liver membrane fractions and was enriched in the PM fraction, which was also enriched in markers for the PM. This distribution was similar to that found for the [^{3}H]Ins(1,4,5)P_3 binding site and the protein recognized by the antibody against the C-terminal end of the receptor [16]. The antibody recognized a doublet structure in the PM fraction enriched in Ins(1,4,5)P_3 receptor. This suggested that a small fraction of the receptors displayed a different electrophoretic pattern. This could be due to the formation of aggregates, to a difference in the glycosylation of the receptors or to the presence of other types of Ins(1,4,5)P_3 receptor recently characterized [18,40,41]. Alternatively, if the band with a molecular mass greater than 260 kDa was not related to the Ins(1,4,5)P_3 receptor, it was much less abundant than the Ins(1,4,5)P_3 receptor even in the PM fraction. Thus this did not

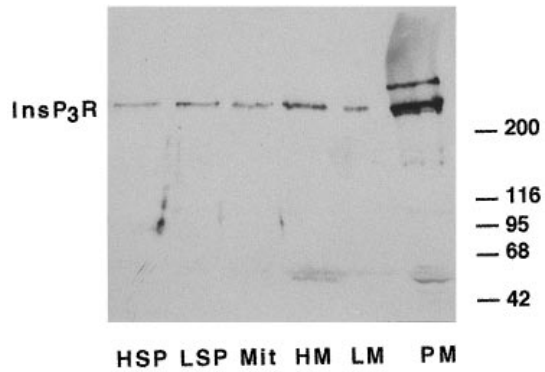

Figure 1 Western blot analysis of the reactivity of the anti-Ins(1,4,5)P_3 receptor antibody with rat liver membranes

The high-speed pellet (HSP), low-speed pellet (LSP), mitochondrial fraction (Mit), heavy microsomal fraction (HM), light microsomal fraction (LM) and PM fraction were prepared. The subcellular fractions (30 μg on each lane) were submitted to SDS/PAGE (4–10% gel gradient) and then transferred to a nitrocellulose membrane. The blot was incubated with the primary antibodies raised against the purified cerebellar Ins(1,4,5)P_3 receptor (3 μg/ml) (InsP_3R) and labelled with secondary antibodies. The blot was developed with the ECL Western blotting system. Molecular mass marker positions (in kDa) are indicated at the right.

preclude the use of the antibody in immunocytochemical experiments.

Immunofluorescence studies

The antibodies against the C-terminal portion of the type 1 receptor and against the entire cerebellar receptor each recognized a single band at 260 kDa in the crude membrane fraction. This protein is distributed in different subcellular fractions such as the Ins(1,4,5)P_3 binding site. Therefore we used these antibodies in immunofluorescence experiments to localize the Ins(1,4,5)P_3 receptor in sections of liver fixed by perfusion. The observation with a confocal microscope of the sections labelled with the antibody against the entire receptor revealed a homogeneous staining of the whole cytoplasm and a more intense punctate labelling appeared along the bile canalicular membrane, the sinusoidal membrane and around the nucleus (Figure 2A). Because the antibody against the entire Ins(1,4,5)P_3 receptor also recognized a minor band in the PM fraction (see Figure 1), we wished to confirm the localization of the receptor in liver sections by using an antibody directed against the C-terminal peptide of the type 1 receptor that does not recognize the 300 kDa polypeptide on a Western blot. Confocal microscopy (Figure 2B) revealed patches of intense labelling at the periphery of the cells and a granular staining in the whole cytoplasm. The difference in the intensity of the staining between the cytoplasm and the periphery of the cells was less apparent with the antibody against the C-terminal peptide than with the antibody against the entire protein. This could be due to the fact that the anti-peptide antibody recognized a single epitope that could be more or less easily reached depending on the localization of the receptor within the cell. The fact that the purified antibodies were directed against different epitopes yet revealed the same intracellular structures strongly suggests that they stained the Ins(1,4,5)P_3 receptor. The incubation of a liver section with non-specific rabbit IgG and secondary antibody did not reveal any cellular structures (Figure 2C). The same negative result was obtained when the sections were incubated with the secondary antibody alone (results not shown).

The localization of the Ins(1,4,5)P_3 receptor at the periphery of the cells and its co-purification with the markers of the PM indicated that the receptor is localized on a specialized subregion of the ER beneath the PM, as has already been found in different tissues [2,16,22,24,42]. In the following experiments we compared the distribution of the Ins(1,4,5)P_3 receptor with the distribution of other markers of the ER, including proteins involved in Ca^{2+} homoeostasis. We used antibodies directed against pancreas RER membranes, against the Ca^{2+} pump SERCA2b, or against the Ca^{2+}-binding protein calreticulin. The two former antibodies have previously been characterized [16]. The anti-calreticulin antibody labelled essentially one band estimated at 60 kDa, in the different liver membrane fractions analysed on a Western blot; the protein was more abundant in the heavy and light microsomal fractions also enriched in the other markers of the ER (Figure 3). The observation with a confocal microscope of the sections labelled with the three different antibodies against ER markers indicated that the cytoplasm was homogeneously stained (Figures 4A–4C). The periphery of the cells was not more intensely labelled, in contrast with the observation with the anti-Ins(1,4,5)P_3 receptor antibody. The incubation of the sections with non-specific IgG and secondary antibody or with secondary antibody alone did not reveal any cellular structures (results not shown).

The Western blot analysis indicated that calreticulin was recovered in all the fractions and was enriched in the heavy microsomal fraction and to a lesser extent in the light microsomal fraction (Figure 3). This distribution was identical with that of the RER markers, SERCA2b and glucose 6-phosphatase activity

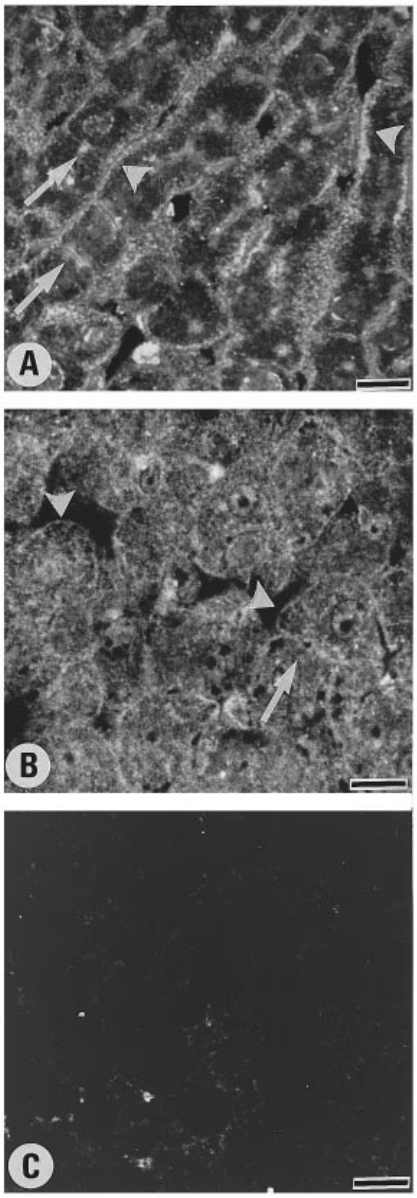

Figure 2 Immunofluorescence labelling of rat liver sections with an anti-Ins(1,4,5)P_3 receptor antibody

The rat liver sections were fixed, permeabilized and stained as described in the Materials and methods section. The sections labelled with the antibody against the 14 C-terminal residues of the type 1 Ins(1,4,5)P_3 receptor or the antibody against the entire cerebellar receptor or non-specific IgG were observed by confocal microscopy. (**A**) The section was labelled with immunopurified antibody against the entire Ins(1,4,5)P_3 receptor (30 μg/ml). A projection of serial optical sections performed at intermediate level showed a punctate labelling along the PM including the canalicular and the sinusoidal domains (arrows indicate transversal and longitudinal sections of the canalicular domain; arrowheads indicate sinusoidal domains). Scale bar, 10 μm. (**B**) The section was labelled with immunopurified antibody against the C-terminal end of the type 1 Ins(1,4,5)P_3 receptor (50 μg/ml). A projection of serial optical sections performed at intermediate level showed the labelling of the cytoplasm and a more intense staining of the periphery of the cells (arrowheads) including the canalicular area (arrow). Scale bar, 10 μm. (**C**) The section was labelled with non-specific IgG (50 μg/ml). No staining was apparent. Scale bar, 10 μm.

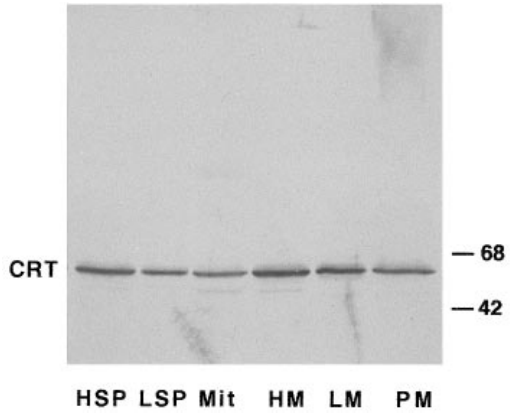

Figure 3 Western blot analysis of the reactivity of the anti-calreticulin antibody with rat liver membranes

The different subcellular fractions (30 μg on each lane) were prepared and immunoblotted as in Figure 1. The blots were incubated with diluted antiserum raised against the human recombinant calreticulin, diluted 1/1000. The blots were developed with the ECL Western blotting system. Molecular mass marker positions (in kDa) are indicated at the right.

[16]. Thus the Ins(1,4,5)P_3 receptor clearly had a different distribution in the hepatocytes than did the Ca^{2+} pump and calreticulin, which behaved like general markers of the ER. These results indicated that the liver Ins(1,4,5)P_3 receptor was localized on specialized subregions of the ER beneath the canalicular and the sinusoidal domains of the PM. These subregions were characterized by a high density of Ins(1,4,5)P_3 receptors, containing a similar density of SERCA2b and calreticulin as found in the remaining ER.

Immuno-isolation of the membrane containing the Ins(1,4,5)P_3 receptor

To further purify and characterize the membranes containing the Ins(1,4,5)P_3 receptor we used an immuno-adsorption approach to extract these membranes more directly from the PM fraction. This fractionation technique with specific antibodies has great potential for the characterization of subcellular compartments [43]. We used the antibody directed against the 14-residue C-terminal peptide of the receptor, which recognized a single band at 260 kDa on a Western blot. We first verified that this antibody also recognized the Ins(1,4,5)P_3 receptor under non-denaturing conditions by testing its ability to immunoprecipitate the solubilized receptor from the liver PM fraction. The Western blot analysis of the Ins(1,4,5)P_3 receptor precipitated from the liver detected a single band at about 260 kDa (Figure 5). The band present on the bottom of lane 3 corresponded to the heavy chain of the antibodies used to immunoprecipitate the receptor. Thus the antibody recognized the native receptor and no other protein. The immunoprecipitated protein specifically bound [^{3}H]Ins(1,4,5)P_3 (results not shown), confirming that the 260 kDa protein recognized by the antibody was actually the Ins(1,4,5)P_3 receptor. Because the Ins(1,4,5)P_3 receptor was the only protein immunoprecipitated from the liver PM, the antibody against the C-terminal part of the type 1 Ins(1,4,5)P_3 receptor can be used to immuno-adsorb the Ins(1,4,5)P_3 receptor-containing membranes specifically, without the risk of adsorbing contaminating membranes.

The liver PM fraction was used either before or after sonication. Sonication of the membranes did not modify the binding

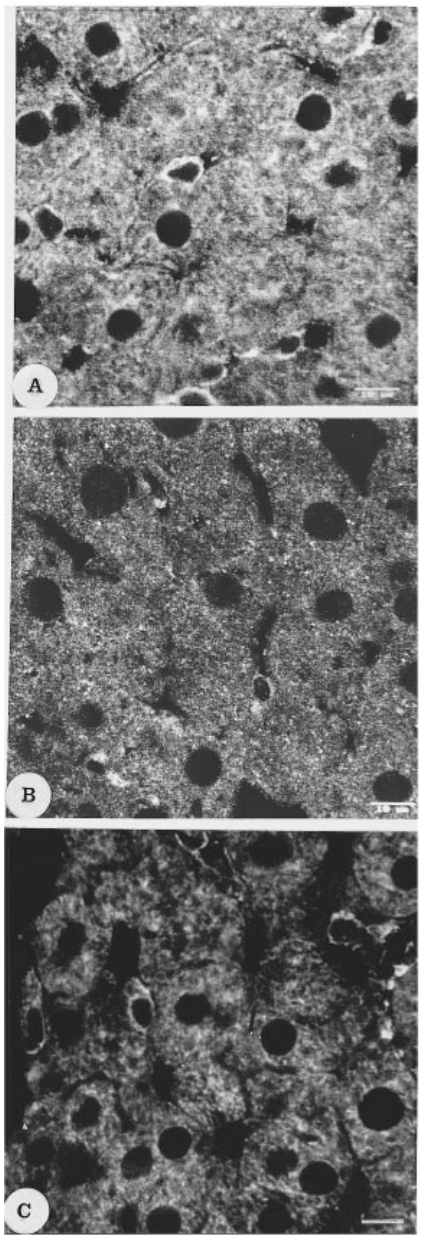

Figure 4 Immunofluorescence labelling of rat liver sections with anti-calreticulin antibody (A), anti-SERCA2b antibody (B) and anti-RER antibody (C)

The rat liver sections were fixed, permeabilized and stained as described in the Materials and methods section. (**A**) The confocal observation of the section stained with antibody against calreticulin (1/50) was performed at intermediate level. Widespread staining appeared in the cytoplasm with the exclusion of the nuclei. Scale bar, 10 μm. (**B**) The confocal observation of the section stained with antibody against SERCA2b (1/50) was performed at intermediate level. Widespread staining appeared in the cytoplasm with the exclusion of the nuclei. Scale bar, 10 μm. (**C**) The confocal observation of the section stained with antibody against RER (1/1000) was performed at intermediate level. Widespread staining appeared in the cytoplasm with the exclusion of the nuclei. Scale bar, 10 μm.

properties of [^{3}H]Ins(1,4,5)P_3 (results not shown). The membrane fractions were incubated with the antibody against the C-terminal end of the receptor or with non-specific antibodies and pelleted with Sepharose protein A. By Western blot analysis, a 260 kDa polypeptide was detected in material adsorbed from sonicated

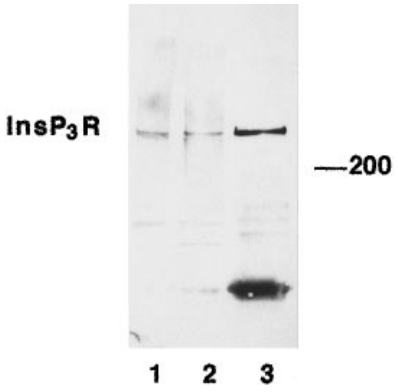

Figure 5 Immunoprecipitation of the solubilized liver Ins(1,4,5)P_3 receptor

The PM fraction was solubilized with 1% Triton X-100. The solubilized proteins (4–5 mg/ml) were incubated with affinity-purified antibodies (7 μg/ml) raised against the 14 C-terminal residues of the type 1 Ins(1,4,5)P_3 receptor, and then pelleted with protein A Sepharose beads. The PM fraction (lane 1), the solubilized proteins (lane 2) and the immunoprecipitated proteins (lane 3) were immunoblotted with the anti-peptide antibodies. A molecular mass marker position (in kDa) is indicated at the right.

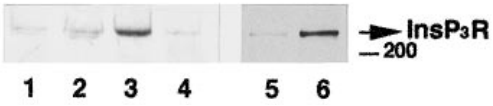

Figure 6 Immuno-adsorption of the Ins(1,4,5)P_3 receptor-containing vesicles

The liver PM fraction, either before (lanes 1 and 2) or after sonication (lane 3 and 4) and the cerebellar membranes (lanes 5 and 6) were incubated with non-specific immunoglobulins (7 μg/ml) (lanes 1, 4 and 5) or with purified antibodies (7 μg/ml) against the C-terminal end of the type 1 receptor (lanes 2, 3 and 6), and then pelleted with protein A Sepharose beads. The immuno-adsorbed material from the sonicated or non-sonicated liver PM fraction and from cerebellar membranes was immunoblotted with purified antibodies against the C-terminal end of the Ins(1,4,5)P_3 receptor (10 μg/ml). A molecular mass marker position (in kDa) is indicated at the right.

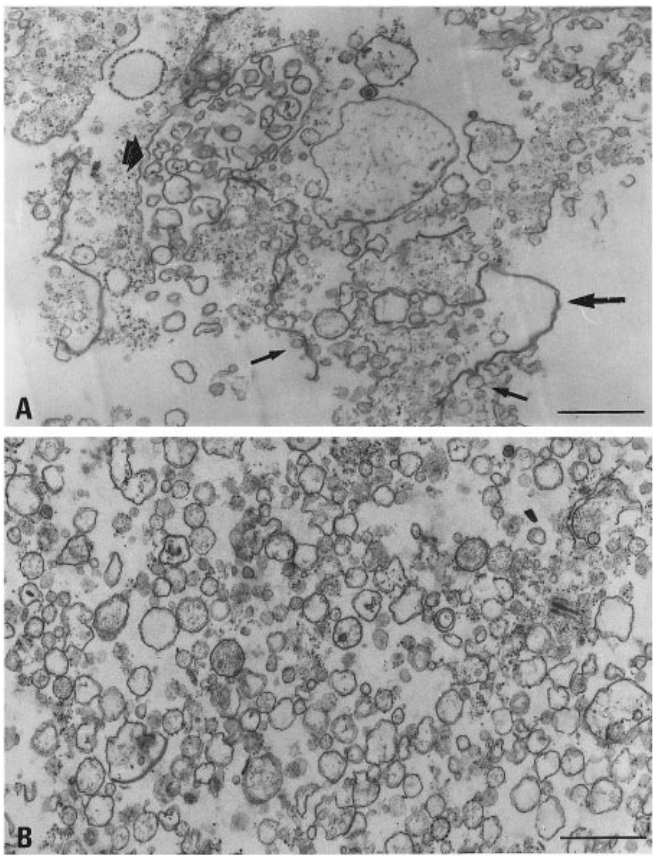

Figure 7 Ultrastructural observation of non-sonicated (A) and sonicated (B) liver PM fractions

The ultrathin sections were treated as described in the Materials and methods section. (**A**) Extended sheets of membranes are apparent (large arrows) and bile canalicular domains are formed by a membrane encircling vesicles (arrowheads). Small adherent vesicles are seen along the membrane sheets (small arrows). (**B**) After sonication, the membrane sheets are no longer apparent and vesicles with diameters ranging from 100 to 500 nm appear randomly dispersed. Scale bar, 1 μm.

membranes but not from non-sonicated membranes (Figure 6, lanes 2 and 3). When non-specific antibodies were used instead of anti-receptor antibodies, no Ins(1,4,5)P_3 receptor was detected in the immuno-adsorbed material even with sonicated membranes (Figure 6, lanes 1 and 4). Thus adsorption of the Ins(1,4,5)P_3 receptor seemed specific for the anti-Ins(1,4,5)P_3 receptor antibody.

The requirement for sonication of the membranes to obtain efficient immuno-adsorption of the receptor suggested that the sonication made the epitope accessible to the antibodies, allowing its precipitation by Sepharose protein A. We tried to reproduce the effect of sonication by pretreating the membrane at pH 11 because this partly releases the Ins(1,4,5)P_3 receptor-containing membranes [16]. We also homogenized the PM fraction with an Ultra-Turrax treatment, which vesiculated the large sheets of membrane [44]. None of these treatments allowed the immuno-adsorption of the Ins(1,4,5)P_3 receptor-containing membranes (results not shown).

Because the co-purification of the Ins(1,4,5)P_3 receptor and the markers of the ER with the plasma membranes is a feature of the liver not found with the cerebellum [45], we examined whether the sonication was also required to immunoprecipitate Ins(1,4,5)P_3 receptor from cerebellar membranes. Western blot analysis of the precipitated material showed that the anti-receptor antibody immunoprecipitated many more receptors than the non-specific antibodies, even if the membranes were not sonicated (Figure 6, lanes 5 and 6). Without sonication, the Ins(1,4,5)P_3 receptor accessibility is probably different between liver membranes and cerebellar membranes.

To understand the effect of sonication on the liver PM fraction we studied the ultrastructures of the native and sonicated membranes. Electron microscopy of the PM fraction revealed the presence of extended sheets of membrane probably containing lateral surface and the sinusoidal front typical of this tissue (Figure 7A, large arrow) [46]. Bile canalicular membranes consisting of small vesicles trapped in encircling membranes were also observed (Figure 7A, arrowhead). Numerous small vesicles, some with ribosomes attached, are associated with the amorphous material observed along the membrane sheets (Figure 7A, small arrows). After sonication, the membrane sheets were no longer visible and a population of vesicles with diameters ranging from 100 to 500 nm appeared randomly dispersed throughout the preparation (Figure 7B). The sonication transformed the membrane sheets into vesicles with homogeneous size and dispersed the vesicles trapped in the amorphous material along

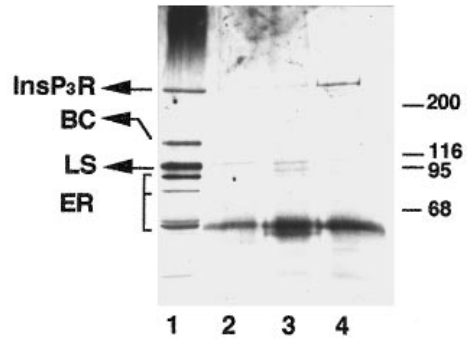

Figure 8 Characterization of the Ins(1,4,5)P_3 receptor-containing vesicles

The sonicated liver plasma-membrane fraction (lane 1) was incubated without immunoglobulins (lane 2) or with non-specific immunoglobulins (lane 3, 7 μg/ml) or with the purified anti-Ins(1,4,5)P_3 receptor antibodies (lane 4; 7 μg/ml), and then pelleted with protein A Sepharose beads. Immuno-adsorbed material was immunoblotted with a mixture of the following primary antibodies: purified antibodies against the C-terminal end of the Ins(1,4,5)P_3 receptor (10 μg/ml); polyclonal antiserum B10 raised against a marker of the bile-canalicular domain of the hepatocyte PM, diluted 1/1000 (BC); polyclonal antiserum B1 raised against a marker of the sinusoidal and the lateral domains of the hepatocyte PM, diluted 1/1000 (LS); and polyclonal antibodies raised against RER from dog pancreas, diluted 1:2000 (ER). Molecular mass marker positions (in kDa) are indicated at the right.

the sheets. Before sonication the Ins(1,4,5)P_3 receptor should have been localized either on the membrane sheets or on the vesicles associated with the amorphous material. In these two cases we assume that the antibodies might not have easy access to its epitope on the C-terminal end of the receptor. The sheets of membrane have been identified as PM domains [46]; therefore it is unlikely that the Ins(1,4,5)P_3 receptor was localized on these sheets but was probably on the vesicles associated with the amorphous material. Sonication released these vesicles and made them accessible to the antibodies and the Sepharose beads.

Characterization of the immuno-adsorbed membranes

To characterize the immunoprecipitated material we used different antibodies against markers of the ER or markers of the PM. None of the markers of the bile canalicular membrane or lateral and sinusoïdal membranes co-purified with the Ins(1,4,5)P_3 receptor (Figure 8). Similarly, antibodies against the RER (Figure 8) or SERCA2b or calreticulin (results not shown) did not recognize any protein in the Ins(1,4,5)P_3 receptor-enriched fraction. We cannot exclude the possibility that small amounts of vesicles were adsorbed and that the other markers were difficult to detect. The results at least indicated that in the purified vesicles the Ins(1,4,5)P_3 receptor has been enriched over the other markers of the PM or the ER. This indicated that the ER vesicles present in the sonicated PM fraction did not display the same ratio of Ins(1,4,5)P_3 receptor over the other markers. This suggests that the subregion of the ER that is rich in Ins(1,4,5)P_3 receptor and associated with the PM is constituted of discrete domains where some receptors could be associated in patches. Patches of Ins(1,4,5)P_3 receptor were seen as points of more intense fluorescence beneath the PM (Figure 2). Such a discontinuous staining with anti-Ins(1,4,5)P_3 receptor antibody has already been observed near the basolateral membrane in MDCK cells [24]. The fragmentation of membranes consisting of discrete domains yields fragments with differing compositions depending on the size of the fragments and the organization of the domains. The present results suggest that during the homogenization process the domains containing the Ins(1,4,5)P_3 receptor concentrated beneath the PM were recovered in specialized vesicles containing small amounts of other markers.

DISCUSSION

We have localized the Ins(1,4,5)P_3 receptor both *in situ* in liver sections by immunofluorescent experiments and by a subcellular fractionation approach. We used an antibody against the C-terminal end of the type 1 receptor and an antibody directed against the entire cerebellar receptor. Different receptor types have been described (at least five so far) [17–20], and it has been found that the mRNA coding for the type 1 receptor represents no more than 30% of the mRNA coding for the Ins(1,4,5)P_3 receptor in the liver. The type 2 receptor is the other predominant form of this tissue [21]. Therefore we considered the possibility that the type 1 and type 2 receptors are localized on different organelles or different subregions of the ER. On that hypothesis, our present data described the localization of the type 1 receptor in the hepatocyte. The other possibility is that the two types of Ins(1,4,5)P_3 receptor are not segregated and are found on the same ER subregions. In support of the latter, we previously found that the type 1 receptor followed the same distribution as the [^{3}H]Ins(1,4,5)P_3 binding sites during fractionation of a liver homogenate [16]. Therefore we concluded that the type 1 receptor is distributed like other types of receptor putatively found in the liver. This is in agreement with the recent identification of heterotetramers of Ins(1,4,5)P_3 receptor in the liver [47]. Our present data confirm the presence of the cerebellar type of the Ins(1,4,5)P_3 receptor in an organelle associated with the PM consistent with radioligand binding experiments [25,26] and identification with an antibody specific for the cerebellar form of the Ins(1,4,5)P_3 receptor [16,28]. These data do not support the recent proposal that the receptor in the liver PM fraction is not identical to the isoform present in the cerebellum and that the different isoforms found in the hepatocytes could be sequestered into separate organelles [48].

Two different antibodies, specific for the C-terminal peptide of the type 1 receptor or for the entire cerebellar receptor, showed a homogeneous staining throughout the cytoplasm and an intense punctate labelling at the periphery of the cells in immunofluorescence experiments *in situ* on liver sections. The results from the two antibodies validated each other and strongly suggested that the antibodies labelled the Ins(1,4,5)P_3 receptor. This confirmed the association of the Ins(1,4,5)P_3 receptor with the PM in the hepatocytes. With the exception of some specific tissues where some form of the Ins(1,4,5)P_3 receptor has been localized on the PM, for example in the T lymphocytes or the olfactory cilia [49,50] or in plasmalemmal caveolae in keratinocytes, endothelial cells and smooth muscle cells [51], it is well accepted that the receptor is localized on ER membranes (see [2]). In the hepatocytes, it is well established that Ins(1,4,5)P_3 mobilized Ca^{2+} from an intracellular compartment [26] and no Ins(1,4,5)P_3-dependent Ca^{2+} channel has been described in the hepatocyte PM. In agreement, we previously found that the Ins(1,4,5)P_3 receptor can be separated with other ER markers from the PM after treatment with alkali [16].

The immuno-adsorption experiments achieved the specific purification of the Ins(1,4,5)P_3 receptor containing vesicles from the PM fraction if it had been previously sonicated. The analysis of the immunopurified material did not reveal any other markers from either the PM or the ER. This could be because the subplasmalemmal ER subregion is highly specialized and contains only high concentrations of Ins(1,4,5)P_3 receptor. Alternatively, this specialized subregion could be organized in discrete domains that are adjacent to each other and contain either

Ins(1,4,5)P_3 receptor or the other markers of the ER. Such an organization of the ER membrane in discrete domains with heterogeneous composition has already been described for liver smooth ER studied by two-phase partitioning [52]. On this hypothesis, the fractionation of the membrane during the homogenization process could give rise to vesicles with different protein compositions, and only vesicles with the Ins(1,4,5)P_3 receptor are recognized by the antibody and are precipitated by Sepharose protein A. The labelling of the Ins(1,4,5)P_3 receptor in immunofluorescence experiments revealed patches of staining that could represent those discrete domains rich in receptors (Figures 2A and 2B). Subcellular fractionation experiments on different tissues also indicated that the Ins(1,4,5)P_3 receptor did not co-purify with the other markers of the ER, for example in chicken or rat cerebella [45,53] or in HL60 leukaemia cells [54]. In the Purkinje cells of the cerebellum, the Ins(1,4,5)P_3 receptor displayed a widespread distribution in the ER, in contrast to that observed in the hepatocytes. During fractionation of cerebellar membranes, the Ins(1,4,5)P_3 receptor did not co-purify with the other markers of the ER, suggesting that in this tissue too the Ins(1,4,5)P_3 receptor was concentrated in discrete domains distributed along the whole ER.

The localization of the Ins(1,4,5)P_3 receptor in different cell types revealed a great diversity in its distribution. The high receptor density in the Purkinje cells of the avian or mammalian cerebella facilitated studies at the ultrastructural level. The receptor was found widely distributed throughout the cytoplasm, in stacks of the ER and in subplasmalemmal cisternae [55]. In smooth muscle, the receptor was found either at the periphery of the cells or in the centre of the cell, depending on the tissue [12]. In polarized epithelial cells, the Ins(1,4,5)P_3 receptor was found at the periphery of the cells but its localization in relation to the cell polarity varied from one cell type to another. The type 1 receptor was localized in basolateral caveolae in rat kidney tubules [24] and along the canalicular [42] and sinusoidal membrane domains of the hepatocyte PM (the present study). The type 3 receptor was localized in a region adjacent to the apical brush border of villus cells and throughout the cytoplasm in crypt enterocytes [22]. In the pancreas, it was localized in the apical region of the acinar cells [23]. These studies revealed that in epithelial cells, the Ins(1,4,5)P_3 receptor was concentrated in specialized regions of the ER, often in proximity to particular domains of the PM. This could correspond to physiological functions requiring local increases in the Ca^{2+} concentration, as in secretion at the apical pole of the cells.

Subcellular fractionation experiments indicated that the distribution of glucose 6-phosphatase, the RER markers and the proteins involved in Ca^{2+} homoeostasis, including SERCA2b and calreticulin, co-purified in the same fractions. This was confirmed by the immunofluorescence experiments, which showed staining throughout the cytoplasm with antibodies against calreticulin, SERCA2b and RER markers, indicating that all the ER markers are distributed along the entire ER membrane. These observations suggested that Ca^{2+} released by Ins(1,4,5)P_3 can be accumulated and stored in the whole ER lumen. Assuming that the ER subregions that are rich in Ins(1,4,5)P_3 receptor are the main site for Ca^{2+} release during hormonal stimulation, this implies that the ER membrane delimits a single luminal compartment or several communicating compartments, and that the mobilizable Ca^{2+} freely and rapidly diffuses inside the ER lumen. Thus the Ca^{2+} release in one specific area of the cytoplasm could lead to a depletion of the Ca^{2+} content in the entire ER lumen and affect the properties at different sites of the cell, i.e. cell proliferation or protein synthesis [3] or the stimulation of Ca^{2+} influx through the PM [4]. It was found recently that the Ca^{2+} content of the nuclear envelope, which is continuous with the rest of the ER, regulates the transport of proteins into the nucleus and passive diffusion through the pore complex [56]. Thus the release of Ca^{2+} in any part of the cytoplasm could regulate some nuclear function. Alternatively, if the sites of high receptor density can release only a small part of the accumulated Ca^{2+}, because the ER lumen is divided into several non-communicating compartments or because Ca^{2+} diffuses slowly in the lumen, the area of low receptor density dispersed along the ER membrane could be used to release Ca^{2+}. The presence of patches of high receptor density concentrated beneath the PM could facilitate signal transduction by putting the Ins(1,4,5)P_3-sensitive Ca^{2+} channels near the site of Ins(1,4,5)P_3 production. The Ins(1,4,5)P_3 receptor could also communicate with a Ca^{2+} channel of the PM and increase Ca^{2+} influx, as hypothesized by Irvine [57]. Alternatively the Ins(1,4,5)P_3 receptor could be involved in another role than Ca^{2+} mobilization, as for example in the Purkinje cells of the cerebellum, where it participates in the formation of the ER stacks [58,59].

Which type of interaction is involved in the segregation of the Ins(1,4,5)P_3 receptor in a special region beneath the PM? The Ins(1,4,5)P_3 receptor interacts with the cytoskeletal proteins ankyrin or actin in such different tissues as the cerebellum, mouse T-lymphoma cells or the liver [27,60,61]. In the present work, the Ins(1,4,5)P_3 receptor was localized beneath the PM, along the canalicular domain and to a lesser extent along the sinusoidal domain. This localization resembled the labelling of F-actin with NBD–phalloidin in liver sections, where the bile canalicular area appeared strongly fluorescent and the sinusoidal and lateral membranes were also moderately positive [62]. Thus the liver Ins(1,4,5)P_3 receptor could be more or less directly associated with the actin filaments, as has been found in cultured keratinocytes [63]. Electron microscopical observations of the membrane fraction enriched in Ins(1,4,5)P_3 receptor and in markers of the PM revealed the presence of vesicles trapped within an amorphous material associated with large sheets of PM. Actin filaments were associated with liver cell membranes and were eliminated by a treatment at alkaline pH [64]. The morphology of the PM fraction was modified by the sonication process, the large sheets of membrane were vesiculated and the vesicles trapped within the amorphous material were released. We specifically immuno-adsorbed the Ins(1,4,5)P_3 receptor-enriched material only when the PM fraction was first sonicated (Figure 6). Two hypotheses can explain the failure to purify the Ins(1,4,5)P_3 receptor-containing vesicles from non-sonicated PM fraction by immuno-adsorption. First, the antibodies might not be able to interact with the epitope at the C-terminal end of the protein. This would occur if it were involved in the interaction with other protein(s) such as ankyrin, for example, which interacts with the C-terminal end of the receptor [60]. In support of this, in liver sections the periphery of the cell appeared less intensely stained with the antibody to the C-terminal end of the receptor than with the antibody to the entire protein, which recognized several other epitopes, suggesting that the antipeptide antibody interacted with more receptors in the whole cytoplasm than beneath the PM (Figures 2A and 2B). Secondly, perhaps the filamentous network around the ER vesicles prevents the free movement of the antibodies to their epitopes, or the large Sepharose beads coated with protein A cannot access the antibodies bound to the receptor. On this hypothesis, sonication would disrupt the filamentous network, making the Ins(1,4,5)P_3 receptor accessible to the antibodies and the beads. Indeed, sonication is known to fragment actin filaments [65] and to solubilize tubulin [66].

Therefore the present data strongly suggest that, in the hepatocyte, some of the Ins(1,4,5)P_3 receptors are localized in discrete domains of the ER membrane concentrated beneath the PM and possibly near the canalicular domain at a higher density. Homogenization of the liver produced large sheets of PM associated with a filamentous network containing trapped ER vesicles. We have so far not characterized the protein(s) involved in interacting with the Ins(1,4,5)P_3 receptor. Ankyrin and actin are putative candidates because they interacted with the receptor, but such interactions have not yet been described in the liver.

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